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Stimulation of β-adrenoceptors activates astrocytes and provides neuroprotection

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Abstract

Our previous studies established that induction of growth factor synthesis and neuroprotection by the β_2-adrenoceptor agonist clenbuterol in vitro and in vivo was associated with the activation of astrocytes, the major source of trophic factors in the brain. In the present study, we further investigated the specificity of β_2-adrenoceptor-mediated effects on astrocyte activation and neuroprotection. In mixed hippocampal cultures neuroprotection against glutamate-induced cell death by clenbuterol (1 μM) was blocked by the $\beta_{1/2}$-adrenoceptor antagonist propranolol and the specific β_2-adrenoceptor antagonists 1-[2,3-(Dihydro-7-methyl-1*H*-inden-4-yl)-oxy]-3-[(1-methylethyl)-amino]-2-butanol (ICI 118,551, 10 μM) and butoxamine (10 μM), while the β_1-adrenoceptor-selective antagonist metoprolol (10 μM) showed no effect. The β_2-adrenoceptor agonists clenbuterol (1–100 μM) and salmeterol (0.01–1 μM) induced profound morphological changes of cultured astrocytes which transformed into activated astroglia with pronounced dendrite-like processes. This phenomenon was blocked by butoxamine (1 mM) and propranolol (10 μM), but not by metoprolol (10 μM). However, similar morphological changes in astrocytes were also observed after stimulation of β_1-adrenoceptors by dobutamine (1–10 μM) and norepinephrine (1–10 μM). This effect was blocked by propranolol (10 μM) and metoprolol (10 μM) but not by butoxamine (1 mM), suggesting that stimulation of either β_1- or β_2-adrenoceptors was sufficient to induce activation of astrocytes. In addition, β_1-adrenoceptor stimulation by dobutamine (1–10 μM) protected hippocampal neurons against glutamate toxicity. In a model of focal cerebral ischemia in mice the cerebroprotective effect of clenbuterol (0.3 mg/kg) was blocked by propranolol (5 mg/kg) and butoxamine (5 mg/kg). Interestingly, the infarct size was reduced after co-treatment with clenbuterol (0.3 mg/kg) and metoprolol (5 mg/kg) as compared to clenbuterol treatment (0.3 mg/kg) alone. In conclusion, activation of astrocytes and neuroprotection can be achieved by stimulation of either β_1- or β_2-adrenoceptors in vitro, whereas in vivo neuroprotection is preferentially mediated through β_2-adrenoceptors.

Keywords: β-Adrenoceptor; Astrocyte; Hippocampal neuron; Clenbuterol; Cerebral ischemia; Middle cerebral artery occlusion

1. Introduction

The pharmacological induction of endogenous growth factor synthesis is a promising strategy for the treatment of neuropathologic disorders including Alzheimer's disease, Parkinson's disease, epilepsy, brain trauma, and stroke (Carswell, 1993; Semkova and Krieglstein, 1999; Zhu and Krieglstein, 1999). Previous studies demonstrated that activation of β_2-adrenoceptors by clenbuterol induced the synthesis of growth factors like nerve growth factor (NGF), basic fibroblast growth factor (bFGF) and transforming growth factor-beta 1 (TGF-β1) in rat brain tissue (Culmsee et al., 1999b; Follesa and Mocchetti, 1993). The induction of endogenous NGF synthesis by the β_2-adrenoceptor agonist clenbuterol was identified as the underlying mechanism for both, the neuroprotective effect against excitotoxic cell death in vitro and protection against ischemic brain damage in rodent models of focal ischemia (Culmsee et al., 1999a; Semkova et al., 1996a). Furthermore, clenbuterol treatment modulated the expression of the oncogenes Bcl-2, Bcl-xl and Bax, and prevented neuronal apoptosis in rat brain after transient forebrain ischemia (Zhu et al., 1998, 1999). Recently, clenbuterol was reported to ameliorate the locomotor and histological outcome after spinal cord injury in rats, suggesting a combined neuroprotective and muscle-supporting effect of the β_2-adreno-

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ceptor agonist (Zeman et al., 1999). Within the brain, astrocytes were identified as the dominant cell type expressing β_1- and β_2-adrenoceptors (Hösli and Hösli, 1993; Mantyh et al., 1995). After brain injury, a pronounced increase in β_2-adrenoceptor expression was detected predominantly in glial cells (Hodges-Savola et al., 1996). Under physiological conditions as well as after an insult, astrocytes are the main source for growth factors in the brain, indicating that astrocyte activation may be an endogenous mechanism of neuroprotection (Oderfeld-Nowak and Bacia, 1994; Schwartz et al., 1993). Activation of astrocytes was associated with increased expression of β_2-adrenoceptors, while astrogliosis and glial scar formation after optic nerve injury were reduced by the unspecific β_1/β_2-adrenoceptor antagonist propranolol (Hodges-Savola et al., 1996). Similarly, the specific β_2-adrenoceptor agonist clenbuterol further enhanced astrocyte activation in ischemic brain tissue (Culmsee et al., 1998, 1999b). Clenbuterol also induced NGF synthesis in cultured astrocytes, suggesting a contribution of these glial cells to the neuroprotective effect of the β_2-adrenoceptor agonist in mixed hippocampal cultures and in rats (Culmsee et al., 1999a). Induction of NGF by clenbuterol in vitro and in vivo and neuroprotection by clenbuterol could be blocked by the unselective $\beta_{1/2}$-adrenoceptor antagonist propranolol demonstrating the involvement of β-adrenoceptors in these effects (Semkova et al., 1996a). However, it has not yet been defined whether the activation of astrocytes and the associated neuroprotection exclusively depend on the specific stimulation of β_2-adrenoceptors. In particular, the proposed mechanism of astrocyte activation and induction of growth factors may involve the activation of adenylate cyclase and enhanced levels of cyclic AMP (c-AMP). It is well established that both, β_1- and β_2-adrenoceptors are linked with stimulatory G-proteins to activate the adenylate cyclase. Therefore, we investigated whether the stimulation of either β_1- or β_2-adrenoceptors exposed comparable effects on astrocyte activation. Furthermore, we applied selective agonists and inhibitors of β_2- and β_1-adrenoceptors in our models of neurodegeneration in vitro and in vivo and in cultured astrocytes to further elucidate the specificity of β_2-adrenoceptor-mediated neuroprotection.

2. Materials and methods

2.1. Primary rat hippocampal cultures

Mixed primary cultures of hippocampal neurons and glial cells were prepared from neonatal Fischer 344 rats as described previously (Semkova et al., 1996a). Briefly, animals were decapitated and hippocampi were isolated under sterile conditions. The extracted hippocampi were incubated in Leibovitz's medium (Gibco BRL) containing 1 mg/ml papain for 20 min at 37 °C. After removing the medium the tissue pieces were triturated carefully in neurobasal medium (Gibco BRL) and transferred to Neurobasal-medium containing trypsin inhibitor. After centrifugation for 20 min at $200 \times g$ the cell pellet was resuspended in neurobasal medium (supplemented with B27, glutamine, streptomycin and penicillin; Gibco BRL) and seeded onto poly-L-lysin-coated multiwells. The cultures were maintained at 37 °C in a 5% CO_2 atmosphere and 95% humidity. Experiments were carried out after 10 days in culture.

