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Clenbuterol induces growth factor mRNA, activates astrocytes, and protects rat brain tissue against ischemic damage

Carsten Culmsee [a], Ralf K. Stumm [b], Martin K.-H. Schäfer [b], Eberhard Weihe [b], Josef Krieglstein [a,*]

[a] *Institut für Pharmakologie und Toxikologie, Philipps-Universität Marburg, Fachbereich Pharmazie, Ketzerbach 63, Marburg, D-35037, Germany*
[b] *Institut für Anatomie und Zellbiologie, Philipps-Universität Marburg, Robert-Koch-Straße 6, Marburg 35032, Germany*

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Abstract

The induction of growth factor synthesis in brain tissue by β_2-adrenoceptor agonists, such as clenbuterol, is a promising approach to protect brain tissue from ischemic damage. Clenbuterol (0.01–0.5 mg/kg) reduced the cortical infarct volume in Long–Evans rats as measured 7 days after permanent occlusion of the middle cerebral artery. Dosages of clenbuterol higher than 1 mg/kg showed no cerebroprotective effect due to a decrease in blood pressure and an increase in plasma glucose level. The increase in the mRNA level of nerve growth factor (NGF), basic fibroblast growth factor (basic FGF), and transforming growth factor-β_1 (TGF-β_1) mRNA in cortical and hippocampal tissue occurred earlier after middle cerebral artery occlusion and was more pronounced in animals treated with clenbuterol than in controls. In addition, glial fibrillary acidic protein (GFAP) mRNA expression was enhanced in astrocytes 6 h after ischemia in clenbuterol-treated animals. The results suggest that growth factor synthesis is enhanced in activated astrocytes and that this could be the mechanism of clenbuterol-induced cerebroprotection after ischemia.

Keywords: NGF (nerve growth factor); FGF (fibroblast growth factor, basic); TGF-β_1 (transforming growth factor-β_1); Cerebral ischemia, focal; Clenbuterol; β_2-Adrenoceptor agonist; Astrocyte; GFAP (glial fibrillary acidic protein)

1. Introduction

Neurotrophic factors are widely distributed in the peripheral nervous system and central nervous system (CNS) where they show specific patterns of distribution during development and through adulthood. Especially the members of the neurotrophin family — nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin 4/5 (NT-4/5) — protected neurons from cell death in animal models of neuropathy, motoneural disorders and cerebral ischemia (Lewin and Barde, 1996; Lindsay, 1996; Yuen and Mobley, 1996; Gao et al., 1997; Hefti, 1997). Similar neuroprotective effects have been demonstrated in numerous studies in vitro and in models of cerebral ischemia in vivo for basic fibroblast growth factor (basic FGF) (Walicke, 1988; Freese et al., 1992; Finklestein et al., 1993; Mattson and Acheff, 1994). Like the neurotrophic growth factors, NGF and basic FGF, the pleiotropic cytokine transforming growth factor β_1 (TGF-β_1) was found to be upregulated in brain tissue after ischemia (Lindvall et al., 1992; Takeda et al., 1993; Wießner et al., 1993; Wang et al., 1995; Iwata et al., 1997). It has been suggested that the increased expression of TGF-β_1 after ischemia is part of an endogenous neuroprotective mechanism. Meanwhile, this hypothesis was supported by many reports demonstrating the neuroprotective potency of TGF-β_1 in vitro and in vivo (reviewed in Krieglstein and Krieglstein, 1998). These findings led to the view that the aforementioned factors were promising candidates for the treatment of acute and chronic neurodegenerative diseases.

However, neurotrophic growth factors are proteins that do not easily cross the blood–brain barrier. The induction of growth factors by systemically administered lipophilic drugs, such as the β_2-adrenoceptor agonist clenbuterol, would be an elegant way to overcome these problems of application (Carswell, 1993; Culmsee et al., 1998a,b). In

* Corresponding author. Tel.: +49-6421-281311; fax: +49-6421-288918; E-mail: krieglst@mailer.uni-marburg.de

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our laboratory, clenbuterol has been demonstrated to protect hippocampal neurons from excitotoxic damage in mixed primary cultures of hippocampal neurons (Semkova et al., 1996b). The neuroprotection was blocked by NGF antibodies, suggesting a protective mechanism of clenbuterol mediated by an increased release of NGF (Semkova et al., 1996b). Propranolol, a β-adrenoceptor antagonist, blocked the induction of NGF and the neuroprotective effect of clenbuterol in hippocampal cells, suggesting the stimulation of β_2-receptors as a general pharmacological principle for the induction of growth factors in the brain (Semkova et al., 1996b). In vivo, we could show that clenbuterol protected brain tissue from ischemic damage in models of permanent focal ischemia in mice and transient forebrain ischemia in rats (Semkova et al., 1996b; Zhu et al., 1998). However, it remained to be clarified whether the induction of NGF or basic FGF (Follesa and Mocchetti, 1993; Hayes et al., 1995; Semkova et al., 1996b) was involved in the protective effect of clenbuterol against ischemic brain damage. In the present study, we attempted to find out whether NGF, basic FGF and TGF-β_1 synthesis could be upregulated in rat brain tissue after permanent focal cerebral ischemia by protective doses of clenbuterol. In addition, we investigated the stimulation of astrocytes by clenbuterol after focal ischemia, because activated astrocytes were suggested to be a major source of neuroprotective factors after brain damage (Lu et al., 1991; Biagini et al., 1994; Hodges-Savola et al., 1996).

2. Materials and methods

2.1. Animals

Male Long–Evans rats (Møllegaard, Denmark; 250–280 g) were used for ischemia experiments. The animals were maintained under controlled light and environmental conditions (12:12 h dark/light cycle, 23° ± 1°C, 55% relative humidity) and had free access to food (Altromin, Lage, Germany) and water.

2.2. Permanent focal cerebral ischemia in rats

Permanent middle cerebral artery occlusion was performed in male Long–Evans rats (Møllegaard, Denmark) according to the method described by Tamura et al. (1981) with modifications as previously described by Semkova et al. (1996c). Briefly, the animals were anesthetized with halothane (1.5% in a mixture of O_2/N_2O 30:70). An incision was made through the left temporalis muscle perpendicular to a line between the external auditory canal and lateral canthus of the left eye. Under direct visualization with the surgical microscope, a burr hole was made with a handheld drill to expose the left middle cerebral artery. After rejecting the dura, the left middle cerebral artery was occluded by microbipolar electrocoagulation. After occlusion, the incisions in the left temporal muscle and the skin were closed by the adhesive histoacryl (Braun-Dexon, Germany) to guarantee the function of the temporal muscle for the uptake of food after surgery. This modified model of middle cerebral artery occlusion produces exclusively cortical infarction. During surgical procedure the body temperature was maintained at 37° ± 0.5°C by means of a heating pad. To prevent a decrease of body temperature, the animals were kept at an environmental temperature of 30°C up to 2 h after middle cerebral artery occlusion. Mean arterial blood pressure and plasma glucose concentration as well as arterial pH, pCO_2 and pO_2 were monitored (Corning 178, Corning, Germany) 3–4 h after clenbuterol administration.

