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Enalapril and moexipril protect from free radical-induced neuronal damage in vitro and reduce ischemic brain injury in mice and rats

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Abstract

Angiotensin-converting enzyme inhibitors have been demonstrated to protect spontaneously hypertensive rats from cerebral ischemia. The present study investigated the protective effect of enalapril and moexipril in models of permanent focal cerebral ischemia in normotensive mice and rats. To elucidate the mechanism of neuroprotection the influence of these angiotensin-converting enzyme inhibitors on glutamate-, staurosporine- or $Fe^{2+/3+}$-induced generation of reactive oxygen species and neuronal cell death in primary cultures from chick embryo telencephalons was studied. Treatment with moexipril or enalapril dose-dependently reduced the percentage of damaged neurons, as well as mitochondrial reactive oxygen species generation induced by glutamate, staurosporine or $Fe^{2+/3+}$. Furthermore, moexipril and enalapril attenuated staurosporine-induced neuronal apoptosis as determined by nuclear staining with Hoechst 33258. In mice, 1 h pretreatment with enalapril (0.03 mg/kg) or moexipril (0.3 mg/kg) significantly reduced brain damage after focal ischemia as compared to control animals. Additionally, moexipril (0.01 mg/kg) was able to reduce the infarct volume in the rat model after focal cerebral ischemia. The results of the present study indicate that the angiotensin-converting enzyme inhibitors enalapril and moexipril promote neuronal survival due to radical scavenging properties.

Keywords: Angiotensin-converting enzyme inhibtor; Neuron, chick; Reactive oxygen specie; Apoptosis; Protection; Ischemia, cerebral

1. Introduction

Angiotensin-converting enzyme inhibitors are commonly used in the therapy of hypertension and chronic heart failure (Govantes and Marin, 1996).

Moreover, it has been shown that angiotensin-converting enzyme inhibitors are able to protect ischemic rat hearts against reperfusion injury (Ferrari et al., 1992; Liu et al., 1992), to block the progression of renal damage (Kohara et al., 1993) and to reduce angiotensin II-induced myocyte and coronary vascular necrosis (Kabour et al., 1995). Furthermore, captopril and quinapril attenuated cardiomyocyte apoptosis in spontaneously hypertensive rats (Diez et al., 1997). These data suggested that angiotensin-converting enzyme inhibitors could have a beneficial influence on cellular apoptosis which is thought to play a role in the pathology of several neurodegenerative diseases such as amyotrophic lateral sclerosis, multiple sclerosis, Parkinson's and Alzheimer's disease as well as in stroke (Bredesen, 1995).

Since the existence of an intracerebral renin–angiotensin system has been revealed (Unger et al., 1988), various studies on the effect of angiotensin-converting enzyme inhibitors on brain function have been performed. Recent data described that angiotensin-converting enzyme inhibitors ameliorated ischemic brain metabolism in spontaneously hypertensive rats by preventing the ischemia-induced increase in tissue lactate concentration and by stabilizing ATP-levels (Sadoshima et al., 1993). Moreover, angiotensin-converting enzyme inhibitors have been shown to reduce mortality in spontaneously hypertensive rats (Fujii et al., 1992; Vacher et al., 1993; Lee et al., 1996), and captopril improved neurologic outcome from incomplete cerebral ischemia in rats (Werner et al., 1991). Nevertheless, it is still unclear whether angiotensin-converting enzyme inhibitors are able to reduce infarct volume after cerebral ischemia in normotensive animals. Although many investigations on the protective capacities of an-

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giotensin-converting enzyme inhibitors were performed, the mechanism of action remained poorly understood. However, there is growing evidence that the protective effects of angiotensin-converting enzyme inhibitors are independent of blood pressure reduction (Gohlke et al., 1996) or inhibition of angiotensin II formation (Takeda et al., 1997).

Reactive oxygen species seem to play an important role in necrotic (Mattson et al., 1995), as well as in apoptotic cell damage (Ratan et al., 1994). Radical scavengers such as 21-aminosteroids, thiols or tocopherol as well as overexpression of antioxidative enzymes could protect against several forms of neuronal damage (Lin and Chang, 1997; Stoyanovsky et al., 1998). Investigators who so far determined radical scavenging properties of angiotensin-converting enzyme inhibitors observed different results. Some authors found that only the angiotensin-converting enzyme inhibitors containing a sulfhydryl (SH)-group such as captopril or zofenopril were capable of scavenging reactive oxygen species (Chopra et al., 1990; Mak et al., 1990; Noda et al., 1997). Others reported that free radical scavenging was independent of the SH-group and that the angiotensin-converting enzyme inhibitors with carboxylat or phosphonic acid structure such as enalapril, lisinopril, ramipril or fosinopril were equally potent antioxidants indicating that also these angiotensin-converting enzyme inhibitors might influence oxidative injury (Mira et al., 1993; Suzuki et al., 1993; Fernandes et al., 1996).

The aim of the present study was to determine whether the angiotensin-converting enzyme inhibitors enalapril and moexipril can protect cultured neurons from glutamate-, $Fe^{2+/3+}$- or staurosporine-induced neuronal damage and whether suppression of reactive oxygen species generation is involved in the mechanism of neuroprotection. Furthermore, the potency of enalapril and moexipril to reduce ischemic brain damage of normotensive rodents when administered 1 h before permanent middle cerebral artery occlusion was investigated.

2. Materials and methods

2.1. Animals

Male Long–Evans rats (200–300 g, Møllegaard, Denmark) and male NMRI mice (20–40 g, Charles River, Germany) were used for ischemia experiments. The animals were kept under controlled light and environmental conditions (12 h dark/light circle, 23 ± 1°C, 55 ± 5% relative humidity) and had free access to food (Altromin, Germany) and water.

2.2. Chemicals

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum and penicillin–streptomycin solution were purchased from Gibco BRL, Life Technologies, Germany. Staurosporine, sodium L-glutamate, Fe_2SO_4, $FeCl_3$, enalapril maleate, Hoechst 33258, poly-L-lysine and dimethylsulfoxide were obtained from Sigma (Germany). Dihydrorhodamine 123 was purchased from Molecular Probes, USA. Moexipril hydrochloride was a kind gift of Schwarz Pharma (Germany).