2.2. Glutamate treatment and cell viability

Cell culture medium was adjusted to a volume of 500 μl. The adrenoceptor antagonists and agonists (butoxamine, 1-[2,3-(Dihydro-7-methyl-1*H*-inden-4-yl)-oxy]-3-[(1-methyl-ethyl-)-amino]-2-butanol (ICI 118,551), metoprolol, propranolol, dobutamine, norepinephrine; Sigma, Deisenhofen, Germany) were applied 20 min before adding the β_2-adrenoceptor agonists salmeterol (Sigma) and clenbuterol (a kind gift of Arzneimittelwerk Dresden, Germany). Cells were treated with the adrenoceptor antagonists/agonists 4 h before, during, and up to 18 h after exposure to glutamate (1 mM). For induction of excitotoxic cell death, medium (conditioned medium) was collected, glutamate-containing medium was added, and then exchanged again after 30 min by the conditioned medium. Eighteen hours after glutamate exposure the cells were stained with Trypan blue for 10 min at 37 °C, washed twice with phosphate-buffered saline (PBS) and fixed with PBS-buffered formalin. Neurons were distinguished from glial cells by common morphological criteria. A total number of 1500–2000 neurons were counted by two investigators without knowledge of treatment history in three randomised subfields of 5 wells per experiment. Trypan blue-stained cells were regarded as non-viable, whereas the non-stained cells with intact neurites and soma were considered as viable.

2.3. Astrocyte cultures

Primary cultures of astrocytes were prepared from cerebral cortices of postnatal day 1 Fisher 344 rats as described previously (Culmsee et al., 1999b). Briefly, brains were removed under sterile conditions, and the cerebral cortices were isolated and dissected in Dulbecco's minimal essential medium (DMEM) containing penicillin–streptomycin (Gibco BRL). The tissue was dissociated through a stainless steel mesh and the resulting cell suspension was centrifuged at $200 \times g$ for 5 min. The pellet was resuspended in culture medium and the cells were then seeded on culture flasks (175 cm^2, Corning) and cultivated in DMEM containing 10% fetal calve serum and penicillin–streptomycin until confluency was reached. Oligodendrocytes and microglial cells growing on the astrocyte monolayer were removed by shaking and washing with cold PBS. Confluent astrocytes were passaged once and used for experiments after further 10 days in culture.

2.4. Immunocytochemistry and immunoblot

The methods were similar to those described previously (Kruman et al., 2000). Cells were fixed in 4% paraformaldehyde, membranes were permeabilized by exposure for 5 min to 0.2% Triton X-100 in PBS, and cells were placed in blocking serum (5% fetal calve serum in PBS) for 30 min. Cells were then exposed to a monoclonal anti-glial fibrillary

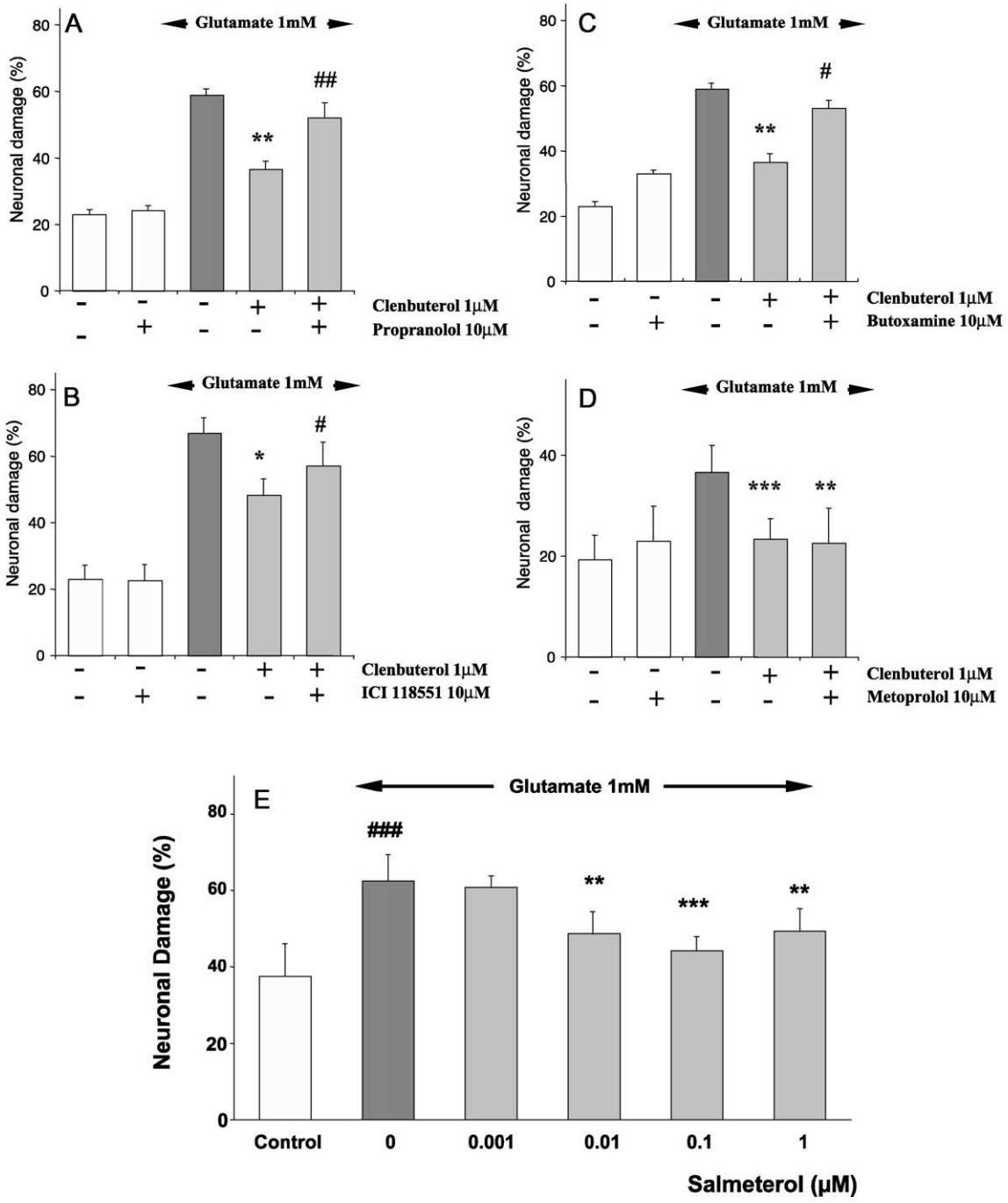


Fig. 1. (A–E) Neuroprotection by clenbuterol in vitro is mediated by specific β_2-adrenoceptor stimulation. After 10 days in vitro, hippocampal cells were incubated with the unspecific β-adrenoceptor antagonist propranolol (A), the specific β_2-adrenoceptor antagonists butoxamine (B) and ICI 118,551 (C), and the β_1-adrenoceptor antagonist metoprolol (D) starting 15 min prior to incubation with clenbuterol (1 μM). Hippocampal cultures were exposed to clenbuterol 4 h before and up to 18 h after glutamate exposure (30 min, 1 mM). Neuronal damage was determined by Trypan blue exclusion. Values are given as means ± S.D. of $n = 5–6$ experiments. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to glutamate treatment; $\#P < 0.05$, $\#\#P < 0.01$ and $\#\#\#P < 0.001$ compared to clenbuterol/glutamate treatment (ANOVA, Scheffé's). (E) Hippocampal cells were incubated with 0.001–1 μM of the specific β_2-adrenoceptor agonist salmeterol 4 h before and up to 18 h after glutamate exposure (30 min, 1 mM). Neuronal damage was determined by Trypan blue exclusion. Values are given as means ± S.D. of $n = 5–6$ experiments.: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ as compared to glutamate treatment (ANOVA, Scheffé's).

acidic protein (GFAP) antibody (1:2000, Sigma) overnight at 4 °C, followed by an incubation for 1 h with biotinylated anti-mouse immunglobuline G (IgG) antibody (1:200), and 30 min in the presence of fluorescein isothiocyanate–avidin (Vector Labs, Burlingame, CA, USA). Images were acquired using a confocal laser scanning microscope (Zeiss, Jena, Germany) with a 60 × oil immersion objective (excitation: 488 nm and emission: 510 nm). All images were acquired using the same laser intensity and photodetector gain to allow comparisons of relative levels of immunoreactivity between cultures.