Seven days after middle cerebral artery occlusion, the rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and decapitated. Brains were removed and frozen in 2-methylbutane at −20° to −30°C (Fluka, Switzerland). For histological evaluation, transversal sections of 20 μm thickness were taken every 0.5 mm using a cryomicrotome (Frigocut, Reichert-Jung, Germany) and stained with 0.5% Cresyl violet to differentiate between intact and damaged brain tissue. Morphometric determination of the manually outlined surface (mm^2) was performed using a computer-based image analysis system (Kontron, Germany). The infarct volume ($V_{infarct}$; mm^3) was calculated from the infarct area (F) of each section and the distance between succeeding sections (Semkova et al., 1996c; Culmsee et al., 1998a,b) using the following formulae:

$$F = k\left(F_{contralateral} - F_{ipsilateral}\right),$$

$$V_{infarct} = \Sigma\left[0.5\,mm \times \frac{1}{3}\left[F_n + F_{n+1} + \left(F_n + F_{n+1}\right)^{1/2}\right]\right],$$

where F: cortical infarct area (mm^2); k: area correction factor; $V_{infarct}$: infarct volume (mm^3); and 0.5 mm: distance between slices.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from brain tissue using the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration was calculated by absorbance at 260 nm and purity was demonstrated by A_{260}/A_{280} ratios between 1.7 and 2.0 (Sambrook et al., 1989). Five micrograms of total RNA were used in an RT reaction followed by PCR (RT-PCR) with aliquots of 500 ng cDNA. RT was performed at a volume of 50 μl. RT solution contained: 1 × PCR buffer, 2.2 mmol/l $MgCl_2$, dNTP mix (dATP, dCTP, dGTP and dTTP, 0.2 mmol/l each), 4 mmol/l dithothreitol, 20 U RNase-inhibitor, oligo-(dT)-primer and RNase-free water. The probes were heated to 65°C for 5 min and chilled on ice. Fifty units of MMLV reverse transcriptase (Gibco Life Technologies, Germany) were added and RT was performed at 37°C for 60 min followed by a 5-min enzyme

denaturation at 95°C. For subsequent PCR, 10 μl cDNA solution was used.

PCR was performed at a volume of 50 μl. PCR solution contained: 1× PCR-buffer, 2.0 mmol/l $MgCl_2$, dNTP mix (dATP, dCTP, dGTP and dTTP, 0.2 mmol/l each), 0.3 μM of each primer (sense and antisense) and 1 U of ThermoprimePlus DNA polymerase (Advanced Biotechnologies, Hamburg, Germany). RT-PCR was carried out in a thermocycler (Omni Gene, Hybaid, Teddington, UK). PCR was performed under conditions optimized for the individual pairs of primers. These conditions were for β-actin (26 cycles): initial template denaturation at 95°C for 30 s; annealing with β-actin primers at 55°C for 60 s; primer extension at 72°C for 120 s; for NGF (28 cycles): initial template denaturation at 95°C for 30 s; annealing with NGF primers at 58°C for 60 s; primer extension at 72°C for 120 s; for basic FGF (30 cycles): initial template denaturation at 95°C for 30 s; annealing with basic FGF primers at 55°C for 60 s; primer extension at 72°C for 120 s; for TGF-β_1 (28 cycles): initial template denaturation at 95°C for 30 s; annealing with TGF-β_1 primers at 60°C for 60 s; primer extension at 72°C for 120 s. In any protocol, the last cycle was followed by a final extension step at 72°C for 5 min. In all experiments, RT-PCR of β-actin was carried out concurrently to control sample loading. Primers for β-actin were as follows with a predicted product size of 380 bp: sense: 5′-atttggcaccacactttctaca-3′; antisense: 5′-tcacgcacgatttccctctcag-3′ (Choi-Lundberg and Bohn, 1995). The oligonucleotide primers for NGF were designed according to the published sequence with a predicted product size of 391 bp (sense: 5′-ctggactaaacttcagcattc-3′; antisense: 5′-tgttgttaatgttcacctcgc-3′). Primers for basic FGF were synthesized as reported by Morrison (1991) — sense 5′-gccttcccgcccggccacttcaagg-3′, antisense 5′-gcacacactcctttgatagacacaa-3′ — and generated an amplification product of 179 bp. The primers used to amplify the TGF-β_1 mRNA were 5′-tggaccgcaacaacgccatctatgagaaaacc-3′ and 5′-tggagctgaagcaatagttggtatccagggct-3′.

Table 1
Clenbuterol reduces dose-dependently the infarct volume in a rat model of permanent focal cerebral ischemia
Clenbuterol was administered intraperitoneally to Long–Evans rats 3 h before permanent occlusion of the middle cerebral artery. Seven days after the surgical procedure, infarct volumes were calculated. Values from two series of experiments are given as means ± S.D. of 9–11 animals.

Clenbuterol (mg/kg, i.p.)	*n*	Infarct volume (mm^3)	*n*	Infarct volume (mm^3)
Control	11	128.8 ± 13.8	10	116.8 ± 14
0.001	10	118.6 ± 21.7	–	
0.01	10	105.6 ± 13.9[b]	–	
0.1	10	75.3 ± 17.3[c,d]	9	90.6 ± 22.3[b]
0.5	–		9	98.2 ± 22.1[a]
1	–		9	114.7 ± 18.1

Different from control: [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$, and different from animals treated with 0.01 mg/kg: [d]$P < 0.01$ (ANOVA followed by Duncan's test).

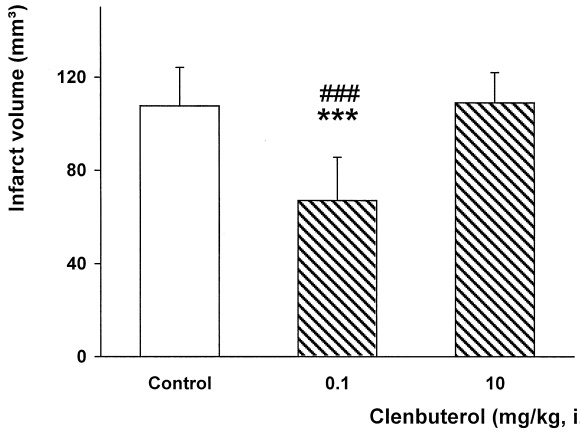


Fig. 1. Clenbuterol reduces the infarct volume in a rat model of permanent focal cerebral ischemia. Clenbuterol was administered intraperitoneally to Long–Evans rats 3 h before permanent occlusion of the middle cerebral artery. Seven days after the surgical procedure, brains were removed and coronal slices were taken every 0.5 mm. The sections were stained with 0.5% Cresyl violet. The infarct volume was calculated from the infarct area of each section and the distance between succeeding slices. Clenbuterol (0.1 mg/kg) reduces the infarct volume when administered 3 h before induction of focal cerebral ischemia. Dosages of clenbuterol higher than 0.5 mg/kg fail to protect rat brain tissue from ischemic damage. Values are given as means + S.D. of 10–11 animals. Different from control ($^{***}P < 0.001$) and different from animals treated with 10 mg/kg ($^{\#\#\#}P < 0.001$; ANOVA followed by Duncan's test).