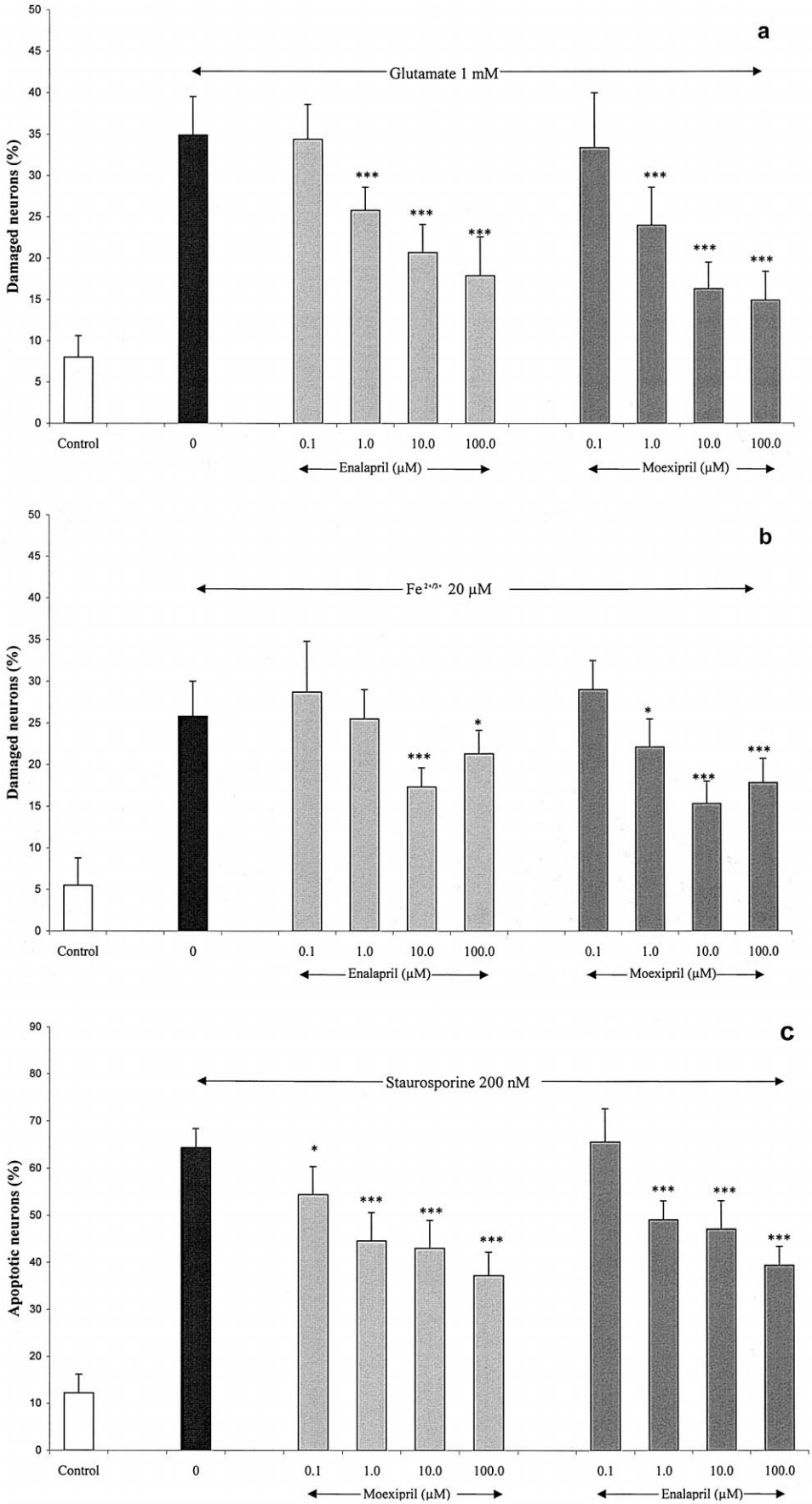
2.3. Cell culture

Primary neuronal cultures were prepared from 7-day old chick embryo telencephalons as described previously (Pettman et al., 1979). The cerebral hemispheres were mechanically dissociated through nylon meshes of 48-μm mesh width. The homogenized cell suspension was seeded into poly-L-lysine coated Petri dishes containing 15-mm glass coverslips or into culture flasks (25 mm^2) with a density of 4×10^4 cells/cm^2. The cells were cultured in DMEM supplemented with penicillin–streptomycin solution and 20% fetal bovine serum at 37°C, 5% CO_2 and 95% relative humidity. Culture medium was replaced every 2 days and the neuronal cells were used for experiments on day 5 after seeding. The percentage of neurons at this time point was > 98% because gliogenesis was known to occur later than 8 days during the development of the chick embryo and the few glial cells in the initial suspension were inhibited by the poly-L-lysine substrate. Neuronal cells were identified by an immunohistochemical demonstration of tetanus toxin binding sites (Pettman et al., 1979).

2.4. Drug treatment

In order to cause neuronal injury the cells were incubated with either 1 mM glutamate for 1 h followed by 23 h of recovery or with 20 μM $FeSO_4$ and 20 μM $FeCl_3$ for 24 h in serum-free DMEM medium. Apoptotic cell damage was induced by incubating the cells with 200 nM

Fig. 1. Effect of enalapril and moexipril on L-glutamate-, $Fe^{2+/3+}$- or staurosporine-induced neuronal damage. After 5 days in culture, neurons from chick embryo telencephalons were treated with glutamate (1 mM; 1 h + 23 h recovery) (a), $Fe^{2+/3+}$ (20 μM; 24 h) (b) or staurosporine (200 nm; 24 h) (c). Cellular viability was determined 24 h after glutamate or Fe $^{2+/3+}$ treatment by Trypan blue exclusion method. Neuronal apoptosis was identified by nuclear staining with Hoechst 33258, 24 h after staurosporine treatment. Moexipril and enalapril were added simultaneously with glutamate, $Fe^{2+/3+}$ or staurosporine and were also present 24 h after treatment. Values are given as means ± S.D. of $n = 8$ experiments. Differences between glutamate-, $Fe^{2+/3+}$- and staurosporine-treated cultures in the presence and absence of enalapril or moexipril: $^{*}P < 0.05$; $^{***}P < 0.001$ using ANOVA 1 with subsequent Scheffé test.


a
Glutamate 1 mM
Damaged neurons (%)
Control
0
0.1
1.0
10.0
100.0
Enalapril (μM)
Moexipril (μM)
b
Fe2+/3+ 20 μM
Damaged neurons (%)
c
Staurosporine 200 nM
Apoptotic neurons (%)
Moexipril (μM)
Enalapril (μM)

staurosporine in serum-free medium for 24 h. The angiotensin-converting enzyme inhibitors enalapril and moexipril were added simultaneously with staurosporine, glutamate or $Fe^{2+/3+}$.