For immunoblot analysis, protein extraction was performed by collecting the cells in ice cold PBS followed by centrifugation at 600 × *g* for 15 min at 4 °C. The pellet was resuspended in ice-cold lysis buffer (50 mM Tris/HCl, pH 7.4, 250 mM mannitol, 50 mM NaF, 1 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride, 5 μg/ml soybean trypsin inhibitor, 1% (v/v) Triton X-100), incubated for 15 min and centrifuged at 14,000 × *g* for 3 min at 4 °C, and the protein content was determined using the Pierce BCA kit (Pierce, USA). Protein samples (100 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel) and transferred to a nitrocellulose membrane. Loading of equal amounts of protein and quality of blotting procedures were controlled by staining of the nitrocellulose membranes with ponceau red for 10 min. Afterwards, the membranes were destained in Tween-containing Tris-buffered saline (TTBS), followed by incubation in 5% nonfat milk for 2 h at room temperature and then overnight at 4 °C in the presence of monoclonal anti-GFAP antibody (1:2000, Sigma). The membrane was washed and afterwards exposed for 1 h to horseradish peroxidase-conjugated secondary antibody (1:5000; Promega), and immunoreactive protein was visualized using a chemiluminescence-based detection kit (ECL kit; Amersham). For semiquantification, the blots were scanned and integrated optical density (IOD) of the signals was determined by using Scion image analysis program (Scion, Frederick, MD, USA).

2.5. Permanent focal cerebral ischemia in mice

Male NMRI mice (Charles River, Germany) were kept under controlled light and environmental conditions (12 h light/dark circle, 23 ± 1 °C, 55 ± 5% relative humidity) and had free access to food (Altromin, Germany) and water. Permanent middle cerebral artery occlusion was performed in 12–17 animals per group (25–29 g) according to the method described by Welsh et al. (1987). Briefly, after the mice were anaesthetized intraperitoneally (i.p.) with tribromoethanol (600 mg/kg), a hole was drilled in the skull to expose the middle cerebral artery. The stem of the middle cerebral artery and both branches were permanently occluded by electrocoagulation. Body temperature was maintained at 37±1 °C with a heating lamp during the surgical procedure. After middle cerebral artery occlusion, mice were kept at an environmental temperature of 30 °C for 2 h. Clenbuterol (0.3 mg/kg)

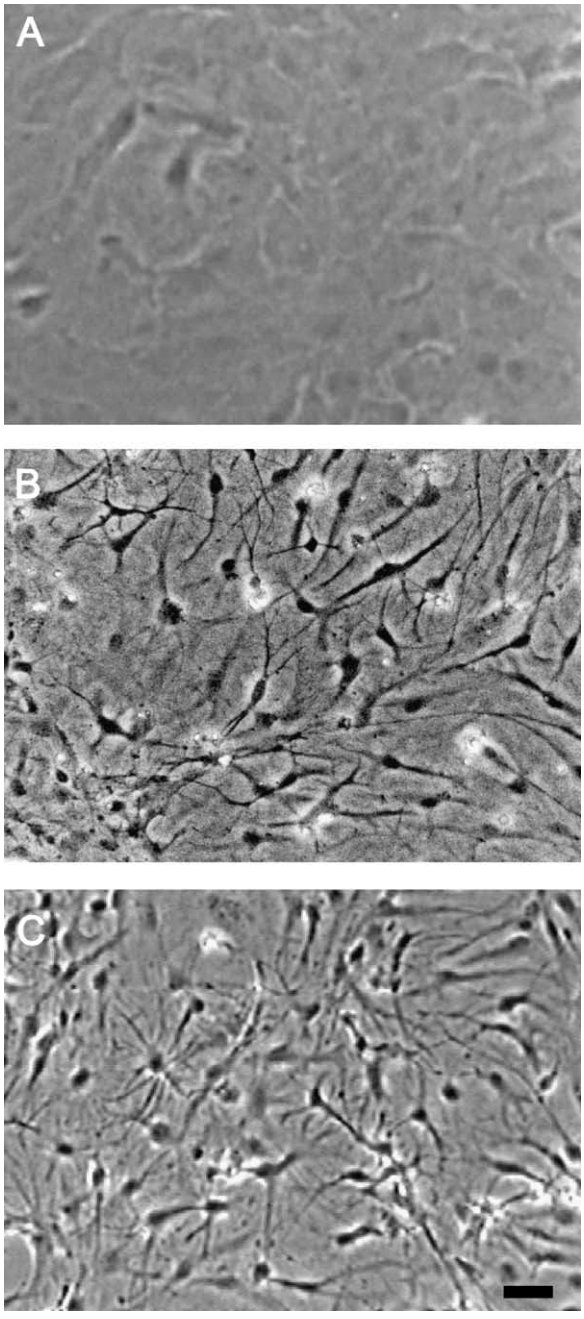


Fig. 2. Activation of cultured rat astrocytes by β_2-adrenoceptor stimulation. Clenbuterol (1–100 μM) or salmeterol (0.01–1 μM) were added to the medium of confluent astrocytes. Phase-contrast photomicrographs (10 × objective) of astrocytes before (A) and after 6 h of β_2-adrenoceptor stimulation by clenbuterol (1 μM, B) or salmeterol (0.01 μM, C) are shown. Note the profound morphological changes of the astrocytes after β_2-adrenoceptor stimulation, including generation of dendritic processes and phase contrast-dense cell bodies, typical for activated astrocytes. Scale bar 40 μM.

or salmeterol (0.1–1 mg/kg) were administered i.p. 5 h before middle cerebral artery occlusion. Propranolol (5 mg/kg), metoprolol or butoxamine (0.5 and 5 mg/kg) were administered i.p. 20 min before injection of clenbuterol. Control animals received vehicle only (0.9% NaCl). Two days after middle cerebral artery occlusion, the mice were anaesthetised and 0.5 ml of a 1.5% neutral red solution was injected i.p. After 30 min, the brains were removed and stored in PBS-buffered formalin (4%) for histological evaluation. The occlusion of the distal part of the middle cerebral artery as performed here predominantly blocks blood supply to the cortex, while other brain regions such as hippocampus and striatum remain sufficiently perfused. Thus, the ischemic infarction is restricted to the cortex and the adjacent parts of the corpus callosum beneath. As shown in previous reports the infarcted area on the brain surface highly correlates with the volume of the cortical infarct (Backhauß et al., 1992). The tissue on the brain surface unstained by neutral red was determined as infarcted area (in mm^2) by means of an image analysing system (Kontron, Germany) according to Backhauß et al. (1992).