To rule out the amplification of genomic DNA, all RT-PCR experiments were routinely controlled by conducting PCR without RT reaction. In addition, a negative control was performed using water instead of RNA for the RT-PCR. PCR products were analyzed using 2% agarose gels and visualized using ethidium bromide staining and UV transillumination. The PCR products were identified at their predicted size by using a 100-bp DNA-ladder (Promega, Germany) as a molecular weight marker.

2.4. In situ hybridization

2.4.1. Probe synthesis

A pGem-T vector (Promega, Germany) containing a part of rat NGF cDNA (390 bp) was linearized in order to generate riboprobes in sense (T7 polymerase-catalyzed

Table 2
Effects of clenbuterol on physiological parameters
Clenbuterol was administered 3 h before permanent middle cerebral artery occlusion in male Long–Evans rats. Mean arterial blood pressure (MABP) and plasma glucose levels were monitored for 1 h during surgical procedure. Values are given as means ± S.D. of four animals.

Clenbuterol (mg/kg, i.p.)	*n*	MABP (mm Hg)	Plasma glucose (mg/dl)
Control	4	89 ± 7.6	102.3 ± 9.5
0.1	4	81.8 ± 6.7	106.5 ± 26.9
10	4	68 ± 2.8[b,c]	129.5 ± 18.9[a]

[a]$P < 0.05$ and [b]$P < 0.01$ compared to controls; [c]$P < 0.01$ compared to 0.1 mg/kg clenbuterol (ANOVA followed by Duncan's test).

transcription) and antisense (SP6 polymerase-catalyzed transcription) orientation, respectively. Restriction endonucleases and RNA polymerases were obtained from Boehringer Mannheim, Germany. In vitro transcription using ^{35}S-UTP and ^{35}S-CTP as radioactive label was performed as described by Melton et al. (1984). To increase tissue penetration of probes, the generated single-stranded riboprobes were subjected to limited alkaline hydrolysis according to Angerer et al. (1987).

2.4.2. In situ hybridization

Brains were rapidly removed and immediately frozen in isopentane cooled at −30° to −40°C on dry ice. Frozen serial sections of the brain were cut at 20 μm thickness on a cryostat (Reichardt Jung, Germany), mounted on gelatine-coated slides with their ventral side facing the glass, and stored at −80°C. Frozen sections were thawed at room temperature for 15 min and processed for in situ hybridization as described previously (Schäfer et al., 1993, 1994). Briefly, the tissues were fixed on slides in 4% phosphate-buffered formaldehyde for 60 min at room temperature and then washed three times in 50 mmol/l phosphate-buffered saline (PBS), pH 7.4, for 10 min each. Slides were briefly rinsed in distilled water, transferred to 0.1 M triethanolamine, pH 8.0, and incubated in the same solution containing 0.25% v/v acetic anhydride for 10 min under rapid stirring. Sections were quickly rinsed in 2× sodium chloride/sodium citrate buffer (SSC), dehydrated in 50 and 70% ethanol and air-dried. Hybridization mix (20 μl) containing 100,000 dpm/μl of ^{35}S-labeled RNA probes in hybridization buffer (3× SSC, 50 mmol/l $NaPO_4$, 10 mmol/l dithiothreitol, 1× Denhardt's solution, 0.25 mg/ml yeast tRNA, 10% dextran sulfate and 50% formamide) was applied to each section. Sections were coverslipped and hybridized in a humidified chamber at 60°C for 14 h. Afterwards, the coverslips were removed in 2× SSC. Sections were treated with 20 μg/ml RNase A and 1 U/ml RNase T1 in RNase buffer (10 mmol/l

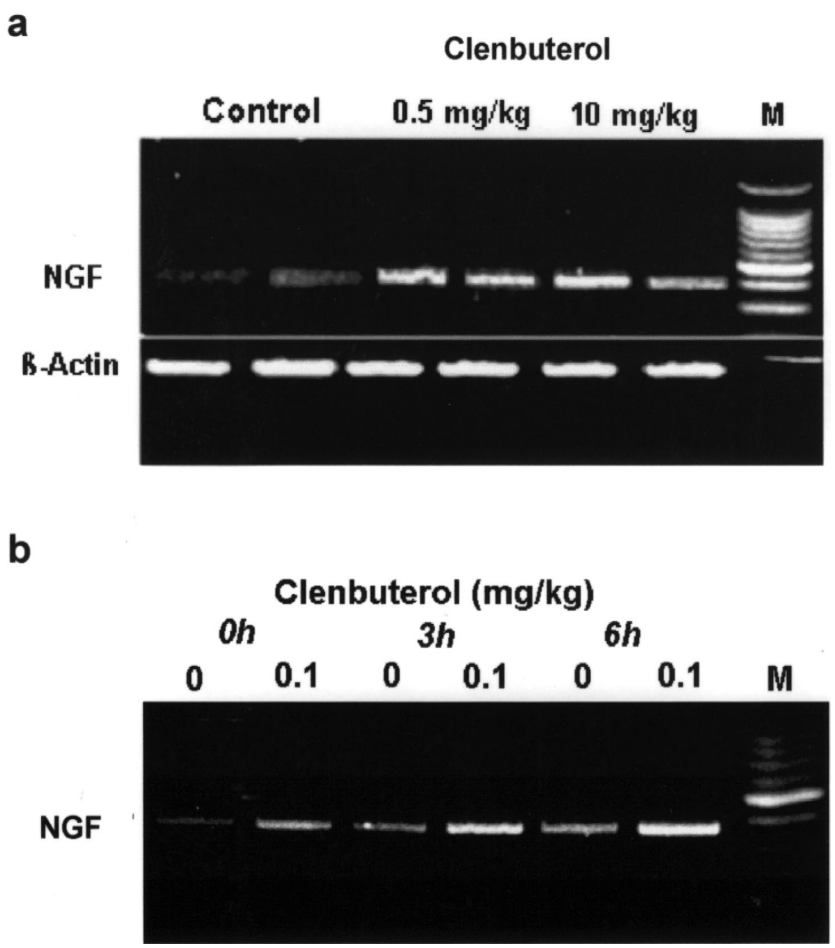


Fig. 2. (a) NGF mRNA induction in the rat cortex by clenbuterol. Rat brains were removed 6 h after intraperitoneal injection of clenbuterol (0.5 and 10 mg/kg). Controls received vehicle (0.9% NaCl) only. Total RNA was extracted from the tissue and reversely transcribed to cDNA. Fractions of this cDNA were separately amplified by PCR with specific primers for NGF and β-actin. The PCR products of two animals per group are presented. (b) Time course of NGF mRNA induction by clenbuterol after focal cerebral ischemia in the rat cortex. Three hours after clenbuterol administration (0.1 mg/kg), the left middle cerebral artery was occluded in male Long–Evans rats. Controls received vehicle only. At the time point of middle cerebral artery occlusion (0 h), and 3–6 h after induction of focal cerebral ischemia, brains were removed for RNA extraction. NGF mRNA was amplified by RT-PCR. The NGF-PCR signals at various time points after ischemia shown here are representative of three animals per group.