2.5. Cellular viability

Cellular viability was determined by the Trypan blue exclusion method which identified damaged neurons on the basis of membrane leakage. For this purpose, cultures were incubated with an 0.4% solution of the hydrophilic dye Trypan blue. After 24 h of treatment the number of stained and unstained neurons was counted in eight randomized subfields of two different flasks containing approximately 80 neurons per subfield. Neuronal damage was expressed as percent ratio of Trypan blue-stained cells vs. the total number of cells.

2.6. Nuclear staining with Hoechst 33258

After fixing the cells in methanol, they were incubated with the DNA fluorochrome Hoechst 33258 (10 μg/ml) for 15 min and nuclear morphology was observed under a fluorescence microscope (Axiovert 100, Zeiss, Germany).

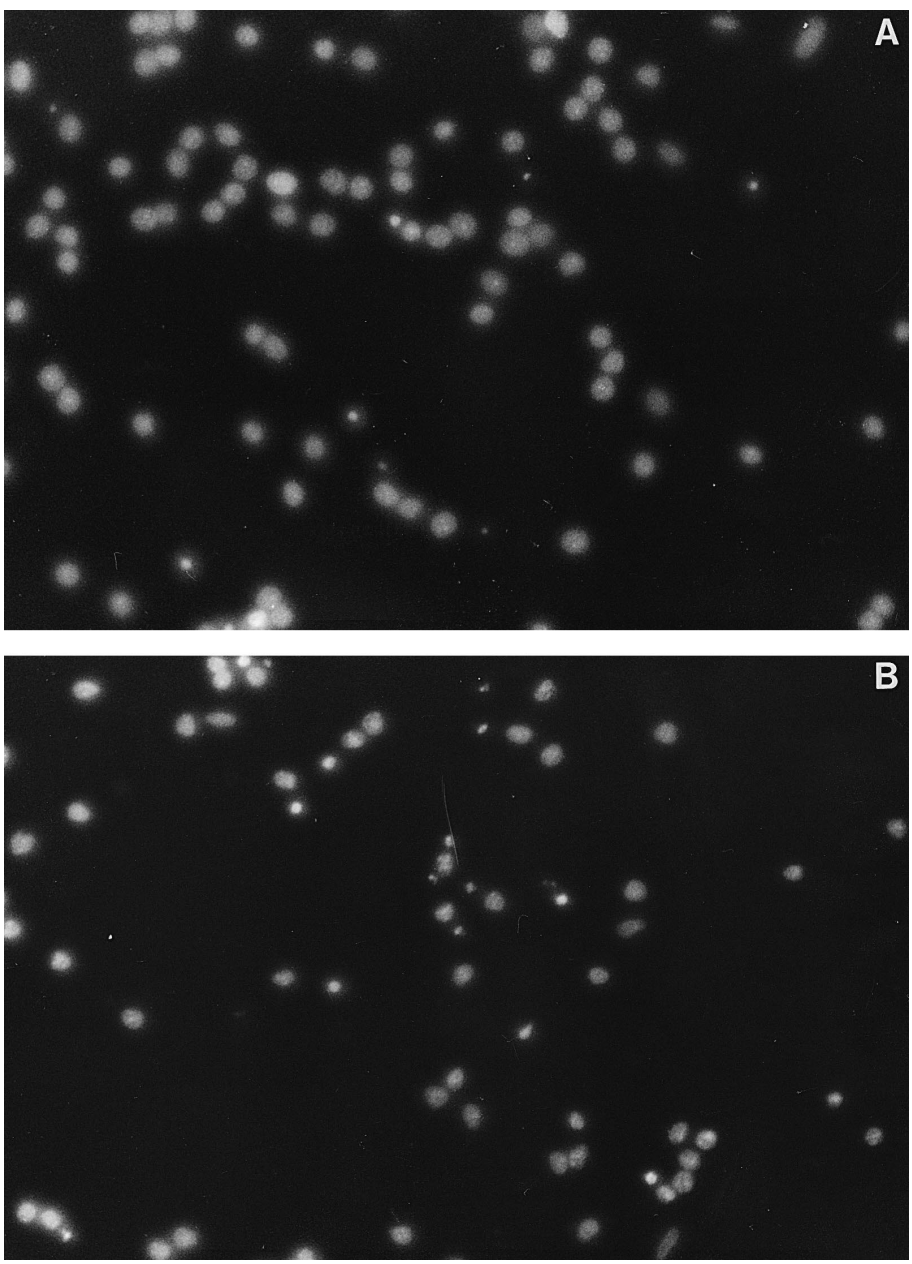


Fig. 2. Nuclear staining of chick embryonic neurons with Hoechst 33258. Representative photomicrographs showing controls (A), staurosporine-treated cultures (B) and staurosporine-treated cultures in the presence of 10 μM enalapril (C) or 10 μM moexipril (D) are demonstrated. Reduced nuclear size, chromatin condensation (visible as an intense fluorescence) and DNA-fragmentation are characteristics of apoptosis.