2.6. Statistical analysis

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

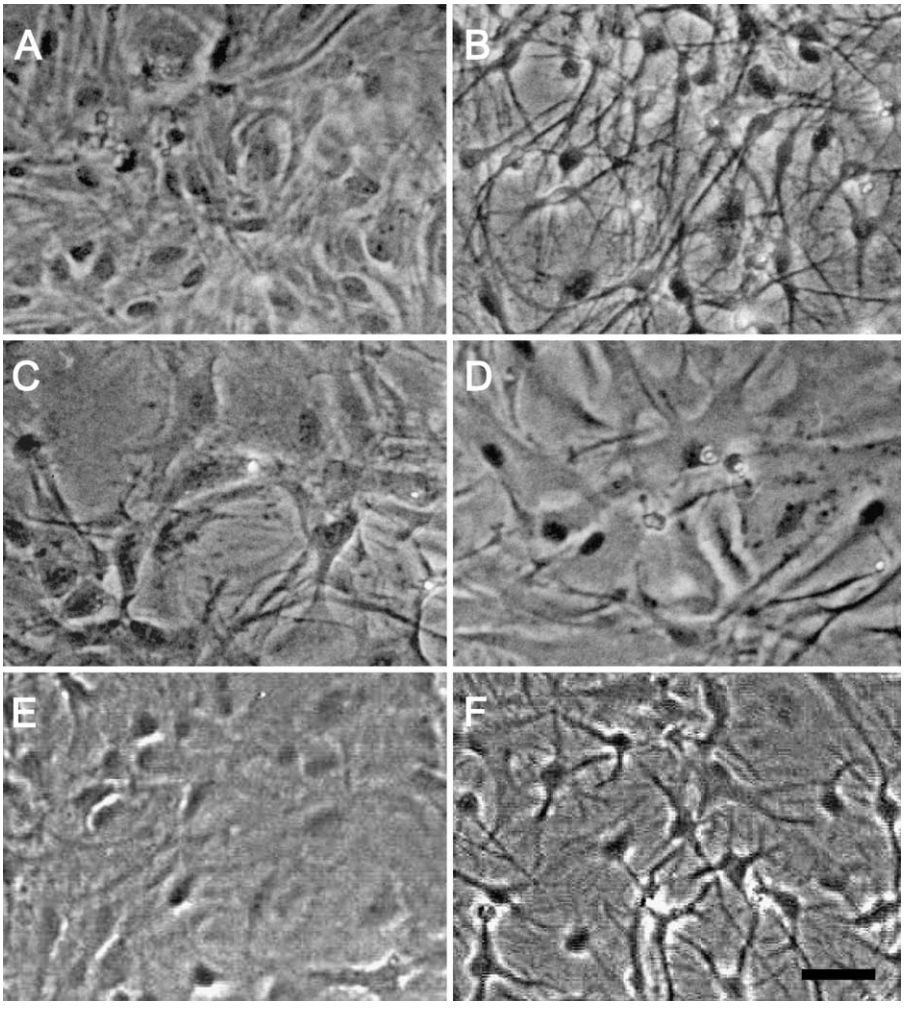
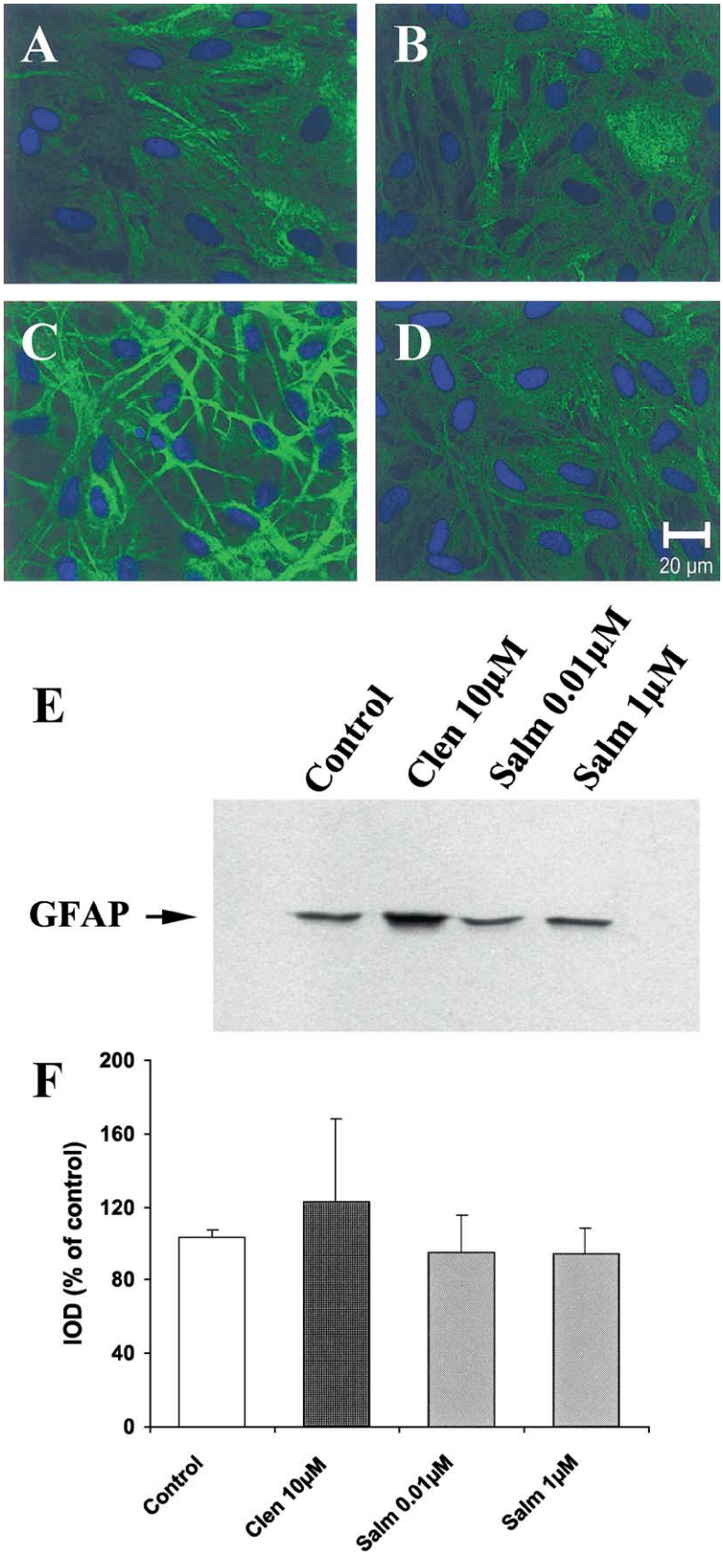


Fig. 3. Effects of the β_2-adrenoceptor antagonist butoxamine and β_1-adrenoceptor antagonist metoprolol on the activation of astrocytes by clenbuterol. Butoxamine (1 mM) or metoprolol (10 μM) were added to the culture medium of confluent astrocytes 30 min before clenbuterol (10 μM). Phase-contrast photomicrographs of astrocytes after 6 h of exposure to clenbuterol are shown. Note the generation of dendritic processes and shrinkage of cell bodies induced by β_2-adrenoceptor stimulation (B) as compared to controls (A). Neither butoxamine (C), nor metoprolol (E) alone affected the astrocytes' morphology. The activation of astrocytes by clenbuterol was blocked by pretreatment with the β_2-adrenoceptor-selective antagonist butoxamine (D). Note that co-treatment with the β_1-adrenoceptor-selective antagonist metoprolol did not affect the activation of astrocytes by clenbuterol (F). Scale bar 20 μM.