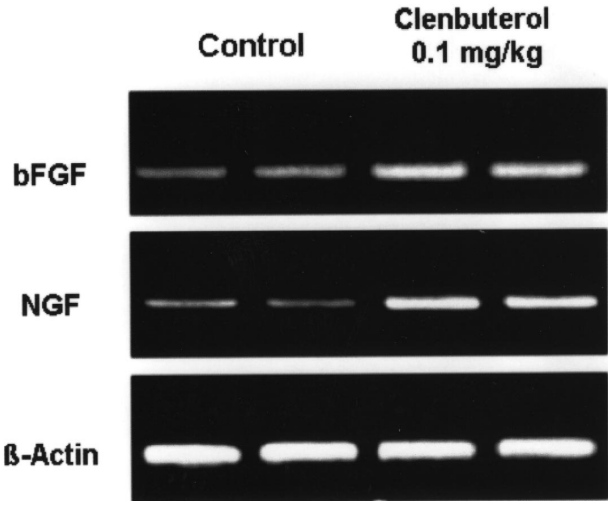


Fig. 3. Induction of basic FGF- and NGF mRNA in the hippocampus by clenbuterol 3 h after focal cerebral ischemia. Clenbuterol (0.1 mg/kg, $n = 3$) was administrated intraperitoneally 3 h before permanent occlusion of the middle cerebral artery in male Long–Evans rats. Control animals ($n = 3$) received vehicles only. Three hours after induction of ischemia, brains were removed for RNA extraction. NGF and basic FGF mRNA were amplified by RT-PCR. From the same cDNA, a β-actin PCR was performed as a control experiment. The representative PCR signals of two animals per group are shown.

Tris, pH 8.0, 0.5 M NaCl, 1 mmol/l EDTA) for 60 min at 37°C. Successive washes were performed at room temperature in 2, 1, 0.5, and 0.2 × SSC for 10 min each and in 0.2 × SSC at 60°C for 60 min. The tissue was then dehydrated and exposed to Amersham β-Max X-ray film for 24–96 h. For quantification analysis, hybridization signals were recorded by means of a CCD-camera and the integrated optical density (IOD) of defined brain areas was evaluated by using the NIH image analyzer. IOD signals were always normalized to the level of the sense probe's unspecific signal.

2.5. Experimental groups

To investigate the cerebroprotective effects of clenbuterol against ischemic brain damage, three series of experiments were performed with different doses of clenbuterol (0.001–10 mg/kg). In any experiment, clenbuterol was administered intraperitoneally 3 h before induction of permanent focal cerebral ischemia. Control animals received the vehicle (0.9% NaCl) only. In the first experiment vehicle, 0.1, 0.5 and 1 mg/kg clenbuterol were administered in 10 animals per group. One animal died in each of the groups treated with 0.1 and 1 mg/kg clenbuterol. In the second experiment vehicle, 0.1 and 10 mg/kg clenbuterol were tested in 10–11 animals per group. In the group treated with 10 mg/kg clenbuterol, one animal died immediately after the surgical procedure. To determine the no-effect level of clenbuterol, we tested 0.001–0.1 mg/kg of the drug in a third series of ischemia experiments.

The time point of middle cerebral artery occlusion was defined as $t_i = 0$ h. Three hours after administration of 0.1 mg/kg clenbuterol (0 h) as well as 3, 6 and 9 h after ischemia, the brains from three animals per group were removed, dissected, immediately frozen in liquid nitrogen and stored at −70°C. From this tissue, RNA was extracted for RT-PCR measurements. Additional three animals per group were treated with 0.5 and 10 mg/kg clenbuterol and the brains from these were removed 3 h after middle cerebral artery occlusion or sham operation to investigate the effect of clenbuterol at these doses.

For the in situ hybridization study, 10 animals per group were treated with clenbuterol (0.1 mg/kg) or vehicle, respectively. Six hours after focal cerebral ischemia, the brains were removed, immediately frozen in 2-methylbutane at −30° to −40°C and then stored at −80°C until coronal slices of 20 μm were prepared.

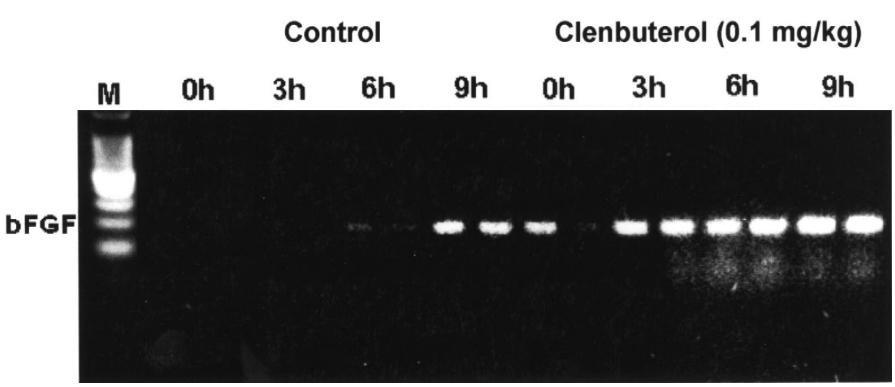


Fig. 4. Induction of basic FGF mRNA by clenbuterol in the rat cortex after focal cerebral ischemia. Three hours after clenbuterol administration (0.1 mg/kg, $n = 3$), the left middle cerebral artery was occluded in male Long–Evans rats. Controls ($n = 3$) received vehicle only. Three to six hours after induction of focal cerebral ischemia, brains were removed for RNA extraction. Basic FGF mRNA was amplified by RT-PCR. Representative basic FGF-PCR signals at different time points after ischemia from two animals per group are shown.

