Combination Therapy in Ischemic Stroke: Synergistic Neuroprotective Effects of Memantine and Clenbuterol

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Background and Purpose—Although excitotoxic overactivation of glutamate receptors has been identified as a major mechanism of ischemic brain damage, glutamate receptor antagonists failed in stroke trials, in most cases because of limited therapeutic windows or severe adverse effects. Therefore, we chose memantine and clenbuterol, both approved safe and efficient in their respective therapeutic categories, and examined combinations of these neuroprotectants for possible therapeutic interactions in ischemic stroke.

Methods—Combinations of the N-methyl-D-aspartate (NMDA) receptor antagonist memantine (20 mg/kg) with the β2-adrenoceptor agonist clenbuterol (0.3 to 3 mg/kg) were tested in a mouse model of permanent focal cerebral ischemia. In addition, combinations of memantine (1 to 10 nmol/L) and clenbuterol (1 to 10 nmol/L) were examined in cultured hippocampal neurons exposed to glutamate (500 μmol/L) or staurosporine (200 nmol/L).

Results—The infarct size was further reduced by combination therapy as compared with effects of the respective neuroprotectants alone. Of note, in combination with memantine, the therapeutic window of clenbuterol was significantly prolonged up to 2 hours after ischemia. Experiments in postnatal cultures of rat hippocampal neurons exposed to glutamate or staurosporine confirmed that neuroprotection by combinations of memantine and clenbuterol exceeded the effects of the individual compounds.

Conclusions—Combinations of memantine with clenbuterol extend the respective therapeutic window and provide synergistic cerebroprotective effects after stroke. (Stroke. 2004;35:1197-1202.)

Key Words: cerebral ischemia ■ N-methyl-D-aspartate ■ neurons ■ glutamates

Previous studies exposed excitotoxic overactivation of N-methyl-D-aspartate (NMDA) glutamate receptors, disruption of the cellular calcium homeostasis, and free radical formation as key mechanisms involved in necrotic brain damage after ischemic stroke.1–3 Increasing evidence based on morphological and molecular studies suggests apoptosis as an additional mechanism of ischemic and excitotoxic cell death,4,5 and pharmacological studies clearly demonstrated the cerebroprotective potency of anti-apoptotic drugs such as inhibitors of p53, caspase inhibitors, or growth factors.6–8 Despite promising results in animal models of ischemia, clinical trials with NMDA receptor antagonists were stopped because of adverse effects, including neuropsychiatric symptoms or hypertension, which occurred in a dose-dependent manner before a neuroprotective plasma level could be achieved.9 Because a single drug that alone acts against the complex series of pathological events after stroke may not exist, the combination of established neuroprotectants with distinct mechanisms has been proposed for stroke therapy. For example, synergistic protective effects have been document
neuroprotective by induction of neurotrophic growth factors such as NGF, bFGF, or TGF-β1 in vitro and in vivo.15–18

The present study in a mouse model of permanent focal cerebral ischemia and in cultured hippocampal neurons demonstrates promising therapeutic interactions of memantine and clenbuterol, including synergistic neuroprotective effects and extended therapeutic windows after ischemic stroke.

Materials and Methods
Permanent Focal Cerebral Ischemia
Male Naval Medical Research Institute (NMRI) mice (Charles River, Salzfeld, Germany) were kept under controlled light and environmental conditions (12-hour light/dark circle, 23±1°C, 55%±5% relative humidity) and had free access to food (Altromin) and water. Permanent middle cerebral artery occlusion (MCAo) was performed in 12 to 16 animals per group (25 to 30 g) according to the method described previously.19 Briefly, after the mice were anesthetized with tribromoethanol (TBE) (350 mg/kg), a hole was drilled in the skull to expose the left MCA. The stem of the MCA and both branches were permanently occluded under visual control by electrocoagulation. Body temperature was maintained at 37±0.5°C with a heating lamp during the surgical procedure. After MCAo, rectal temperature was controlled every 30 minutes, and normothermia (37±0.5°C) was maintained by keeping the mice at an environmental temperature of 30°C for 6 hours.