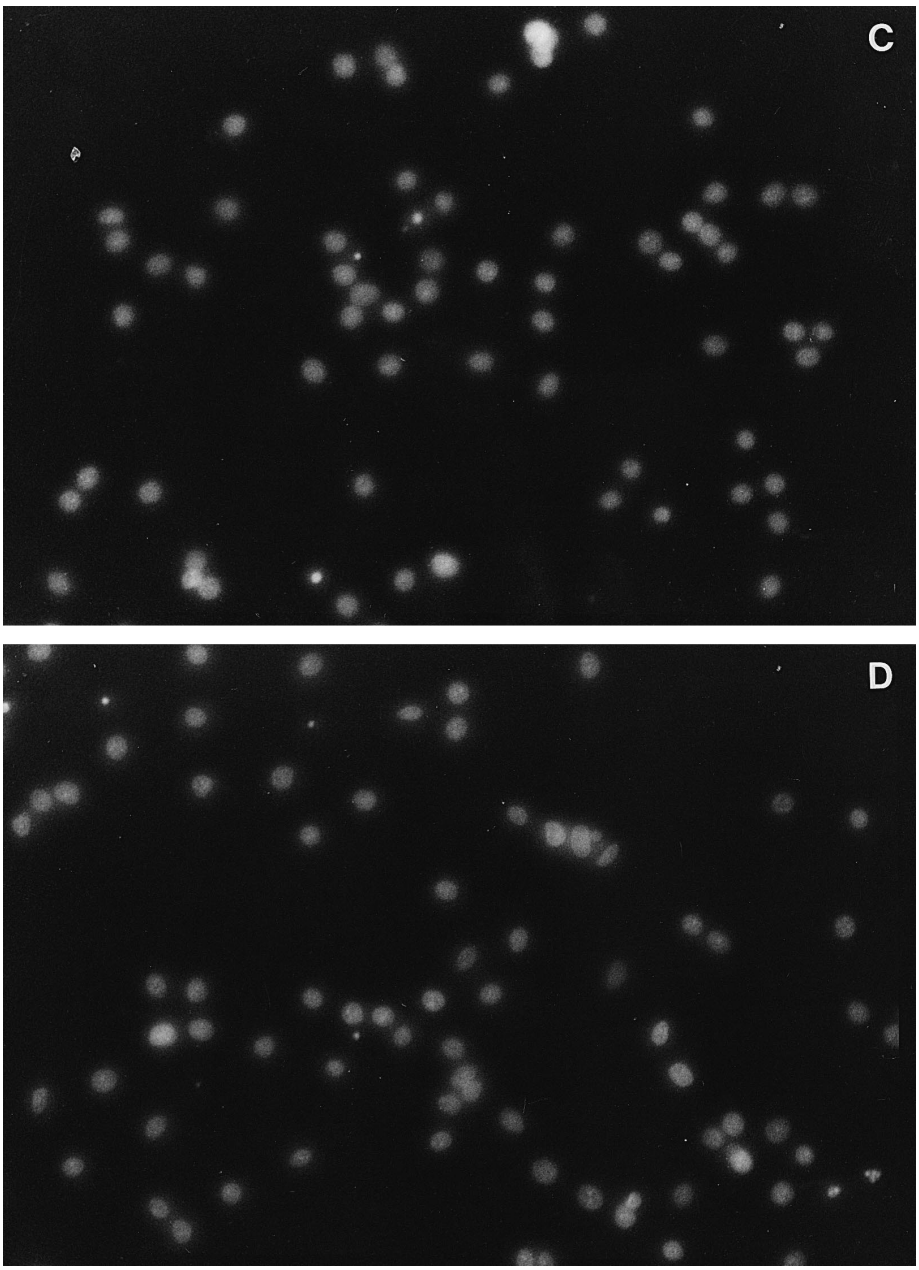


Fig. 2 (continued).

Cells which exhibited reduced nuclear size, chromatin condensation (visible as an intense fluorescence) and nuclear fragmentation were considered to be apoptotic neurons. The number of cells with apoptotic features and total cell number was counted in eight randomized subfields of two different culture flasks containing approximately 60 neurons per subfield. Neuronal apoptosis was expressed as percent ratio of cells with apoptotic features vs. the total number of cells.

2.7. Measurement of reactive oxygen species

Generation of reactive oxygen species was determined using the lipophilic non-fluorescent dye dihydrorhodamine 123 which accumulates in mitochondria and is oxidized by oxygen radicals to the positively charged fluorescent rhodamine 123. To record fluorescence, cells were stained with 5 μM dihydrorhodamine 123 for 15 min. Digital video imaging of rhodamine 123 fluorescence was conducted using a fluorescence microscope (Axiovert 100, Zeiss, Germany) with attenuated UV illumination from a 75-W xenon lamp. Fluorescence intensity was measured at an excitation wavelength of 490 nm and an emission wavelength of 510 nm. An electronic shutter which opened during image acquisition only, minimized photobleaching and phototoxicity. Images were taken by the use of a CCD camera (C 2400-87, Hamamatsu, Germany) and were digitalized as 256×256 pixels. Before measurement of fluo-

Table 1
Effect of the angiotensin-converting enzyme inhibitors enalapril and moexipril on $Fe^{2+/3+}$-, glutamate- or staurosporine-induced formation of reactive oxygen species

Treatment	Fluorescence (Fl.U.)					
	Control	Enalapril (μM)				
		0	0.1	1.0	10.0	100.0
$Fe^{2+/3+}$ (20 μM $FeSO_4$ + 20 μM $FeCl_3$; 3 h)	10 ± 2	57 ± 11	45 ± 5	34 ± 7[b]	38 ± 9[c]	19 ± 3[c]
Glutamate (1 mM; 1 h)	5 ± 1	36 ± 7	32 ± 5	25 ± 4[b]	21 ± 7[b]	6 ± 3[c]
Staurosporine (200 nM: 3 h)	7 ± 1	40 ± 4	37 ± 2	26 ± 4[b]	19 ± 3[c]	17 ± 2[c]
	Control	Moexipril (μM)				
$Fe^{2+/3+}$ (20 μM $FeSO_4$ + 20 μM $FeCl_3$; 3 h)	10 ± 2	57 ± 11	45 ± 9	29 ± 6[c]	30 ± 5[c]	30 ± 7[c]
Glutamate (1 mM; 1 h)	5 ± 1	36 ± 7	32 ± 5	23 ± 3[c]	15 ± 5[c]	10 ± 2[c]
Staurosporine (200 nM; 3 h)	7 ± 1	40 ± 4	39 ± 5	27 ± 4[b]	17 ± 3[c]	15 ± 3[c]

Five days after seeding, primary cultures of chick neurons were treated with $Fe^{2+/3+}$ (20 μM; 3 h), glutamate (1 mM; 1 h) or staurosporine (200 nM; 3 h). The angiotensin-converting enzyme inhibitors enalapril and moexipril were added simultaneously. To determine the formation of reactive oxygen species, cells were incubated with 5 μM of the non-fluorescent dye dihydrorhodamine 123 for 15 min. Fluorescent intensities of the oxidized rhodamine 123 are expressed as arbitrary units (Fl.U.). Values are given as means ± S.D. for $n = 5–7$ neurons in six to eight separate experiments. Statistics were performed by ANOVA 1 with subsequent Scheffé test. Differences between $Fe^{2+/3+}$-, glutamate- and staurosporine-treated cultures in the presence and absence of enalapril or moexipril: [b]$P < 0.01$, [c]$P < 0.001$.

rescent values, a background picture was taken that was later subtracted from the images. Data were analyzed using Argus 50 software (Hamamatsu, Germany). Fluorescence intensities were given as arbitrary units (Fl.U.).