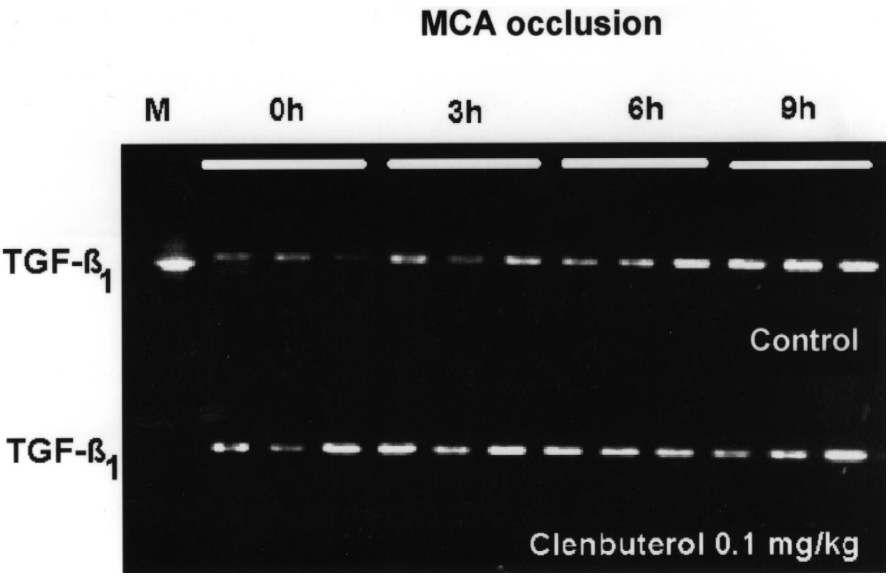


Fig. 5. Enhanced TGF-β_1 mRNA expression in the rat hippocampus by clenbuterol. Rat brains were removed up to 9 h after middle cerebral artery occlusion. Clenbuterol (0.1 mg/kg) was injected intraperitoneally 3 h before induction of ischemia. Controls received vehicle (0.9% NaCl) only. Total RNA was extracted from the hippocampi of both hemispheres and the TGF-β_1 mRNA was amplified by RT-PCR. The PCR products of three animals per group are presented.

2.6. Statistics

All values were calculated as means ± S.D. One-way analysis of variance (ANOVA) combined with Duncan's test were used for multiple comparisons of infarct volumes 7 days after middle cerebral artery occlusion. Mann–Whitney *U*-test was used for analysis of IOD values from in situ hybridization signals.

3. Results

3.1. Effect of clenbuterol on the infarct volume after permanent middle cerebral artery occlusion in the rat

Clenbuterol (0.001–10 mg/kg) was administered to Long–Evans rats 3 h before permanent occlusion of the middle cerebral artery in three different experiments. Seven days after the induction of permanent focal cerebral ischemia, the infarct volumes were calculated. Our experiments revealed a dose-dependent cerebroprotective effect of clenbuterol at doses of 0.01 to 0.5 mg/kg (Table 1). In all experiments, clenbuterol reduced the infarct volume at the dose of 0.1 mg/kg (Fig. 1; Table 1). In contrast to the cerebroprotective effect of 0.01–0.5 mg/kg clenbuterol, 1 and 10 mg/kg clenbuterol failed to protect rat brain tissue from ischemic damage (Fig. 1; Table 1). Dosages lower than 0.01 mg/kg did not reduce the infarct volume after middle cerebral artery occlusion (Table 1).

3.2. Physiologic parameters

The physiologic parameters were measured 3–4 h after administration of clenbuterol (0.1 and 10 mg/kg) during the surgical procedure of middle cerebral artery occlusion. Clenbuterol (10 mg/kg) reduced the mean arterial blood pressure measured 3 h after administration in a dose-dependent manner to 68 mm Hg (Table 2).

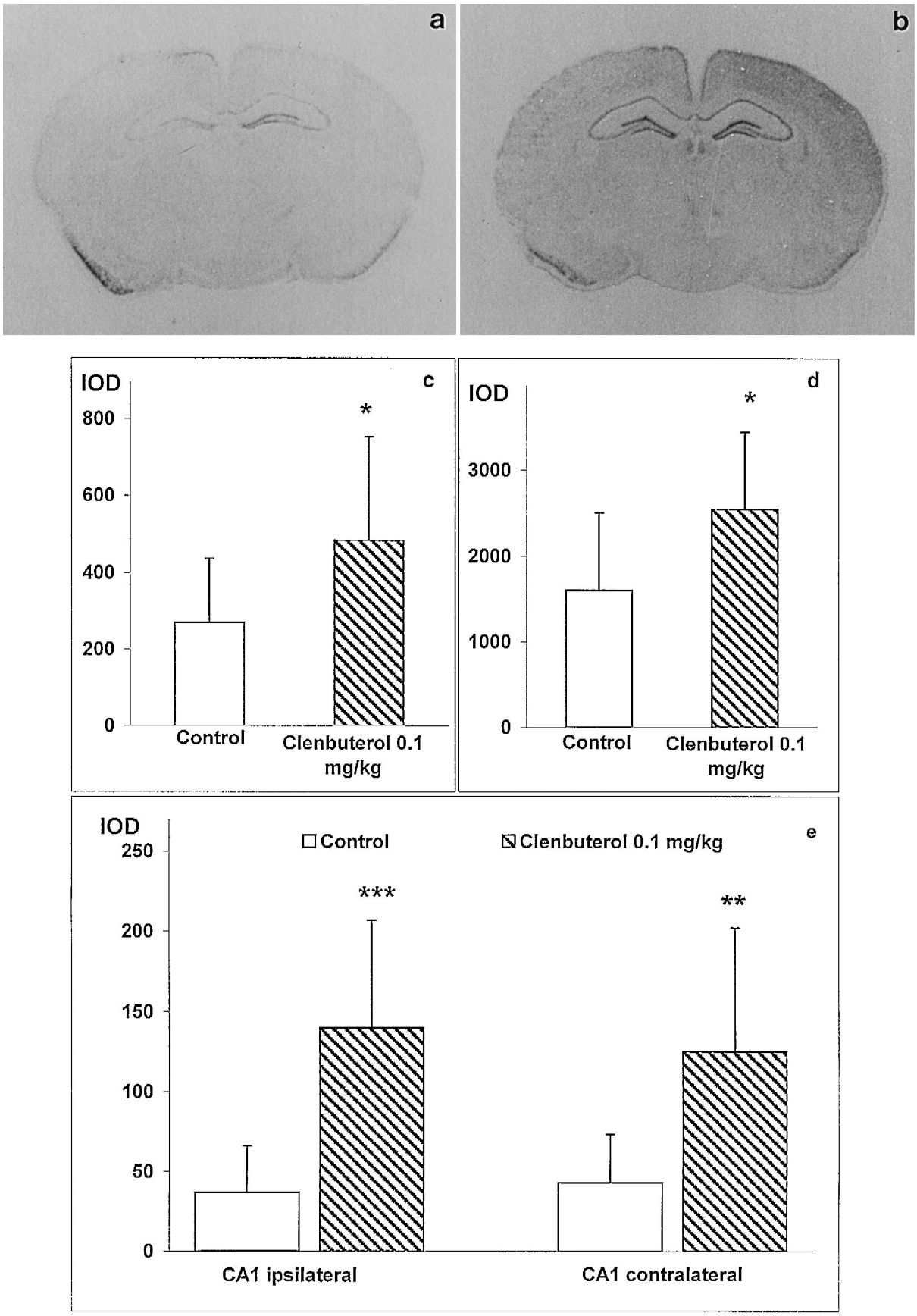
In the same group treated with 10 mg/kg clenbuterol, a significant increase in blood glucose levels was observed 3 h after the administration. The values of arterial pH, pCO_2 and pO_2 monitored simultaneously were not influenced by clenbuterol (data not shown).

