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In vivo neuroprotective effects of ACEA 1021 confirmed by magnetic resonance imaging in ischemic stroke

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Abstract

The neuroprotective activity of ACEA 1021 (5-nitro-6,7-dichloro-1,4-dihydro-2,3-quinoxalinedione; licostinel), a selective antagonist at the strychnine-insensitive glycine site associated with the NMDA receptor complex, has been investigated in various models of focal cerebral ischemia. In isoflurane-anaesthesised Wistar rats with permanent ipsilateral carotid artery ligation and transient middle cerebral artery occlusion (duration of occlusion, 2 h) followed by reperfusion (24 h), intravenous administration of ACEA 1021 (bolus: 10 mg/kg, 15 min after the onset of middle cerebral artery occlusion; infusion: 7 mg/kg/h for 6 h beginning 30 min after occlusion of the artery) produced a 32% reduction in infarct volume. Similarly, in Sprague–Dawley rats with transient middle cerebral artery occlusion (2 h) followed by 24 h of reperfusion, identical treatment with ACEA 1021 decreased infarct size by 39%. Magnetic resonance imaging (MRI) confirmed these effects in the transient model, in that infarct volume observed using apparent diffusion coefficient (ADC) maps was significantly smaller after 24 h in the ACEA 1021-treated rats compared with Tris-treated controls. Furthermore, the increase in perfusion signal intensity after reperfusion was more pronounced in the ACEA 1021-treated rats than in controls. In Fisher 344 rats with permanent occlusion of the middle cerebral artery, ACEA 1021 induced a dose-related decrease in infarct volume, which was associated with an improvement in neurological outcome as measured by the rope suspension procedure. Administration of the same dose regimen, as above, in Fisher rats with permanent middle cerebral artery occlusion reduced infarct volume by 68%. This dose was as effective when administration was delayed for 2 h. In mice with permanent middle cerebral artery occlusion, ACEA 1021 (5 mg/kg, i.v., 5 min after occlusion; 30 mg/kg, s.c., 1 and 4 h post-middle cerebral artery occlusion) decreased infarct size by 42%. The consistent anti-ischemic effects of ACEA 1021 make it a valuable compound for exploratory stroke research.

Keywords: Middle cerebral artery occlusion; Ischemia; Reperfusion; Body temperature; Neurological deficit; Diffusion–perfusion magnetic resonance imaging

1. Introduction

Acute stroke affects more than 780,000 people annually in the USA, being the third leading cause of death and one of the leading causes of disability. The treatment of stroke remains an important unmet medical need and, at present, there is no treatment that reduces the neurological symptoms of stroke, except for tissue plasminogen activator, in a small percentage of patients when administered within 3 h of symptoms onset.

The development of neuronal damage following ischemia is thought to be predominantly due to the excessive increase of glutamate in the synaptic cleft (Butcher et al., 1990; Choi et al., 1987; Meldrum and Garthwaite, 1990). Several studies have indicated that many compounds acting at excitatory amino acid receptors have beneficial effects in conditions of cerebral ischemia, including noncompetitive (Gill et al., 1987; McCulloch, 1992) and competitive NMDA receptor antagonists (Boast et al., 1988; Grotta et al., 1990; Park et al., 1992). However, early NMDA antagonists have had little clinical value for stroke due to their side effect profile, including learning impairment, neurotoxicity, and psychotomimetic actions. Another approach to the reduction of ischemic brain damage is to target the glycine recognition site of the NMDA receptor complex. Compounds that are

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potent and selective antagonists at this strychnine-insensitive glycine site have been synthesized. 7-Chlorokynurenic acid has been reported to be neuroprotective in ischemia-induced neuronal damage in hippocampal slices in vitro (Newell et al., 1995), to reduce ischemia-induced CA1 cell loss in the gerbil (Pelligrini-Giampietro et al., 1994; Wood et al., 1992), and to attenuate ischemia-induced learning deficits (Wood et al., 1993). More recently, newer glycine site antagonists have been reported to be neuroprotective in models of focal cerebral ischemia (Gill et al., 1995; Warner et al., 1995; Takaoka et al., 1997; Bordi et al., 1997), global cerebral ischemia (Hicks et al., 1999), and ischemic brain damage resulting from acute subdural hematoma (Tsuchida and Bullock, 1995).

ACEA 1021 (5-nitro-6,7-dichloro-1,4-dihydro-2,3-quinoxalinedione; licostinel) is a systemically active antagonist of the NMDA receptor glycine site. It is a potent competitive antagonist of glycine at NMDA receptors in *Xenopus* oocytes expressing rat brain mRNA ($K_b = 6-8$ nM), in rat cortical neurons ($K_b = 5-7$ nM), and in *Xenopus* oocytes expressing cloned NMDA receptors ($K_b = 2-13$ nM; Keana et al., 1995; Woodward et al., 1995). Although quinoxalinediones are also known to block non-NMDA receptors, ACEA 1021 displays ~250-fold selectivity for NMDA over non-NMDA receptors in vitro (Keana et al., 1995; Woodward et al., 1995). With respect to subtypes of non-NMDA receptors, ACEA 1021 has similar activity at cortical AMPA and dorsal root ganglion kainate receptors (K_b 2.5 and 0.9 μM, respectively) (Wilding and Huettner, 1996). In the present study, the anti-ischemic effects of ACEA 1021 have been investigated in different rodent models of cerebral ischemia to examine if its neuroprotectant effects may be species- or strain-specific. Secondly, the anti-ischemic effects of ACEA 1021 have been examined in different stroke models to determine if its neuroprotectant effects are dependent on reperfusion. The therapeutic window of ACEA 1021 has also been evaluated in permanent focal ischemia.