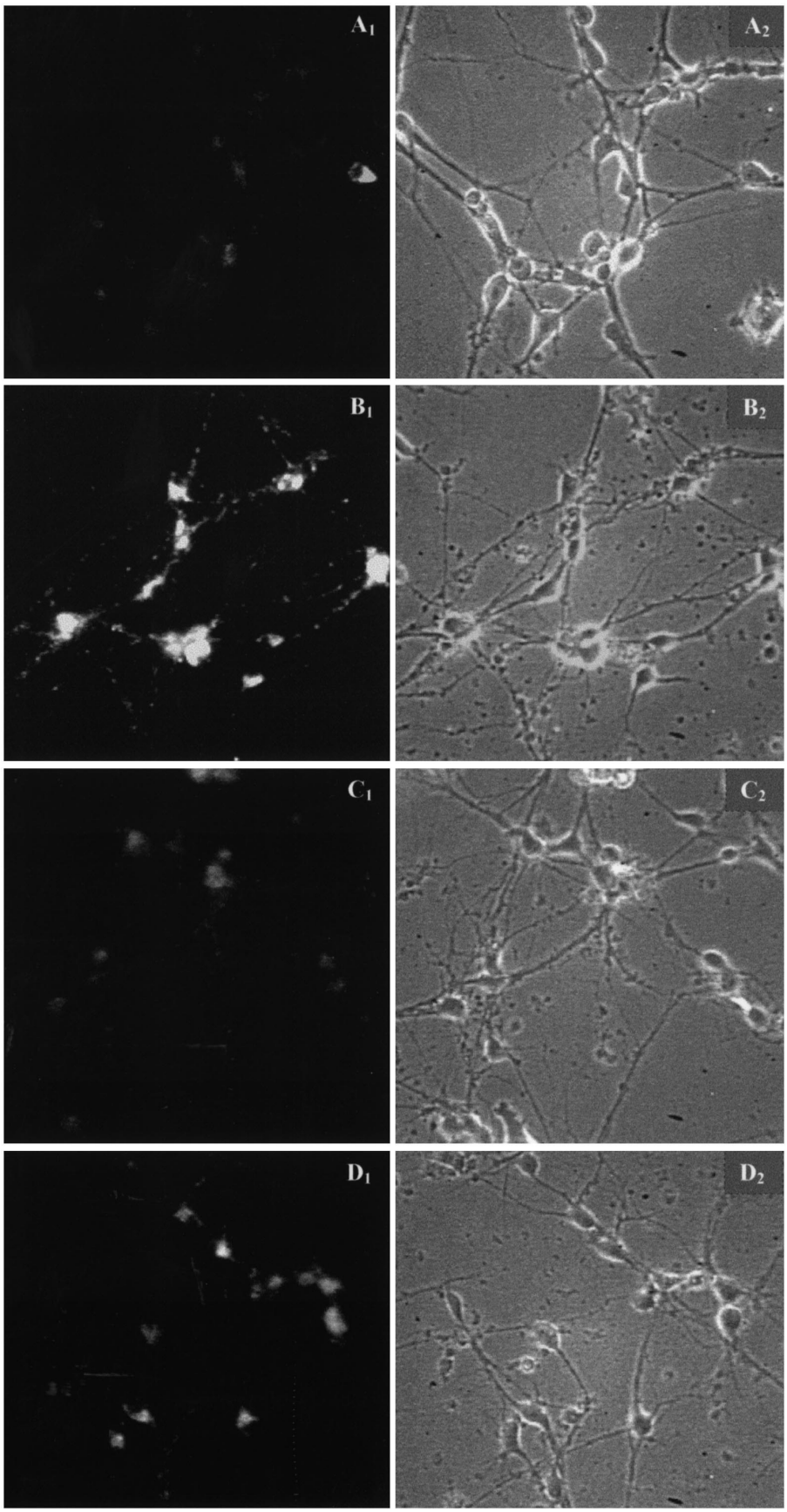
2.8. Permanent focal ischemia in mice

Permanent middle cerebral artery occlusion was performed in male NMRI mice according to the method described by Welsh et al. (1987). The mice were anesthetized with tribromoethanol (600 mg/kg i.p.) and the middle cerebral artery was permanently occluded by electrocoagulation. Body temperature was maintained at 37°C with a heating lamp during the surgical procedure. Afterwards, the mice were kept under an environmental temperature of 30°C for 2 h followed by an additional period of 2 days at 23 ± 1°C where the behaviour of the animals was observed. After 2 days, the mice were anesthetized again with tribromoethanol and i.p. perfused with a solution of the dye neutral red (1.5%). The brains were removed and fixed in a phosphate-buffered (pH 7.4) formalin solution (4%). The unstained tissue region was calculated as infarct area (mm^2) using an image analyzing system (Kontron, Germany) according to Backhauß et al. (1992). Doses of 0.03 and 3 mg/kg enalapril and moexipril were i.p. administered 1 h before middle cerebral artery occlusion. Control animals received vehicle only.

2.9. Permanent focal ischemia in rats

Permanent middle cerebral artery occlusion was performed in male Long–Evans rats as described by Tamura et al. (1981) with minor modifications according to Semkova et al. (1996). Animals were anesthetized with a mixture of 70% N_2O/30% O_2 containing 1.5% halothane. An incision was made between the external auditory canal and lateral canthus of the right eye to expose the skull. Under visual control with a surgical microscope a craniotomy was performed to approach the left middle cerebral artery. The stem of the middle cerebral artery was irreversibly occluded by microbipolar electrocoagulation. After surgery, incisions were closed with adhesive histoacryl (Braun–Dexon, Germany) to guarantee the vital function of the animals. This modified technique of middle cerebral artery occlusion causes mainly cortical infarction. Mean arterial blood pressure (Recomed, Hellige, Germany) and plasma glucose concentration (Glucose analyzer 2, Beckmann, USA) as well as arterial pH, pCO_2, pO_2 (Blood gas analyzer, Chiron, Germany) were monitored up to 30 min after surgery. Body temperature was maintained at 37°C by means of a heating pad. Afterwards, the animals were kept at an environmental temperature of 30°C for 2 h and then 7 days in their home cages at 23 ± 1°C. Seven days after middle cerebral artery occlusion the rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and

Fig. 3. Effect of the angiotensin-converting enzyme inhibitors enalapril and moexipril on $Fe^{2+/3+}$-induced formation of reactive oxygen species. Five days after seeding primary neuronal cultures were treated with 20 μM $Fe^{2+/3+}$ for 3 h. Enalapril and moexipril were added simultaneously with $Fe^{2+/3+}$. To determine fluorescence the cultures were incubated with the dye dihydrorhodamine 123 (5 μM; 15 min). Representative rhodamine 123 fluorescence images of controls (A_1) and cultures treated with 20 μM $Fe^{2+/3+}$ in the absence (B_1) and in the presence of 10 μM enalapril (C_1) or 10 μM moexipril (D_1) are demonstrated. Correlating phase-bright images are shown in A_2, B_2, C_2 and D_2.