3.3. Effect of clenbuterol on the expression of growth factor mRNA measured by RT-PCR

3.3.1. NGF mRNA expression

The mRNA expression of the growth factors NGF, basic FGF and TGF-β_1 in rat brain tissue was determined

Fig. 6. In situ hybridization of NGF mRNA. Representative slices of 10 animals per group are presented (magnification 5.5 ×). Saline (a) or clenbuterol (0.1 mg/kg, b) were administered 3 h before permanent occlusion of the left middle cerebral artery. Brains were removed 6 h after middle cerebral artery occlusion and cut to coronal slices. In situ hybridization was performed with 20 μl of a hybridization mix containing 10^5 dpm/μl of ^{35}S-labeled riboprobes at 60°C for 14 h. After RNase A treatment and successive washing procedures, the tissue was dehydrated and exposed to X-ray films for 45 h. IOD of specific hybridization signals for NGF mRNA was measured in the ipsilateral cortex (c), contralateral cortex (d), and the hippocampus (e). Values are given as means + S.D. of 10 animals. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to controls.


a
b
IOD
c
800
600
400
200
0
*
Control
Clenbuterol 0.1 mg/kg
IOD
d
3000
2000
1000
0
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Control
Clenbuterol 0.1 mg/kg
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e
Control
Clenbuterol 0.1 mg/kg
250
200
150
100
50
0
***
**
CA1 ipsilateral
CA1 contralateral

3–12 h after clenbuterol treatment (0.1–10 mg/kg). At 3 h after clenbuterol administration, the left middle cerebral artery of the rats was permanently occluded. As shown in Fig. 2a and b, clenbuterol induced NGF mRNA expression in the non-ischemic cortical tissue in all doses investigated. An increase in NGF mRNA was observed in vehicle-treated rats within 9 h after middle cerebral artery occlusion (data not shown). In ischemic animals pretreated with clenbuterol (0.1 mg/kg), an enhanced expression of NGF mRNA was observed already at 3 h after administration of the β_2-adrenoceptor agonist (0 h) as well as 3–6 h after the ischemic lesion (Fig. 2b). In hippocampal tissue, a similar induction of NGF mRNA expression 3 h after ischemia was found in animals treated with 0.1–10 mg/kg clenbuterol (Fig. 3).

3.3.2. Basic FGF mRNA expression

As measured by RT-PCR, focal cerebral ischemia induced the expression of basic FGF mRNA in rat cortical tissue 9 h after middle cerebral artery occlusion. However, this induction of basic FGF mRNA occurred earlier in the ipsilateral cortex when the rats were pretreated with 0.1 mg/kg clenbuterol. As shown in Fig. 4, one of three animals showed an enhanced expression already 3 h after clenbuterol treatment at the time point of middle cerebral artery occlusion. At 6 to 12 h after clenbuterol administration, i.e., 3 to 9 h after middle cerebral artery occlusion, a pronounced increase in basic FGF mRNA levels as compared to vehicle-treated controls was observed in cortical tissue ipsilateral to focal cerebral ischemia (Fig. 4). In hippocampal tissue, RT-PCR revealed a similar induction of basic FGF mRNA by clenbuterol as shown before for the NGF mRNA. However, the enhancement of basic FGF mRNA was less pronounced as compared to the NGF mRNA induction by clenbuterol in the hippocampus (Fig. 3).

3.3.3. TGF-β_1 mRNA

TGF-β_1 mRNA was not found to be significantly changed in the cortex neither by clenbuterol nor by ischemia within 9 h after middle cerebral artery occlusion. In contrast, an induction of TGF-β_1 mRNA expression was observed in hippocampal brain tissue already 3 h after clenbuterol administration. The enhancement of TGF-β_1 mRNA expression in hippocampal tissue by clenbuterol was also found 3–6 h after induction of focal cerebral ischemia (Fig. 5). This TGF-β_1 mRNA induction by clenbuterol was less pronounced at 6 h after ischemia and not detectable at 9 h after middle cerebral artery occlusion (Fig. 5).

3.4. Effect of clenbuterol on the expression of NGF and GFAP mRNA measured by in situ hybridization

In situ hybridization was performed to further characterize the localization of clenbuterol-induced NGF mRNA expression in the rat brain tissue at 6 h after ischemia. Furthermore, the effect of clenbuterol on GFAP mRNA expression was investigated. In order to determine the effect of clenbuterol on the levels of NGF or GFAP mRNA, the IOD of the specific signal in a defined brain area was measured.

3.4.1. NGF mRNA

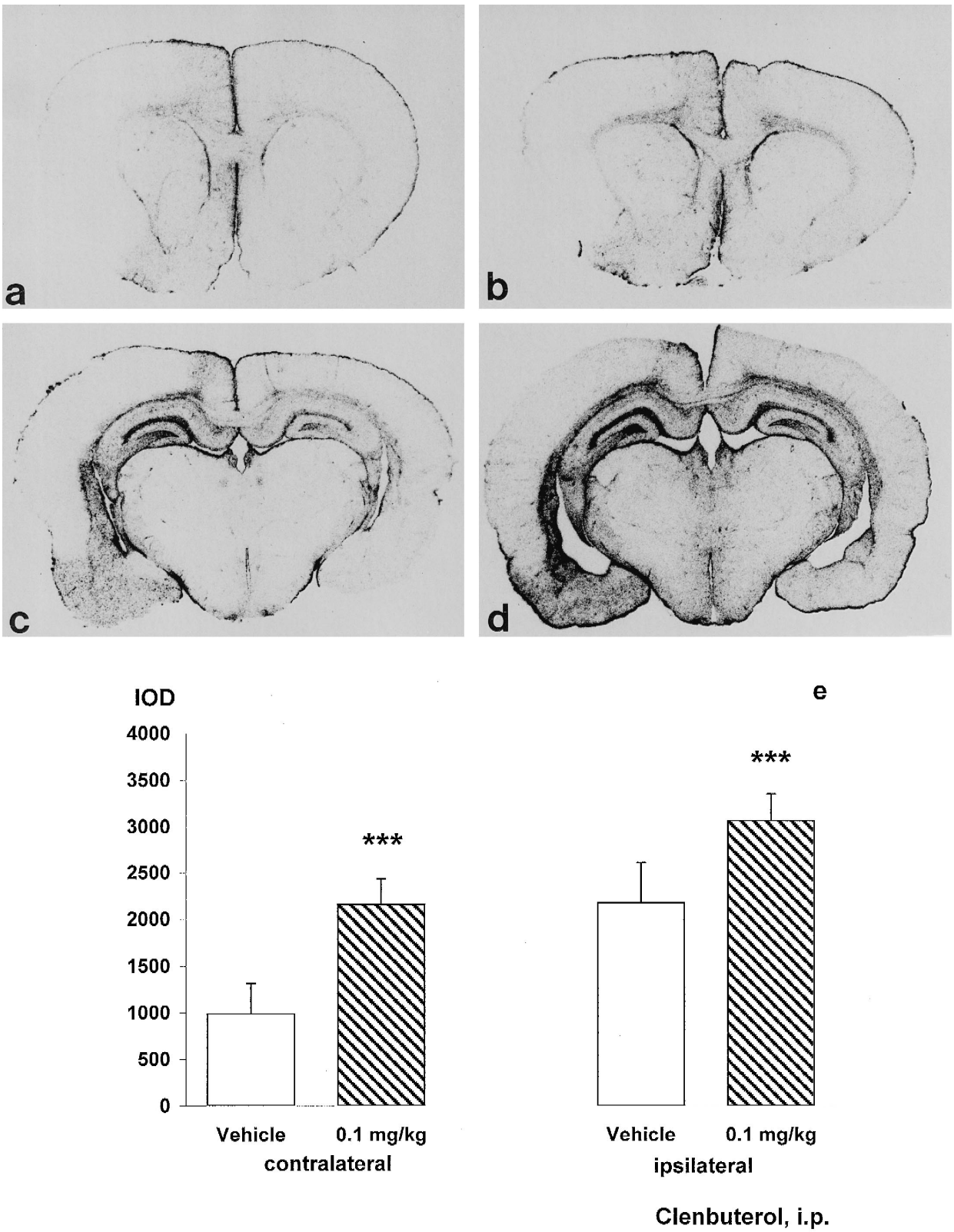
Specific signaling from the NGF antisense riboprobe was observed predominantly in the CA1 to CA3 subfield of the hippocampus and in the dentate gyrus. In addition, a strong NGF mRNA signal was detected in the piriform cortex that was increased in the hemisphere ipsilateral to the ischemic insult compared to the contralateral side in every animal investigated. The measurement of NGF mRNA by in situ hybridization confirmed the results obtained in the RT-PCR study. IOD of specific NGF mRNA signals was increased in cortical areas ipsilateral (by 80%, $P < 0.05$) and contralateral (by 70%, $P < 0.05$) to the ischemic lesion in clenbuterol-treated animals as compared to vehicle-treated controls (Fig. 6). Ipsilateral to the middle cerebral artery occlusion, very low NGF mRNA signaling was observed within the ischemic tissue in every animal. Following clenbuterol treatment, a significant increase in the NGF mRNA signal was observed in CA1 to CA3 subfields of the hippocampus in the ipsilateral hemisphere (IOD increased to 300%, $P < 0.001$) and the contralateral hemisphere (IOD increased to 200%, $P < 0.001$) (Fig. 6).