In the first series of experiments memantine (20 mg/kg) was administered intraperitoneally 30 minutes before MCAO or up to 90 minutes after MCAO. Clenbuterol (0.3 to 3 mg/kg) was administered intraperitoneally before (5 hours) or after onset of ischemia (5 hours, 1 hour, 2 hours, 3 hours, or 6 hours after MCA occlusion). Control animals received vehicle only (0.9% saline). Seven days after MCAO, the mice received 0.5 mL of a 1.5% neutral red solution intraperitoneally; 30 minutes later, the brains were removed and the unstained tissue on the brain surface was determined as infarcted area (mm2) by means of an image analyzing system (Kontron).19 Infarct areas on the cortical surface of neutral red-stained brains highly correlated with infarct volumes determined by standard volumetry on crenyl violet-stained histological sections (r=0.99, P<0.001), and such a correlation was also found 7 days after MCAO in vehicle-treated versus memantine-treated animals (infarct area: 21.24±2.36 versus 18.43±3.28, P=0.017; infarct volume: 23.79±3.35 versus 19.14±2.95; P=0.001).

Physiological Parameters
The left external carotid artery was cannulated for blood sampling, monitoring of blood gases, electrolyte status, and mean arterial blood pressure (MABP) at 15 and 30 minutes during TBE anesthesia. In addition, systolic blood pressure was measured at 2 hours and 3 hours after surgery by a tail pressure cuff to evaluate drug effects on blood pressure. As established in a separate experiment in mice, values of MABP correlated well with values of systolic blood pressure (MABP) at 15 and 30 minutes during TBE anesthesia. In addition, systolic blood pressure was measured at 2 hours and 3 hours after surgery by a tail pressure cuff to evaluate drug effects on blood pressure. As established in a separate experiment in mice, values of MABP correlated well with values of systolic blood pressure (r=0.956, P<0.001).

Neuronal Cultures
Mixed primary cultures of hippocampal neurons and glial cells were prepared from neonatal Fischer 344 rats as described previously.17 Experiments were performed after 10 days in culture. The NMDA antagonist memantine (kind gift of Merz, Germany) and clenbuterol (kind gift of Arzneimittelwerk Dresden, Germany) were applied 1 hour or 5 hours before exposure of the cells to glutamate (500 µmol/L, 1 hour), respectively. For induction of excitotoxic cell death, medium (conditioned medium) was collected and glutamate-containing medium was added, and then exchanged again after 1 hour by the conditioned medium. Memantine and clenbuterol were present in the culture medium during and after exposure to glutamate. For induction of apoptosis, neurons were exposed to staurosporine (STS; 200 nmol/L, Sigma) for 18 hours. Cell death was determined by the trypan blue exclusion method or after staining the nuclei with the DNA-binding fluorochrome Hoechst 33258 as described previously.17,20

Statistics
All values are given as means±SD. One-way analysis of variance (ANOVA) combined with Scheffé test were used for multiple comparisons for in vitro experiments and combined with Duncan test for in vivo studies.

Results
Physiological Parameters
Physiological parameters measured at 15 and 30 minutes during TBE anesthesia are presented in Table 1. In addition, systolic blood pressure was measured at 2 hours and 3 hours in animals treated with vehicle, memantine (20 mg/kg; 15 minutes after surgery), clenbuterol (0.3 mg/kg; 2 hours after surgery), or the combination of both drugs (Table 2). There was no difference in physiological parameters between the treatment groups. Functional analysis did not reveal significant changes in general activity or exploratory behavior at 1 to 7 days after ischemia. All animals revealed slight pathological changes in the postural reflex without significant differences between groups. Moreover, all animals showed similar weight loss of 2 g within 48 hours after ischemia, followed by recovery that resulted in a gain of weight to a value exceeding the initial weight by ~1 g.

Therapeutic Interactions of Memantine and Clenbuterol in Cerebral Ischemia
On the basis of previous studies in rodent models of cerebral ischemia, we administered memantine at a dose of 20 mg/kg and clenbuterol at 0.3 mg/kg, and evaluated the respective therapeutic windows of the individual compounds in a model

| TABLE 1. Physiological Parameters During Tribromoethanol Anesthesia in Mice |
|-------------------------------|---------------------|
| | 15 Minutes | 30 Minutes |
| pH | 7.32±0.03 | 7.33±0.05 |
| pCO2 | 43.0±5.0 | 39.9±7.8 |
| pO2 | 158.2±12.3 | 155.3±4.1 |
| Na (mmol/L) | 142.4±1.5 | 142.1±1.8 |
| K (mmol/L) | 4.33±0.35 | 4.72±0.43 |
| Ca (mmol/L) | 1.27±0.04 | 1.26±0.04 |
| Cl (mmol/L) | 105.8±2.2 | 107.4±1.7 |
| MABP (mm Hg) | 65.0±8.9 | 66.6±12.6 |

| TABLE 2. Systolic Blood Pressure |
|-------------------------------|---------------------|
| | 2 Hours | 3 Hours |
| Control | 92.7±6.3 | 90.1±17.5 |
| Memantine | 86.0±11.2 | 87.8±18.6 |
| Clenbuterol | 82.3±15.1 | 87.1±14.8 |
| Memantine + clenbuterol | 81.8±16.2 | 82.1±8.8 |