A
B
C
D
20 μm
E
Control
Clen 10μM
Salm 0.01μM
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GFAP
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40
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Control
Clen 10μM
Salm 0.01μM
Salm 1μM

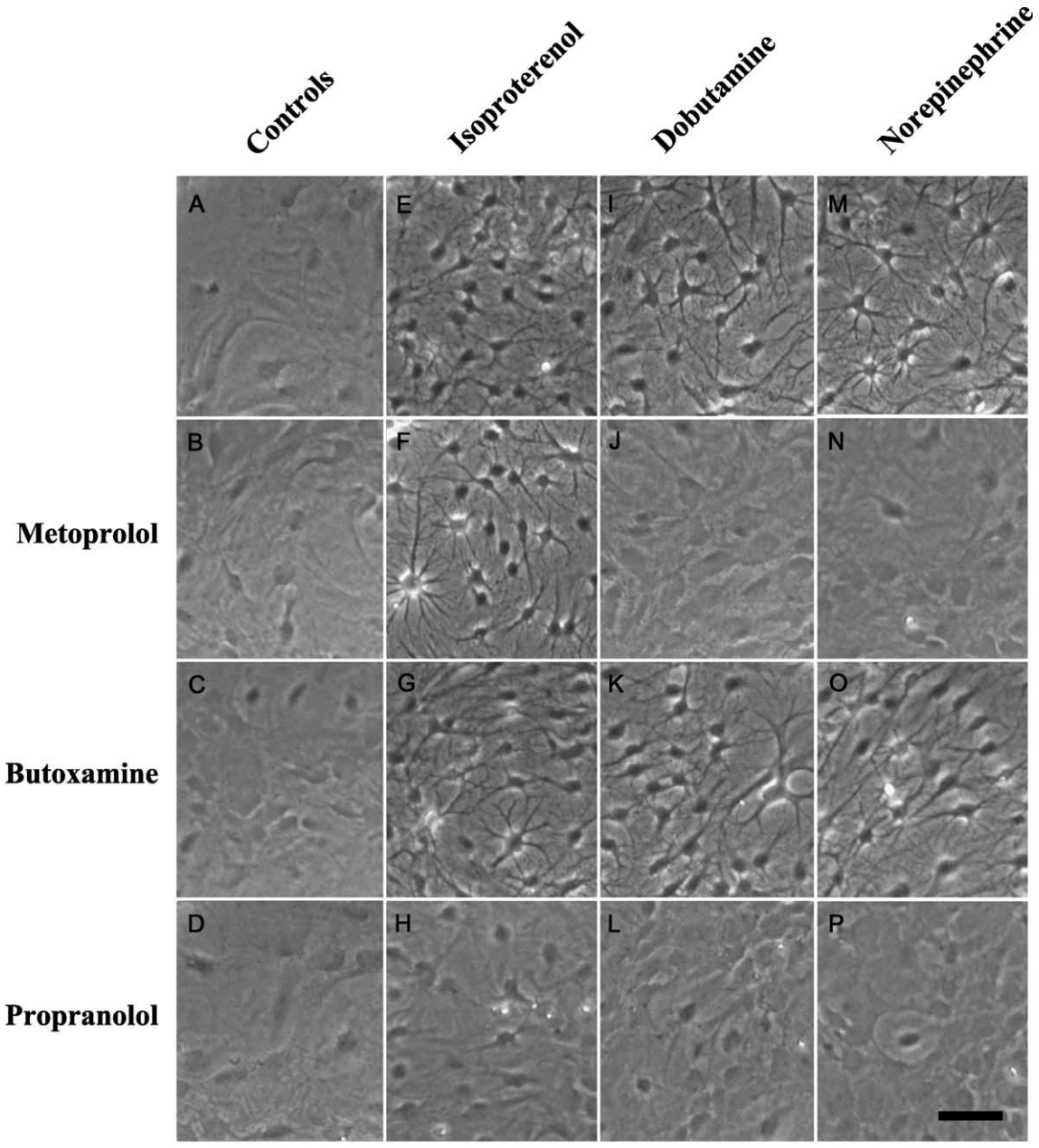

Fig. 5. Activation of cultured rat astrocytes by β_1-adrenoceptor stimulation. Isoproterenol (10 μM, E–H), dobutamine (10 μM, I–L) and norepinephrine (10 μM, M–P) were added to the medium of confluent astrocytes. Phase-contrast photomicrographs (10x objective) of astrocytes after 6 h of exposure to the β-adrenoceptor agonists are shown. Untreated Controls (A) contained flat, amoeboid shaped astrocytes. Note the profound morphological changes of the astrocytes after β-adrenoceptor stimulation, including generation of dendritic processes and shrinking of cell bodies, typical for activated astrocytes. Metoprolol (10 μM, B, F, J, N), butoxamine (1 mM, C, G, K, O) or propranolol (10 μM, D, H, L, P) were added to the culture medium of confluent astrocytes 30 min before exposure to isoproterenol, dobutamine and norepinephrine. Phase-contrast photomicrographs of astrocytes after 6 h of exposure to the β-adrenoceptor agonists are shown. Note that the effects of isoproterenol on astrocyte activation were blocked by the $\beta_{1/2}$-adrenoceptor antagonist propranolol but neither by the β_1-adrenoceptor antagonist metoprolol nor by the β_2-adrenoceptor antagonist butoxamine alone. Activation of astrocytes by dobutamine and norepinephrine were blocked by metoprolol and propranolol, but not by butoxamine. Scale bar 40 μM.

Fig. 4. Increase in GFAP immunoreactivity in astrocytes activated by β_2-adrenoceptor stimulation is due to changes in cell volume. Fluorescence laser scanning photomicrographs show GFAP immunoreactivity in astrocytes under control conditions (A) and 6 h after exposure to 1 mM butoxamine (B), 10 μM clenbuterol (C) or co-treatment with both (D). Note the pronounced increase in GFAP immunoreactivity associated with the morphological changes in clenbuterol-treated astrocytes (C). Activation of astrocytes and changes in GFAP immunoreactivity were abolished by pretreatment with the selective β_2-adrenoceptor antagonist butoxamine (D). Increased immunoreactivity of GFAP was detected because of the reduced cell volume in activated astrocytes and not due to increased protein synthesis as immunoblot analysis (E) revealed only moderate or no changes in GFAP protein levels in astrocytes 6 h after exposure to clenbuterol (10 μM) or salmeterol (0.01–1 μM), respectively. The relative integrated optical densities (IOD) of GFAP signals were quantified from three blots (F).