3.4.2. GFAP mRNA

The expression of GFAP mRNA was increased in the non-ischemic brain tissue of vehicle-treated animals from 6 h up to 7 days after middle cerebral artery occlusion. This increase in GFAP mRNA levels by the focal ischemic lesion was spread all over the brain, also to the contralateral hemisphere and peaked after 2 days (data not shown). GFAP expression was not observed in the infarct core (Fig. 7). As shown in Fig. 7, a global increase in GFAP mRNA expression was observed in clenbuterol-treated ani-

Fig. 7. GFAP mRNA expression in the rat brain after middle cerebral artery occlusion and clenbuterol administration. In situ hybridization was performed with 20 μl of a hybridization mix containing 100 000 dpm/μl of ^{35}S-labeled riboprobes at 60°C for 14 h. After RNase A treatment and successive washing procedures, the tissue was dehydrated and exposed to X-ray films for 3 h. (a) and (c) show slices of different brain regions from saline-treated animals at 6 h after permanent focal cerebral ischemia (magnification 6×). Clenbuterol (0.1 mg/kg) was administered 3 h before permanent middle cerebral artery occlusion and enhanced the GFAP mRNA expression in the rat brain tissue after induction of cerebral ischemia (b, d). IOD of specific GFAP mRNA signals was measured in non-ischemic tissue of contralateral and ipsilateral hemispheres. Values are given as means S.D. of 10 animals (e). $^{***}P < 0.001$ compared to controls (Mann–Whitney *U*-test).

mals as compared to controls at 6 h after middle cerebral artery occlusion. In the ipsilateral hemisphere, clenbuterol increased the GFAP mRNA signal to 140% ($P < 0.001$) in the ipsilateral hemisphere and to 200% in the contralateral hemisphere, as determined by quantitative in situ hybridization (Fig. 7).



4. Discussion

In this study, we provide evidence for the view, that the induction of growth factor synthesis within the brain tissue by low molecular weight drugs is a promising approach to use the neuroprotective potential of these peptides in vivo. As we reported previously, the β_2-adrenoceptor agonist, clenbuterol, has been shown to protect neurons against glutamate-induced cell death due to increased NGF synthesis by β_2-adrenoceptor stimulation in mixed hippocampal cultures (Semkova et al., 1996b). There is evidence from our initial studies using models of permanent focal ischemia in mice and transient forebrain ischemia in rats that clenbuterol acts as an neuroprotectant in vivo (Culmsee et al., 1996; Semkova et al., 1996b; Zhu et al., 1998). In the present study, we could demonstrate that an enhanced synthesis of NGF and basic FGF mRNA in the neocortex as well as an accelerated activation of astrocytes accompanied the protective effect of clenbuterol (0.01–0.5 mg/kg) in a rat model of focal cerebral ischemia. Doses of clenbuterol higher than 0.5 mg/kg did not reduce the infarct volume under our experimental conditions. This finding is in line with our results in a mouse model of focal ischemia where a reduction of the infarct area by clenbuterol was observed only at doses lower than 5 mg/kg (Culmsee et al., 1996; Semkova et al., 1996b). Most likely, the significant reduction of the mean arterial blood pressure and increased blood glucose levels are two side effects of the β_2-mimetic drug that suppresses the cerebroprotective effects of clenbuterol at doses of 1–10 mg/kg (Table 2, Semkova et al., 1996a). It has been shown that a reduction of the mean arterial blood pressure to 60 mm Hg increased the ischemic damage after focal cerebral ischemia (Osborne et al., 1987). A similar lesioning effect has been discussed for high blood glucose levels (Bomont and MacKenzie, 1995; Duverger and MacKenzie, 1988). High glucose levels led to a decrease in tissue pH in the ischemic penumbra because of anaerobic metabolism to lactate and, thus, to a further increase in the size of the developing infarct (Nagai et al., 1993).

After cerebral ischemia, an increase in the expression of growth factors, such as NGF, basic FGF, and TGF-β_1, has been described by several authors (Lindvall et al., 1992; Wang et al., 1995; Iwata et al., 1997). In line with these findings, we demonstrate an induction of NGF mRNA and basic FGF mRNA within 9 h after the ischemic lesion. The upregulation of growth factor mRNA after ischemia has been proposed as an endogenous mechanism to protect neurons from ischemic cell death (Nieto-Sampedro et al., 1982). The mechanism of this postischemic induction of NGF or basic FGF remains unclear. Spreading depression as well as the increased release of excitatory amino acids in brain tissue after ischemia have been suggested to induce the transcription of immediate early genes, such as c-*fos* and c-*jun* or HSP72 that could mediate the expression of NGF or other growth factors after ischemia (Hughes et al., 1993; Takeda et al., 1993; Arai et al., 1996; Belayev et al., 1996). However, the induction of growth factors occurred too late after ischemia to protect brain tissue from damage effectively.