Memantine (20 mg/kg) was administered 15 minutes after onset of anesthesia and clenbuterol was administered 2 hours later. Control animals received vehicle only (0.9% NaCl).
of permanent MCAO in mice.\textsuperscript{13,14,17} Memantine (20 mg/kg) reduced the infarct size by 20% or 10% only when administered 30 minutes before or immediately after MCAO, respectively (Figure 1A). When administered at 30 or 90 minutes after onset of ischemia, memantine failed to protect brain tissue from ischemic damage (Figure 1A). Clenbuterol reduced the infarct area by 17.3% only when administered 5 hours before MCAO, whereas administration of clenbuterol immediately after ischemia or 1 to 6 hours later did not show any effect as compared with controls (Figure 1B).

The combination of memantine (20 mg/kg), administered 30 minutes before ischemia, and clenbuterol (0.3 mg/kg), administered 1 or 2 hours after MCAO, reduced the ischemic brain damage by 29% or 27.7% as compared with vehicle-treated animals (Figure 2A, B). Of note, the protective effect of the combination therapy with clenbuterol administered 1 or 2 hours after MCAO was also more pronounced than the effect achieved with memantine alone (reduction of infarct area by 15.4% or 12%, respectively; Figure 2A, 2B). The combination therapy still exposed significant cerebroprotection with clenbuterol administered 3 hours or 6 hours after ischemia as compared with vehicle controls or clenbuterol treatment alone (Figure 2C, 2D).

We next examined whether a synergistic effect of memantine and clenbuterol was also observed when clenbuterol was administered at a dose of 1 mg/kg 2 hours after onset of ischemia. In animals receiving the combination therapy, the infarct size was reduced by 23%, whereas memantine alone reduced the infarct area by 14.7% as compared with vehicle-treated controls (Figure 3A). However, further acceleration of clenbuterol doses to 3 mg/kg did not result in a reduction of the infarct size by the combination therapy as compared with controls (Figure 3B).

After optimizing the treatment regimen for clenbuterol administered intraperitoneally 2 hours after MCAO at a dose of 0.3 mg/kg, we next attempted to evaluate the therapeutic...
window for memantine in the combination therapy. When administered 5 minutes after onset of ischemia, memantine exposed a similar protective effect as previously observed with pretreatment, now reducing the infarct area by 14%, and by 20% in combination with 0.3 mg/kg clenbuterol (Figure 4A). The synergistic effect of the compounds was still detectable when memantine was administered 30 minutes after onset of ischemia, with clenbuterol administered 2 hours after MCAO, whereas memantine alone had no protective effect when administered at 30 minutes after ischemia (Figure 4B). However, when administered later than 30 minutes, neither memantine alone nor the combination therapy with clenbuterol resulted in a reduction of the ischemic brain damage (Figure 4C).

Synergistic Effects of Memantine and Clenbuterol in Cultured Neurons
Using cultured hippocampal neurons, we wanted to clarify whether the combination of memantine and clenbuterol protected the cells rather from glutamate-induced excitotoxic cell death or if their synergistic activity also affected apoptotic mechanisms. In line with earlier results, clenbuterol significantly reduced glutamate toxicity in hippocampal neurons (Figure 5A). Surprisingly, a similar synergistic effect was observed when a combination of both drugs was tested against staurosporine-induced apoptosis. When administered at concentrations of 10 nmol/L, the single compounds did not have any effect on staurosporine-induced apoptosis, whereas the combination of both drugs significantly reduced the number of apoptotic nuclei (Figure 5B).