3. Results

3.1. Evidence for neuroprotection by β_2-adrenoceptor stimulation

In mixed hippocampal cultures clenbuterol protected neurons from glutamate-induced excitotoxic cell death at concentrations of 1–100 μM (Fig. 1A–D; Semkova et al., 1996). We further employed different β_1- or β_2-adrenoceptor antagonists to provide evidence that neuroprotection by clenbuterol was mediated via specific stimulation of β_2-adrenoceptors. The $\beta_{1/2}$-adrenoceptor antagonist propranolol (10 μM) as well as the selective β_2-adrenoceptor inhibitors butoxamine (10 μM) or ICI 118,551 (10 μM) abolished the protective effect of clenbuterol against glutamate excitotoxicity (Fig. 1A–C). Propranolol or ICI 118,551 alone had no effect on neuronal viability (Fig. 1A,C) while butoxamine treatment resulted in a slight damage of the cells (Fig. 1B). The specific β_1-adrenoceptor agonist metoprolol (10–100 μM) neither influenced the neuroprotective effect of clenbuterol (1–10 μM) against glutamate toxicity nor affected neuronal viability under control conditions (Fig. 1D). These results indicated that neuroprotection by clenbuterol was mediated via stimulation of β_2 but not β_1-adrenoceptors. To exclude substance-specific effects of clenbuterol, we next tested whether salmeterol (0.001–1 μm), another lipophilic β_2-adrenoceptor agonist, also provided neuroprotection against glutamate toxicity. Salmeterol prevented excitotoxic neuron death at concentration as low as 0.01 μM (Fig. 1E). At concentrations higher than 1 μM, salmeterol did not affect glutamate toxicity in hippocampal cultures, and at 10 μM a slight increase in neuron cell death was detected (data not shown).

3.2. Astrocyte activation induced by β_2-stimulation

In culture, confluent astrocytes appeared in a flat, amoeboid shape without dendrites (Fig. 2). When astrocytes were exposed to clenbuterol (1–100 μM) or salmeterol (0.01–10 μM) for 6 h they became activated and revealed profound changes in the morphology, such as small, phase contrast-dense cell bodies with several dendritic processes (Figs. 2 and 3). Clenbuterol-induced morphological changes in astrocytes were blocked by butoxamine (Figs. 3 and 4), but not by metoprolol (Fig. 3), indicating that activation of astrocytes was mediated by β_2-adrenoceptors. Fluorescence laser scanning microscopy revealed enhanced immunoreactivity of GFAP in astrocytes after β_2-adrenoceptor stimulation, suggesting increased GFAP expression in activated astrocytes (Fig. 4). Surprisingly, the corresponding increase in GFAP protein levels detected by western blot analysis in protein extracts of clenbuterol-treated astrocytes was only moderate and not detectable in extracts from salmeterol-treated glia after 6 h of treatment, suggesting that in fluorescence microscopy enhanced GFAP immunoreactivity at this timepoint appeared rather due to reduction in cell volume than enhanced protein synthesis (Fig. 4). However, it cannot be excluded that GFAP levels were enhanced at timepoints after 6 h of β_2-adrenoceptor stimulation, and in some experiments clenbuterol induced such an increase in GFAP as also shown in the immunoblot presented in Fig. 4. Our findings suggest that morphological changes induced by β_2-adrenoceptor agonists preceded increases of GFAP protein levels which are usually associated with astrocyte activation.

3.3. Astrocyte activation by β_1-adrenoceptor agonists

To further elucidate the specificity of astrocyte activation via stimulation of β-adrenoceptors, we exposed cultured astrocytes to the $\beta_{1/2}$-adrenoceptor agonist isoproterenol and to the more selective β_1-adrenoceptor agonists dobutamine and norepinephrine. All adrenoceptor agonists induced the morphological changes described above transforming the glial cells into activated astrocytes with pronounced dendrite formation (Fig. 5). The effect of isoproterenol was only blocked by the $\beta_{1/2}$-adrenoceptor antagonist propranolol but neither by metoprolol nor by butoxamine alone, suggesting the involvement of both β_1- and β_2-adrenoceptors in astrocyte activation (Fig. 5E–H). This result was confirmed by experiments using the more selective β_1-agonists dobutamine (Fig. 5I–L) and norepinephrine (Fig. 5M–P). Both adrenoceptor agonists induced pronounced morphological changes in astrocytes within 6 h after exposure. This effect was abolished by propranolol and metoprolol, respectively, but not by the β_2-adrenoceptor antagonist butoxamine (Fig. 5).

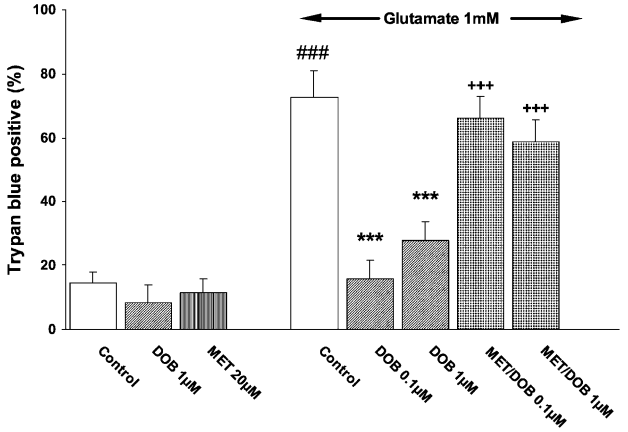

Fig. 6. Neuroprotection by dobutamine is mediated by β_1-adrenoceptor stimulation. After 10 days in vitro, hippocampal cells were incubated with the β_1-antagonist metoprolol starting 15 min prior to incubation with dobutamine (0.1–1 μM). Hippocampal cultures were exposed to the β_1-adrenoceptor agonist 4 h before and up to 18 h after glutamate exposure (30 min, 1 mM). Neuronal damage was determined by Trypan blue exclusion. Values are given as means±S.D. of $n=4$ experiments. ###$P<0.001$ compared to control, ***$P<0.001$ compared to glutamate treatment, and +++ $P<0.001$ compared to dobutamine/glutamate treatment (ANOVA, Scheffé's).

3.4. Neuroprotection by β_1-adrenoceptor agonists

On the basis of our results obtained with β_1-adrenoceptor agonists in cultured astrocytes, we next tested whether dobutamine could also protect neurons against glutamate toxicity in mixed hippocampal cultures. Dobutamine (0.1–10 μM) protected hippocampal neurons against the excitotoxic insult (Fig. 6). The protective effect of dobutamine was blocked by metoprolol suggesting that β_1-stimulation was sufficient to provide neuroprotection in mixed hippocampal cultures (Fig. 6).

3.5. Cerebroprotective effects by β_2-adrenoceptor stimulation

To investigate the specificity of β_2-adrenoceptor-mediated neuroprotection by clenbuterol in vivo, we tested the effect of the different β-adrenoceptor antagonists in a mouse model of focal cerebral ischemia. Clenbuterol (0.3 mg/kg, i.p.) applied in a single dose 5 h before middle cerebral artery occlusion significantly reduced the infarct area on the mouse brain surface (Fig. 7). Co-treatment with the $\beta_{1/2}$-antagonist propranolol (5 mg/kg) or the selective β_2-antagonist butoxamine (5 mg/kg) abolished the protective effect of clenbuterol. As expected, the β_1-adrenoceptor antagonist metoprolol (0.5–5 mg/kg) did not block protection against ischemic brain damage by clenbuterol, suggesting a cerebroprotective mechanism mediated by β_2-adrenoceptors. Surprisingly, mice treated with a combination of clenbuterol (0.3 mg/kg) with metoprolol (5 mg/kg) developed smaller infarcts than animals receiving clenbuterol (0.3 mg/kg) alone. Neither propranolol or butoxamine nor metoprolol affected the infarct size when administered alone (Fig. 7).