Pharmacological dosages of growth factors were injected directly into brain tissue, and genetically engineered cells that constantly secreted growth factors for a limited time period were implanted into the brain in order to demonstrate their neuroprotective potential in vivo (Shigeno et al., 1991; Fisher et al., 1995; Pechan et al., 1995). Although the results from these experimental studies were encouraging, the necessity to administer neurotrophic factors directly into the brain remained an important obstacle against their therapeutical application. Interestingly, intravenously injected basic FGF reduced the infarct size in a model of focal cerebral ischemia in rats even when administered after the induction of ischemic brain damage (Finklestein et al., 1994; Fisher et al., 1995). However, the pharmacological doses that were used to demonstrate this neuroprotective effect of basic FGF caused tissue levels much higher than those under physiologic conditions. Clinical studies revealed severe side effects of neurotrophic factors such as pain caused by NGF and liver toxicity by CNTF that do not allow to use pharmacological dosages of these growth factors for the therapy of neurodegenerative disorders (Lewin et al., 1993; Petty et al., 1994; Miller et al., 1996).

We found that the endogenous induction of NGF and basic FGF mRNA after ischemia was markedly increased by clenbuterol in cortical brain tissue after middle cerebral artery occlusion. In previous studies, doses of 10 mg/kg clenbuterol were used to demonstrate the induction of NGF and basic FGF mRNA in the non-ischemic brain tissue (Follesa and Mocchetti, 1993; Hayes et al., 1995). As 10 mg/kg of clenbuterol did not reduce the ischemic brain damage under our experimental conditions, it was necessary to investigate whether growth factors were also upregulated in the ischemic brain tissue by clenbuterol doses lower than 10 mg/kg. As we could demonstrate in the rat model of global ischemia, the upregulation of NGF mRNA was followed by an upregulation of NGF protein, indicating that changes of growth factor's mRNA levels were also relevant for the neuroprotective effect of clenbuterol (Zhu et al., 1998). Our findings from the present study indicate that clenbuterol induces early upregulation of various growth factors, i.e., not only NGF may be responsible for its protective effect against ischemic brain damage. The factors found to be upregulated by clenbuterol have been shown in many studies to protect neurons from insults including axotomy, oxidative stress and excitotoxicity (Williams et al., 1986; Anderson et al., 1988; Hefti et al., 1989; Sendtner et al., 1992; Frim et al., 1993; Lindsay, 1996).

The upregulation of TGF-β_1 mRNA by ischemia or clenbuterol showed a different pattern as compared to NGF or basic FGF mRNA. We could not find any upregu-

lation of TGF-β_1 mRNA by clenbuterol in the cortex. In addition, TGF-β_1 mRNA levels were not elevated in the cortex of saline-treated animals up to 9 h after ischemia. This observation is in line with previous findings demonstrating a delayed increase in TGF-β_1 mRNA levels 24 h to 3 days after focal cerebral ischemia (Wießner et al., 1993; Wang et al., 1995; Krupinski et al., 1996). Interestingly, we observed a marked increase in TGF-β_1 mRNA already 3 h after clenbuterol administration in the rat hippocampus. This early upregulation of TGF-β_1 in the hippocampus could be the mechanism of the anti-apoptotic effect of clenbuterol that was observed in the CA1 region after transient forebrain ischemia (Knuckey et al., 1996; Zhu et al., 1998). TGF-β_1 infused intraventricularly reduced the percentage of damaged neurons in the hippocampal CA1 subfield in a model of transient forebrain ischemia (Henrich-Noack et al., 1996). In vitro, TGF-β_1 protected neurons from cell death induced by glutamate or cyanide (Prehn et al., 1993; Henrich-Noack et al., 1994). Although it remains unclear which of the growth factors up-regulated by clenbuterol may be most important for the reduction of ischemic brain damage observed in our study, there is increasing evidence that the upregulation of NGF may be a key event in the underlying mechanism of neuroprotection by the β_2-adrenoceptor agonist. Recently, we could demonstrate that NGF-antisense oligonucleotides injected into the cortex prior to the lesion blocked the protective effect of clenbuterol against ischemic brain damage induced by middle cerebral artery occlusion in the rat (Culmsee et al., 1998a,b, 1999). However, TGF-β_1, basic FGF, as well as activated astrocytes, may contribute essentially to the neuroprotective effect of clenbuterol in vivo.

Astrocytes have been widely discussed as a possible source of neurotrophic factors that support neuronal survival under lesioning conditions (Nieto-Sampedro et al., 1982; Lu et al., 1991; Rudge, 1993; Mantyh et al., 1995). It has been suggested, e.g., that specific manipulation of growth factor expression in glial cells by so-called astrocyte-kinetic drugs could become a new therapeutic strategy for neurodegenerative diseases and stroke (Lu et al., 1991; Biagini et al., 1994; Hodges-Savola et al., 1996; Semkova et al., 1996c). In our study, we could detect an activation of astrocytes after cerebral ischemia by measuring GFAP mRNA expression. The parallel increase of GFAP mRNA levels and enhanced synthesis of NGF and basic FGF indicated that astrocytes were involved in the upregulation of growth factor synthesis. This finding was in line with published data showing a similar activation of astrocytes after brain lesions (Chen et al., 1993; Schroeter et al., 1995; Martin and O'Callaghan, 1996; Yamashita et al., 1996). Here, the increase in the expression of GFAP mRNA was further accelerated by clenbuterol, supporting the hypothesis that the stimulation of astrocytes could be pharmacologically regulated by a manipulation of β_2-receptors. Astrocytes in culture, as well as in various regions of the mammalian central nervous system (CNS), have been shown to express β_2-adrenergic receptors (Salm and McCarthy, 1989; Shao and Sutin, 1992; Mantyh et al., 1995). Furthermore, there is evidence that expression of β_2-receptors increases in astrocytes in response to CNS injury or disease (Hodges-Savola et al., 1996). In line with these findings, our previous studies demonstrated an increase in NGF synthesis and release in primary cultures of rat cortical astrocytes after β_2-adrenoceptor stimulation (Semkova et al., 1996a; Culmsee et al., 1998a,b, 1999). Thus, it was suggested that the pharmacological stimulation of NGF synthesis in astrocytes contributed substantially to the neuroprotective effects of clenbuterol. There is evidence that an early activation of astrocytes leads to a reduction in the damage of brain tissue (Biagini et al., 1994). On the other hand, the suppression of astrocyte activation by the β-adrenoceptor antagonist, propranolol, led to an increase in neuronal damage (Hodges-Savola et al., 1996).

5. Conclusion

Our findings suggest that NGF, as well as other neuroprotective growth factors such as basic FGF and TGF-β_1, are involved in the cerebroprotective effect of clenbuterol in vivo. Activated astrocytes could be a major source of the growth factor synthesis accelerated by the β_2-mimetic drug. Induction of growth factor synthesis and activation of astrocytes could become new therapeutic strategies for the treatment of chronic neurodegenerative disorders and stroke.

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