A1
A2
B1
B2
C1
C2
D1
D2

decapitated. Brains were removed from the skull and frozen in 2-methylbutane (Fluka, Swizerland) on dry ice. Transversial sections of 20 μm thickness were taken every 500 μm using a cryomicrotome (Frigocut, Reichert-Jung, Germany). The sections were stained with Cresyl violet solution (0.5%) to differentiate between intact and damaged brain tissue. Morphometric determination of the manually outlined surface area was performed using a computer based image analyzing system (Kontron). The infarct volume was calculated from the infarct area of each section and the distance between succeeding sections. Doses of 0.01 and 0.1 mg/kg moexipril were i.p. administered 1 h before middle cerebral artery occlusion. Control animals received vehicle only.

2.10. Statistics

All values were calculated as means ± standard deviation (S.D.). For in vitro data one-way analysis of variance (ANOVA 1) with subsequent Scheffé test was employed. ANOVA 1 combined with Duncan's test was used for in vivo data.

3. Results

3.1. Effect of enalapril and moexipril on glutamate- or $Fe^{2+/3+}$-induced neuronal damage

The protective effect of enalapril and moexipril against glutamate- (1 mM; 1 h + 23 h recovery) or $Fe^{2+/3+}$ (20 μM $FeSO_4$ and 20 μM $FeCl_3$; 24 h)-induced neurotoxicity was determined by Trypan blue exclusion. In glutamate-treated cultures the percentage of Trypan blue-stained neurons increased from 5.5% (controls) to 34.9%. Enalapril and moexipril significantly reduced the percentage of damaged neurons in a dose-dependent manner (Fig. 1a). $Fe^{2+/3+}$ exacerbated neuronal damage to a level of 25.8% Trypan blue-stained neurons compared to 5.5% in controls. $Fe^{2+/3+}$-induced neurotoxicity was also significantly attenuated by enalapril or moexipril (Fig. 1b). Enalapril or moexipril alone had no effect on neuronal viability (data not shown).

3.2. Effect of enalapril and moexipril on staurosporine-induced neuronal apoptosis

To investigate the influence of enalapril and moexipril on neuronal apoptosis, the cultures were treated with 200 nM staurosporine for 24 h. Staurosporine markedly increased the percentage of neurons with apoptotic features from 12.2% in controls to 61.4%. Enalapril and moexipril did not cause significant changes in the percentage of apoptotic neurons when added under control conditions (data not shown) but exerted a dose-dependent antiapoptotic effect when given simultaneously with staurosporine (Fig. 1c and Fig. 2).

3.3. Radical scavenging properties of enalapril and moexipril

To monitor the formation of reactive oxygen species a fluorescence microscope combined with a digital video imaging system was used which allowed measurement of reactive oxygen species-fluorescence within single neurons. The neuronal cultures were loaded with dihydrorhodamine 123 and baseline fluorescence was recorded to evaluate the control level. After exposing the cultures to $Fe^{2+/3+}$ (20 μM $FeSO_4$ and 20 μM $FeCl_3$) for 3 h there was an increase in fluorescence from 10 Fl.U. in controls to 57 Fl.U. Addition of enalapril or moexipril decreased the $Fe^{2+/3+}$-induced elevation of mitochondrial fluorescence to maximally 19 and 29 Fl.U., respectively. Similar radical scavenging effects could be observed when cultures were exposed to 1 mM glutamate for 1 h or 200 nM staurosporine for 3 h which resulted in a burst of oxygen radical generation in each case. Again, enalapril as well as moexipril significantly reduced neuronal reactive oxygen species-fluorescence (Table 1 and Fig. 3).

3.4. Effect of enalapril and moexipril on the ischemic damage induced by middle cerebral artery occlusion in mice

To investigate the effect of moexipril and enalapril in NMRI mice, focal cerebral ischemia by permanent occlusion of the middle cerebral artery was performed. This caused ischemic cortical injury evaluated 2 days later as infarcted brain surface area (mm^2). Moexipril (0.3 mg/kg), i.p. injected 1 h before middle cerebral artery occlusion, significantly reduced the infarct area on the mouse brain surface. In addition, 0.03 mg/kg enalapril was able to decrease the infarct size. Other doses were not effective (Fig. 4).

3.5. Physiological variables

In the rat model physiological parameters such as mean arterial blood pressure, blood glucose, arterial pH, pCO_2 and pO_2 were monitored. Blood glucose levels as well as arterial pH, pCO_2 and pO_2 were not changed by moexipril when monitored up to 30 min after the surgical procedure. In contrast, a dose-dependent reduction in mean arterial blood pressure by moexipril was found. Administration of 0.01 mg/kg moexipril did not alter mean arterial blood pressure. In contrast, 0.1 mg/kg significantly reduced the blood pressure level from 99.5 mm Hg (controls) to 87.3 mm Hg 45 min before middle cerebral artery occlusion and from 95.0 mm Hg (controls) to 81.8 mm Hg when measured 30 min before middle cerebral artery occlusion.