Discussion
The use of potent neuroprotectants such as the NMDA receptor antagonist memantine and even more so the β2-adrenoceptor agonist clenbuterol for stroke therapy appeared to be limited, because until now, both drugs were found inactive when applied after ischemia in experimental models of stroke. Here, we showed that combination therapy not only resulted in a further reduction of brain damage as compared with effects of the individual compounds but also significantly extended the therapeutic window. For memantine, the extension of the therapeutic window to 30 minutes after onset of ischemia may reflect the fact that the massive release of glutamate inducing excitotoxic cell death is an early event that triggers further pathological mechanisms involved in ischemic brain damage. Although neither memantine nor clenbuterol alone was protective when applied later than 30 minutes after MCAO, the combination of both drugs...
In comparison to memantine, the extension of the therapeutic window for clenbuterol in the combination therapy was remarkable, because the therapeutic window was shifted from 5 hours pretreatment to 2 hours after ischemia. Our previous work in various ischemia models established a requirement for pretreatment with clenbuterol, because the underlying mechanism of neuroprotection, ie, the induction of neurotrophic growth factors, had to be triggered hours before ischemia to achieve therapeutic effects.15–18 By blocking glutamate toxicity, memantine probably slowed crucial pathological mechanisms of ischemic brain damage, hence extending the therapeutic window for drug-induced growth factors. In addition, memantine has been reported to induce brain-derived neurotrophic factor (BDNF) and its receptor TrkB in brain tissue.21 Enhanced BDNF signaling could likely act synergistically with clenbuterol-induced neurotrophic factors NGF, bFGF, or TGF-β1,18,16,18

Such synergistic effects of induced neurotrophic growth factors may also explain why the combination of memantine and clenbuterol exceeded neuroprotection against staurosporine-induced apoptosis in cultured hippocampal neurons as compared with the individual compounds. Notably, memantine alone at a concentration of 10 μmol/L was able to protect cultured neurons against apoptosis when applied 5 hours before exposure to STS (not shown). Such a period of pretreatment would be sufficient for the induction of BDNF and TrkB receptors in cultured neurons similar to the reported effects of memantine in brain tissue.22 Therefore, the combination of memantine and clenbuterol provides enhanced neuroprotective effects not only against glutamate-induced excitotoxic neuronal cell death but also against apoptosis. Our previous studies provided evidence for the anti-apoptotic effect of clenbuterol,18,23 and clenbuterol-induced growth factors such as NGF or TGF-β1 prevented apoptosis in cultured neurons and in animal models of cerebral ischemia.20,23,24,25

It is therefore likely that also in the present study ischemia-induced apoptotic cell death was inhibited by the β2-adrenoceptor agonist. Similar synergistic protection against ischemic brain damage was found when memantine was combined with the antiapoptotic p53 inhibitor PFT,26 supporting the view that concomitant targeting of excitotoxicity and mechanisms of apoptosis is a promising strategy for stroke therapy. Moreover, recent data also implied that mild hypothermia could further improve therapeutic efficiency of such neuroprotective drug combinations.14,27,28 Like many other NMDA-antagonists, memantine provides additional protective effects against ischemic brain damage by induction of hypothermia.14 Most likely, memantine-induced hypothermia could further extend cerebroprotection by the combination therapy demonstrated here in normothermic animals. Other adverse effects of NMDA receptor antagonists, such as hypertension, were not observed in the present study; a possible moderate stimulation of motoric activity by memantine could also support recovery of stroke patients.9

Overall, the present findings demonstrated synergistic effects of potent neuroprotectants with different modes of action directed against glutamate toxicity and apoptotic signaling, two major pathological mechanisms involved in

reduce the infarct size when administered 30 minutes (memantine) and 2 hours (clenbuterol) after ischemia. Moreover, cerebroprotection by the combination therapy also exceeded the effects of memantine (20 mg/kg) or clenbuterol (0.3 mg/kg) alone administered repeatedly intraperitoneally 30 minutes before and 2 hours after MCAO (not shown). These results demonstrated that the synergistic protective effect was indeed a result of the combination of different neuroprotectants at low doses.

With regard to stroke therapy, such combinations may thus be superior to elevated dosages or repeated injections of single drugs, because combinations with drugs at low doses have lower risks for adverse effects as compared with prolonged high-dose therapy. In particular, increased clenbuterol doses have been found to be ineffective in models of stroke in our previous studies, because a strong stimulation of β2-adrenoceptors induced significant reduction of arterial blood pressure.16 In line with earlier results,15,16,18 clenbuterol did not alter blood pressure at cerebroprotective doses. Possible severe adverse effects of β2-adrenoceptor agonists such as induction of (heart) muscle toxicity are most likely negligible in the treatment regimen used here, because such toxic effects were only observed after repeated application of high doses of clenbuterol or other β2-adrenoceptor agonists over days.21
stroke. Because memantine and clenbuterol together provided synergistic neuroprotection and considerable extension of the individual therapeutic windows at safe doses, this combination could be highly effective in stroke therapy.

References
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