A significant reduction of the infarct area could also be achieved by salmeterol (0.3 mg/kg), indicating protection against ischemic brain damage by lipophilic β_2-adrenoceptor agonists as a general mechanism independent from the particular structure (Fig. 7).

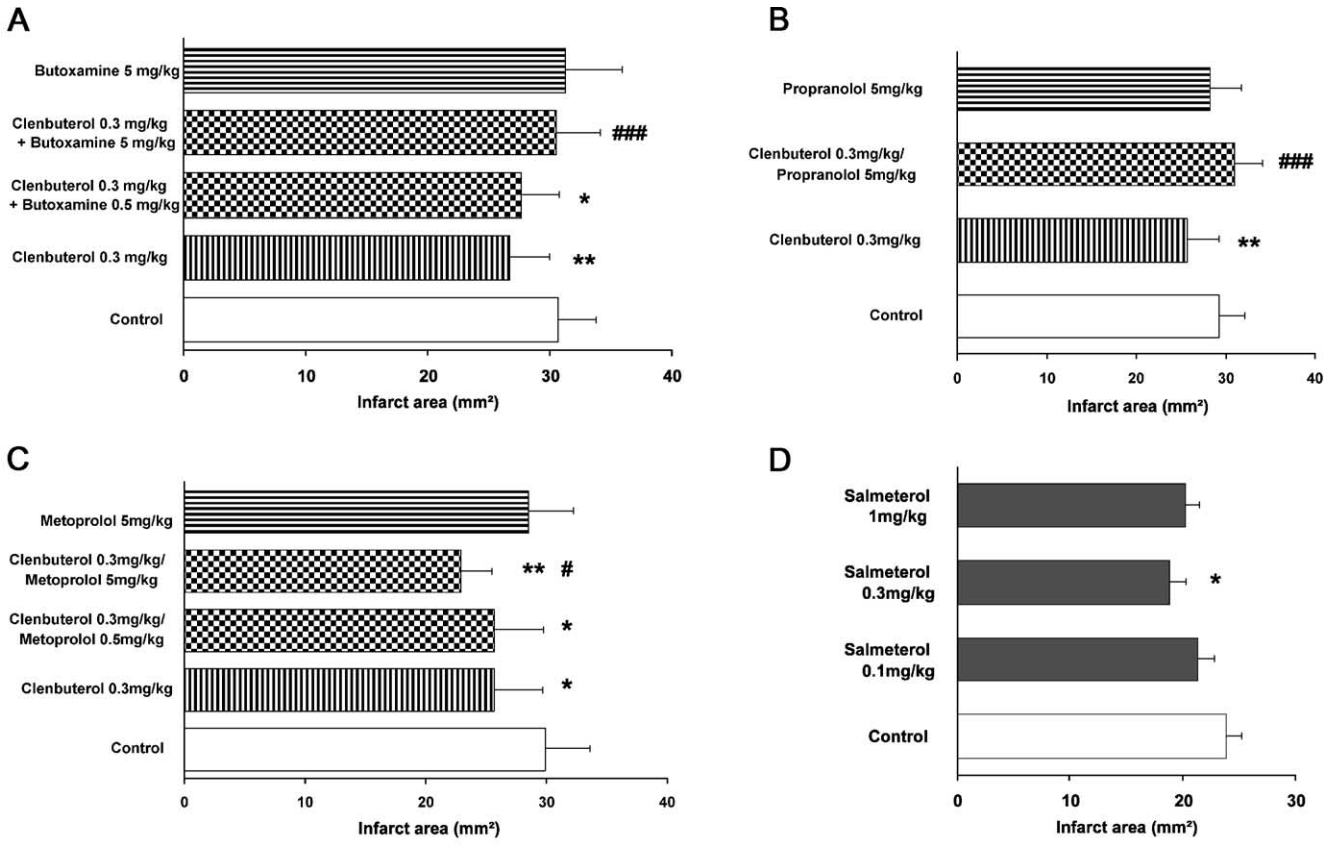

Fig. 7. (A–D) Effect of β-adrenoceptor agonist on cerebroprotection by clenbuterol in a mouse model of focal cerebral ischemia. NMRI mice were treated with butoxamine (A), propranolol (B) and metoprolol (C) 20 min before administration of 0.3 mg/kg clenbuterol. Five hours after clenbuterol treatment middle cerebral artery occlusion was performed. Two days after induction of ischemia the mice were perfused with a solution of neutral red to stain the brain tissue. After fixing the isolated brain, the unstained tissue region on the cortical surface was evaluated by measurement with an image processing system. Data are given as means±S.D of 15 animals. ***$P<0.001$, **$P<0.01$, *$P<0.05$ as compared to controls; ###$P<0.001$, ##$P<0.01$, #$P<0.05$ as compared to clenbuterol treatment (ANOVA, Duncan's). (D) Salmeterol reduces infarct area in the mouse model of permanent occlusion. NMRI mice were treated with salmeterol (0.1, 0.3 and 1 mg/kg, i.p.) 5 h before middle cerebral artery occlusion. Data are given as means±S.D. of 16 animals. Statistically different from control group: *$P<0.05$ (ANOVA, Duncan's).

4. Discussion

In the present study we found that activation of astrocytes and the associated neuroprotective effect in mixed hippocampal cultures by β-adrenoceptor agonists are not exclusively linked to β_2-adrenoceptor stimulation. We demonstrated that either β_1- or β_2-adrenoceptor stimulation alone is effective and sufficient to induce activation of astrocyte. Moreover, β_1-adrenoceptor agonists were also capable to protect neurons from glutamate toxicity in vitro, suggesting that the intracellular signaling pathways activated by β_1- and β_2-adrenoceptors exert similar effects on neurons and astrocytes.