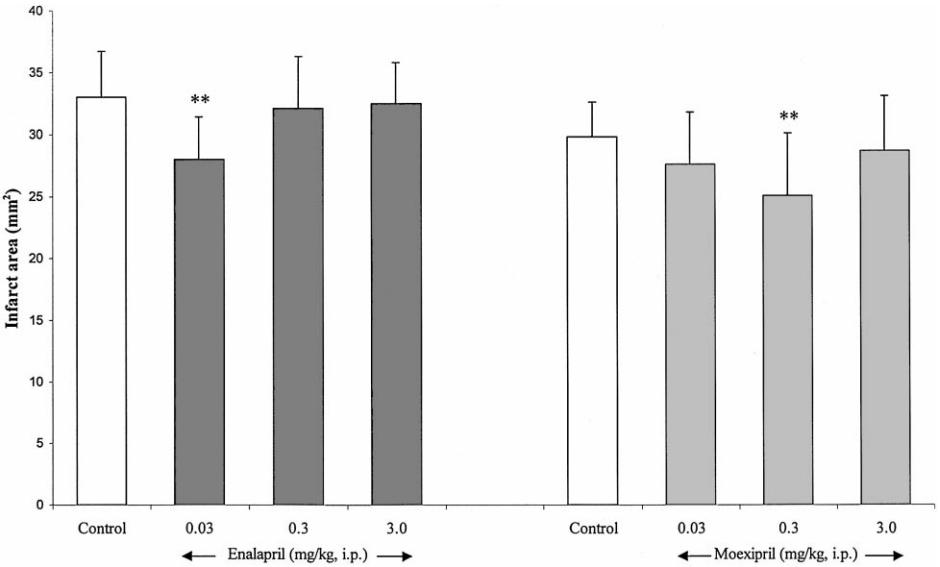


Fig. 4. Effect of the angiotensin-converting enzyme inhibitors enalapril and moexipril on focal cerebral ischemia in mice. Focal ischemia in mice was induced by permanent occlusion of the middle cerebral artery. A total of 48 h after middle cerebral artery occlusion, the mice were i.p. perfused with a solution of the dye neutral red (1.5%) to stain the brain tissue. After fixing the isolated brains in formalin phosphate buffer (pH 7.4) for 24 h, the unstained tissue region on the cortical surface was calculated as infarct area by means of an image analyzing system. One hour before middle cerebral artery occlusion, enalapril or moexipril were administered i.p. at doses of 0.03 to 3 mg/kg. Data are given as means ± S.D. of 15–18 animals. Different from control: ** $P < 0.01$ by ANOVA 1 with subsequent Duncan's test.

3.6. Effect of moexipril in a rat model of focal cerebral ischemia

The neuroprotective activity of moexipril was tested in a rat model of focal cerebral ischemia. The infarct volume (mm^3) was determined 7 days after middle cerebral artery occlusion. Moexipril (0.01 mg/kg), administered i.p. 1 h before middle cerebral artery occlusion, significantly attenuated the cortical infarct volume from 114.4 to 98.2 mm^3 ($P < 0.05$) as compared to non-treated animals. At dosages higher than 0.01 mg/kg moexipril did not reduce the infarct volume of the rat brain (data not shown).

4. Discussion

In this study we demonstrated that the angiotensin-converting enzyme inhibitors enalapril and moexipril were protective against glutamate-, staurosporine- or $Fe^{2+/3+}$-induced damage in cultured neurons although the neurotoxic mechanisms of the damaging agents were partially different. Overactivation of glutamate receptor was known to entail excitotoxic cell damage via various processes including Ca^{2+} overload and activation of proteases, phospholipases and endonucleases (Tymianski et al., 1993; Prehn and Krieglstein, 1996). Ferrous ions could trigger DNA strand breaks (Hartwig and Schlepegrell, 1995). The mycotoxin staurosporine has been shown to induce apoptosis activating a cell death program common to nearly all cells and the proapoptotic effect of staurosporine involved activation of the sphingomyelin–ceramide pathway with an onset of caspase cascade (Bertrand et al., 1994; Wiesner and Dawson, 1996; Krohn et al., 1998). Besides these different mechanisms, glutamate-, staurosporine- and $Fe^{2+/3+}$-induced elevation of intracellular reactive oxygen species is a common mechanism of these agents which seems to play an important role in ongoing cell death (Lafon-Cazal et al., 1993; Prehn et al., 1997). Oxygen radicals, containing only a single electron in the outer electron orbital, cause high chemical reactivity capable of damaging lipids, proteins and nucleic acids (Barber and Harris, 1994). In addition, an increased release of excitatory amino acids could be observed in the presence of oxygen free radicals indicating that reactive oxygen species reinforce the deleterious cascade of excitotoxic damage (Pellegrini-Giampietro et al., 1988). Several studies revealed that antioxidants like tocopherol, ascorbic acid and glutathione as well as the support of enzymatic defense mechanisms against oxidative damage promote cellular survival in various in vitro and in vivo models (Liu et al., 1989; Clemens and Panetta, 1994; Stoyanovsky et al., 1998). Our data showed that enalapril and moexipril were equally potent free radical scavengers inhibiting staurosporine-, glutamate- and $Fe^{2+/3+}$-induced reactive oxygen species generation. Both angiotensin-converting enzyme inhibitors were also able to improve cellular viability of chick embryonic neurons when simultaneously given to the culture medium with the damaging agents L-glutamate or $Fe^{2+/3+}$. In addition, enalapril and moexipril significantly reduced staurosporine-induced neuronal apoptosis. Because the antioxidant activities of the angiotensin-converting enzyme inhibitors paralleled their neuroprotective capacities, we conclude that in our models the radical scavenging properties of the angiotensin-converting en-

zyme inhibitors enalapril and moexipril represent the key event in promoting neuronal survival. This is in accordance with other authors who observed protective effects of angiotensin-converting enzyme inhibitors in myocardial cells due to their ability to exert radical scavenging activities (Anderson et al., 1996; Satoh and Matsui, 1997). The antioxidant properties of angiotensin-converting enzyme inhibitors could be caused by a direct chemical interaction of angiotensin-converting enzyme inhibitors and reactive oxygen species but also intracellular antioxidant pathways may play a role. It has been described that captopril and enalapril were able to enhance antioxidant defenses by upregulation of superoxide dismutase or glutathione peroxidase in different mouse tissues (De Cavanagh et al., 1997).

To clarify whether the neuroprotective properties of the angiotensin-converting enzyme inhibitors enalapril and moexipril obtained in vitro were also relevant in vivo we investigated the influence of these drugs in a mouse model of permanent focal cerebral ischemia. We demonstrated that 1 h pretreatment with 0.3 mg/kg moexipril as well as 0.03 mg/kg enalapril could reduce the infarct volume caused by middle cerebral artery occlusion. Neither lower nor higher doses were effective indicating a therapeutic window for angiotensin-converting enzyme inhibitor treatment. Angiotensin-converting enzyme inhibitors are well known to reduce blood pressure, and although long-term blood pressure control is thought to reduce the incidence of stroke, acute blood pressure reduction after stroke is an event which worsens neurological outcome (Kelley, 1996; Lees and Dyker, 1996). Therefore, we presumed that doses of enalapril and moexipril higher than 0.3 mg/kg which did not protect mice against cerebral ischemia could decrease mean arterial blood pressure. To address this hypothesis and to confirm the protective effects observed in the mouse model we additionally tested moexipril in a rat model of focal cerebral ischemia. Here, we monitored the physiological variables mean arterial blood pressure, pCO_2, pO_2, pH and glucose levels. Again, moexipril was able to reduce the brain infarct volume after ischemia caused by middle cerebral artery occlusion. Interestingly, only the dose of 0.01 mg/kg which did not affect blood pressure levels was effective whereas 0.1 mg/kg moexipril that significantly decreased mean arterial blood pressure of normotensive rats when measured 30 and 45 min after drug administration failed to exert protection against ischemic damage. Therefore, it seems to be important to achieve a plasma concentration of the angiotensin-converting enzyme inhibitor which does not cause hypotension in order to gain acute protection against stroke.

Many pathophysiological events responsible for neuronal degeneration like activation of excitatory processes with subsequent accumulation of intracellular Ca^{2+} are triggered by cerebral ischemia (Choi, 1988). These events initiate a cascade with deleterious consequences, the mechanism of which are not yet completely understood. However, the formation of reactive oxygen species seems to represent a final pathway of ischemic neuronal damage (Siesjö et al., 1989; Christensen et al., 1994). Especially in the penumbra zone of the developing infarct after middle cerebral artery occlusion a persisting blood flow with concomitant oxygen supply is maintained (Ginsberg and Pulsinelli, 1994). In the penumbra region, free radicals were suggested to be the major mediator of increased neuronal cell death. It was shown that radical scavengers protected penumbral brain tissue and reduced cerebral damage after ischemia (Krieglstein and Wolz, 1996). Thus, we suggest that also in vivo the radical scavenging properties of the angiotensin-converting enzyme inhibitors enalapril and moexipril which we clearly demonstrated in vitro could be an important mechanism of the observed cerebroprotective effect.

However, in our study the effective concentrations of the angiotensin-converting enzyme inhibitors used in vitro were relatively high as 1 μM was necessary to achieve reactive oxygen species scavenging as well as neuroprotection. In contrast, the presumable plasma concentration of the angiotensin-converting enzyme inhibitors in vivo following administration of the effective dose (0.01 mg/kg) in the rat model was expected to be lower. As we have no data about the concentration of enalapril and moexipril within brain tissue after i.p. administration it is difficult to compare the doses used in vivo with in vitro concentrations. Nevertheless, the differences in the effective doses of the angiotensin-converting enzyme inhibitors between the in vivo and the in vitro experiments indicate that a participation of the free radical scavenging properties in the apparent neuroprotective effect in vivo remains unclear. Furthermore, the necessary concentrations of the prodrugs enalapril and moexipril to exert radical scavenging activities in vitro were above the range of the IC_{50}-values for inhibition of plasma angiotensin-converting enzyme and purified angiotensin-converting enzyme from rabbit lung (Edling et al., 1995). Thus, we cannot exclude that other mechanisms of action could also contribute to the observed protective capacities of angiotensin-converting enzyme inhibitors. Trandolapril and quinapril, for instance, have been demonstrated to protect spontaneously hypertensive rats from stroke by inhibiting fibrinoid necrosis (Vacher et al., 1993; Richer et al., 1994). Furthermore, the role of angiotensin-converting enzyme inhibitor-induced increase in endogenous bradykinin concentration was considered to be involved in the protection of guinea pig heart by ramipril (Massoudy et al., 1994).

The question arises whether inhibition of the cerebral renin–angiotensin system is involved in angiotensin-converting enzyme inhibitor-mediated neuronal protection. Recently, some studies have focused on the effect of angiotensin II on cellular viability. There is evidence that exogenous angiotensin II precedes deleterious events within several types of cells. For example, angiotensin II was shown to induce apoptosis in cardiac myocytes and human

endothelial cells, and different mechanisms like activation of caspases and p53 protein as well as elevation of intracellular Ca^{2+} levels and inactivation of the antiapoptotic protein Bcl-2 have been discussed (Dimmeler et al., 1997; Horiuchi et al., 1997; Leri et al., 1998). However, it seems unlikely that in our in vitro studies the angiotensin-converting enzyme inhibitor mediated suppression of angiotensin II formation is involved in neuroprotection, because even if angiotensin II was secreted by the neurons under damaging conditions it would have been rapidly diluted in the culture medium and thus not reach concentrations high enough to induce neuronal damage. In vivo, it has been shown that angiotensin II is a potent cerebral vasoconstrictor of smooth muscle cells directly and indirectly by elevating the release of catecholamines and excitatory neurotransmitters from sympathetic neurons (Dzau, 1988). Angiotensin II may impair cerebral blood flow during ischemia and therefore trigger ischemic damage (Haas et al., 1985). In contrast, there is evidence that angiotensin II may also have protective effects and recent data showed that angiotensin II promoted the regeneration of retinal neurons (Lucius et al., 1998). Moreover, an increase in blood pressure and collateral blood flow has been suggested to mediate the protective effect of angiotensin II in a model of focal cerebral ischemia in gerbils (Kaliszewski et al., 1988). We assume that although angiotensin-converting enzyme inhibition probably occurred, neither of the abovementioned mechanisms related to angiotensin II is a dominant factor in our in vivo models because we showed acute effects while in previous studies a prolonged pretreatment was examined which is necessary for a constant reduction of angiotensin II levels in the central nervous system (Stier et al., 1989). However, the functional role of cerebral angiotensin II in ischemic processes remains unclear. Because angiotensin-converting enzyme inhibitors have been shown to cross the blood-brain barrier under ischemic conditions and to inhibit angiotensin-converting enzyme in cerebral brain tissue (Werner et al., 1991; Jouquey et al., 1995) further studies have to clarify whether this inhibition of brain renin–angiotensin system might contribute to the angiotensin-converting enzyme inhibitor-mediated acute protection against stroke in normotensive animals.

Taken together, we conclude that the angiotensin-converting enzyme inhibitors enalapril and moexipril possess neuroprotective properties most importantly due to their ability to scavenge reactive oxygen species. Beside blood pressure reduction, the neuroprotective capacities of angiotensin-converting enzyme inhibitors could be an important additional benefit for the treatment of hypertensive patients with an elevated risk of stroke.

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