In our study, activation of astrocytes and neuroprotection by clenbuterol was mediated exclusively by β_2-adrenoceptor stimulation. In hippocampal cells, the neuroprotective effect of clenbuterol against glutamate toxicity could be blocked by the $\beta_{1/2}$-antagonist propranolol, and by the β_2-adrenoceptor-selective antagonists butoxamine and ICI 118,551. In contrast, clenbuterol-induced neuroprotection in vitro was not reversed by the β_1-adrenoceptor antagonist metoprolol proposing a protective mechanism mediated by β_2-adrenoceptor stimulation. Salmeterol, the other lipophilic β_2-agonist used in the present study, also protected hippocampal neurons against the excitotoxic insult and reduced the infarct area after permanent middle cerebral artery occlusion in mice. Though both adrenoceptor agonists clenbuterol and salmeterol predominantly bind to β_2-adrenoceptors and are lipophilic, hence able to cross the blood–brain barrier, substance-specific differences have been reported. For clenbuterol anabolic effects have been documented that are independent of β_2-adrenoceptor stimulation (Maltin et al., 1989; Reeds et al., 1986). The mechanism of these anabolic properties or a possible contribution to neuroprotective effects of clenbuterol are unknown. On the other hand, salmeterol reveals different receptor binding kinetics showing a higher affinity to the β_2-adrenoceptor combined with a continuous stimulation of the active site of the receptor as compared to clenbuterol (Johnson et al., 1993; Isogaya et al., 1998). In line with differences in EC_{50} values neuroprotective concentrations of salmeterol in vitro (0.01–1 μM) were also lower than those of clenbuterol (1–100 μM). However, our data now demonstrate that β_2-adrenoceptor stimulation provides neuroprotective effects independent of additional anabolic properties or different receptor binding kinetics. As shown for clenbuterol in our previous studies (Culmsee et al., 1999a,b; Semkova et al., 1996, Zhu et al., 1998), salmeterol also revealed a U-shaped dose–response curve in vitro and in vivo. At high concentrations, the neuroprotective effect of salmeterol may be abolished by unspecific, receptor-independent mechanisms such as membrane interactions or by β-adrenoceptor-mediated pathways. For example, hyperglycemia and hypotension induced by systemic β_2-adrenoceptor stimulation have been documented to counteract the protective effect of clenbuterol at high doses in rodent models of cerebral ischemia (Culmsee et al., 1998, 1999b; Zhu et al., 1998). Hyperglycemia may exacerbate ischemic brain damage because of an enhanced decrease of pH values in the ischemic tissue due to anaerobic metabolism of glucose to lactate (Osborne et al., 1987; Nagai et al., 1993). An acute drop of blood pressure after stroke could further reduce the perfusion in the penumbra region, hence increase the ischemic area (Kelley, 1996; Lees and Dyker, 1996). In addition, arterial hypotension could cause tachycardia and enhanced release of renin, both leading to cardiovascular dysfunction. In the present study, a reduction of the infarct area was found for clenbuterol and salmeterol, suggesting that at 0.3 mg/kg the protective effect was dissociated from the counteracting unwanted effects of β_2-stimulation. Using the lipophilic β_2-specific adrenoceptor antagonist butoxamine we could further demonstrate the β_2-specificity of cerebroprotection by clenbuterol. Interestingly, the protection of brain tissue against ischemic damage was more pronounced in mice treated with a combination of metoprolol and clenbuterol as compared to mere clenbuterol treatment. Metoprolol alone did not influence the ischemic brain damage or neuronal survival in hippocampal cultures, showing that the β_1-antagonist by itself did not provide neuroprotection. The synergism of clenbuterol and metoprolol observed in vivo but not in neuron cultures indicated that metoprolol blocked the systemic side effects associated with β_2-stimulation, such as tachycardia or hyperglycemia.

Results from previous studies suggested an involvement of activated astrocytes in β_2-adrenoceptor-mediated neuroprotection (Culmsee et al., 1999a; Hayes et al., 1995). Glial cells are the main source for trophic factors in the brain supporting function and maintenance of neurons, and stimulation of astrocytes by β-adrenoceptor agonists increases synthesis and release of growth factors including NGF, TGF-β_1 and bFGF in vitro and in vivo (Culmsee et al., 1999a,b; Follesa and Mocchetti, 1993; Zhu et al., 1998). Furthermore, neuroprotection against ischemic brain damage by clenbuterol is associated with increased growth factor synthesis and enhanced astrocyte activation (Culmsee et al., 1999a,b; Zhu et al., 1998). Increased growth factor synthesis of astrocytes in response to brain injury has been reported (Oderfeld-Nowak and Bacia, 1994; Schwartz et al., 1993) suggesting that activation of astrocytes is an endogenous mechanism of neuroprotection that could be further enhanced by so-called astrocyte-kinetic drugs (Biagini et al., 1994). For example, Biagini et al. (1994) demonstrated enhanced GFAP and bFGF immunoreactivity in astrocytes after brain injury in rats pretreated with the monoamine oxidase B inhibitor selegiline. Here we demonstrated in vitro that activation of astrocytes after exposure to clenbuterol and salmeterol was mediated by stimulation of β_2-adrenoceptors. After exposure to clenbuterol or salmeterol the confluent astrocytes changed profoundly from flat, amoeboid astrocytes to phase contrast-dense cells with a shrunken cell soma and several dendritic processes consistent with the morphology of activated astrocytes. Astrocytes

express high levels of β_2-adrenoceptors and β_2-adrenoceptor expression is further increased when astrocytes become activated after cerebral insults, suggesting a major role of these receptors in astrocyte activation (Rainbow et al., 1984; Mantyh et al., 1995).

However, the data in the present study suggest that astrocyte activation and neuroprotection can also be achieved by dobutamine and norepinephrine which preferentially stimulate β_1-adrenoceptors. Indeed, the effect of both β_1-adrenoceptor agonists on astrocytes was abolished by the β_1-antagonist metoprolol but not by the β_2-antagonist butoxamine, demonstrating the involvement of β_1-adrenoceptor stimulation in this effect. Moreover, the transformation of astrocytes into activated stellate cells by the $\beta_{1/2}$-adrenoceptor agonist isoproterenol was neither affected by metoprolol nor by butoxamine alone and could only be blocked by the $\beta_{1/2}$-antagonist propranolol. This result confirmed the hypothesis that the stimulation of either subtype of β-adrenoceptors was sufficient to induce activation of astrocytes. Similar results were obtained in mixed hippocampal cultures where β_1-adrenoceptor stimulation by dobutamine provided neuroprotection against glutamate toxicity. However, the use of β_1-adrenoceptor agonists in vivo, particularly after cerebral ischemia, is highly questionable because of expected cardiovascular effects including tachycardia and a severe increase in blood pressure.

Our results suggest that in cultured cells the stimulation of both β_1- and β_2-adrenoceptors exert similar effects that might be linked to similar signal transduction pathways, such as the G-protein coupled activation of adenylate cyclase. For both receptor subtypes, such a link to the adenylate cyclase leading to increased levels of cAMP in the cell has been described. As we reported before, enhanced cAMP-signaling stimulated by forskolin induced an activation of astrocytes (Semkova and Krieglstein, 1999) that was similar to the activation demonstrated here after the exposure to β-adrenoceptor agonists. Furthermore, growth factor synthesis in astrocytes depends on cAMP-dependent signalling pathways downstream β-adrenoceptor stimulation, such as the CCAAT/enhancer binding proteins (Colangelo et al., 1998; Riva et al., 1996; Rosenberg and Li, 1995). Interestingly, NGF also activates CCAAT/enhancer binding proteins and the activation of astrocytes could be further enhanced by NGF in an autocrine activation loop (Hutton and Perez-Polo, 1995; Sterneck and Johnson, 1998; Yokoyama et al., 1993). On the other hand, we cannot entirely exclude from the present study that neuroprotection by β-adrenoceptor agonists could be in part achieved by a direct stimulation of neuronal β-adrenoceptors. Indeed, we observed similar neuroprotective effect of dobutamine in embryonic hippocampal cultures which contained less than 5% astrocytes (Culmsee and Krieglstein, unpublished results). However, in the mixed hippocampal cultures used here and more so in brain tissue astrocytes represent the dominant cell population expressing β-adrenoceptors. Thus, a substantial contribution of activated astrocytes to the neuroprotective effect of β-adrenoceptor agonists in vitro and in vivo is concluded.

In summary, our data show that β-adrenoceptor stimulation provides activation of astrocytes associated with neuroprotection. The stimulation of either β_1- or β_2-adrenoceptors was sufficient to exert these effects in vitro. We conclude that β-adrenoceptor agonists protect neurons due to enhanced growth factor synthesis in activated astrocytes in vitro. In vivo, the stimulation of β_2-adrenoceptors is most likely the predominant mechanism underlying the neuroprotective effect of β-adrenoceptor agonists as β_2-adrenoceptors are up-regulated after brain injury, particularly in activated astrocytes. Co-treatment with β_2-adrenoceptor agonists and β_1-antagonists could be a useful strategy to enhance the cerebroprotective properties of β_2-adrenoceptor agonists in vivo by reducing counteractive systemic effects.

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