The goal of emerging treatment strategies is to minimize the progression of tissue damage in the acute phase of stroke. To this end, diffusion-weighted imaging (DWI) and perfusion-weighted imaging (PWI), two magnetic resonance technologies, have been increasingly used in recent years for the evaluation of acute ischemic stroke. Acute DWI abnormalities are markers of focal ischemic brain injury (Moseley et al., 1990), whereas PWI indicates the presence of disturbances in microcirculatory perfusion. Hence, the effects of ACEA 1021 have been investigated on DWI and PWI images to determine if the DWI images confirm the histological assessment of infarct volume with tetrazolium and to examine if ACEA 1021's effect on infarct volume is reflected by a change in perfusion.

Many studies have demonstrated that the extent of ischemic damage is influenced by brain temperature during the ischemic period, and, particularly, the beneficial effects of hypothermia on infarct size (Morikawa et al., 1992; Barone et al., 1997). In the present study, the effects of ACEA 1021 on body temperature were also examined since hypothermia could influence an anti-ischemic activity of ACEA 1021.

2. Methods

Male Fisher 344 rats weighing 260–300 g, Sprague–Dawley and Wistar rats weighing 280–320 g, and CD-1 mice weighing 35–40 g were obtained from Charles River Laboratory (Wilmington, MA, USA) 1 week before surgery. Rats were housed two per cage and mice five per cage in a climate-controlled colony and kept on a standard 12-h light/dark cycle with free access to food and water.

All animal use procedures were conducted according to NIH guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Aventis Pharmaceuticals.

2.1. Animal preparation

Rats, fasted from 3:00 p.m. on the day prior to surgery, were anaesthesised with 5% isoflurane (Aerrane, Fort Dodge, IA, USA) in a mixture of 30% O_2 and 70% N_2O. Body temperature was maintained normothermic (37 °C) by means of a heating pad placed under the animal, and temperature was monitored by means of a rectal temperature probe. Mice were anaesthesised with chloral hydrate (400 mg/kg, i.p.) and body temperature was maintained normothermic as described above. All the major surgical procedures were carried out using aseptic conditions and sterilized instruments. A femoral artery catheter was inserted for measurement of blood gases, pH, and haemodynamic parameters. The right external jugular vein was cannulated for drug administration with PE 50 tubing, which was exteriorized at the previously shaven scruff of the neck and sutured in place. Wound clips closed the thorax incision.

2.2. Permanent model of focal ischemia

2.2.1. Fisher 344 rats

A small incision was made in the previously shaved upper thorax of the rat. The right common carotid artery was isolated and occluded by means of two ligatures. The permanent model of middle cerebral artery occlusion was performed based on a modified technique of Tamura et al. (1981). Rats were placed in a lateral position under an operating microscope. A curved vertical incision approximately 3 mm in length was made between the animal's right orbit and external auditory canal. The temporalis muscle was deflected to allow access for the craniotomy, which was made 3 mm anterior and 1 mm lateral to the foramen ovale with a dental drill. The dura was incised and the right middle cerebral artery exposed and coagulated approximately 2 mm proximal to the olfactory tract by bipolar electrocoagulation with fine forceps (Vetroson, V-10 Bipolar

Electrosurgical Unit; Summit Hill Laboratories, Navesink, NJ, USA). After coagulation, the artery was cut to avoid recannulization. The craniotomy was covered with bone wax, the muscle was allowed to fall back into place, and the wound was sutured. Two hours following occlusion of the artery, the rats were tested on a standard neurological battery to confirm the presence of a neurological deficit.

2.2.2. CD-1 mice

Under an operating microscope, a 3-mm vertical skin incision was made 2 mm behind the right orbit and the temporal muscle deflected. A small craniotomy was carried out at the point where the rostral end of the zygoma fuses to the temporal bone. The dura was incised and deflected, and the distal part of the right middle cerebral artery was exposed. The artery was occluded upstream to the main bifurcation by bipolar electrocoagulation with fine forceps. After surgery, the mice were returned to their common cage, the temperature of which was kept between 31 and 33 °C until the following morning.

2.3. Transient model of focal ischemia in Wistar and Sprague–Dawley rats

In Wistar rats, the right common carotid artery was ligated. In both Wistar and Sprague–Dawley rats, the transient model of middle cerebral artery occlusion was based on techniques described by Zea Longa et al. (1989) and Belayev et al. (1996). By means of an operating microscope, the right common carotid artery was exposed through a midline neck incision. The right superior thyroid, occipital, and pterygopalatine arteries were ligated and cut. A poly-L-lysine-coated 3-0 nylon monofilament with a heat-blunted tip was inserted through the proximal external carotid artery into the internal carotid artery and pushed forward a distance of 19 or 20 mm from the carotid bifurcation, depending on the weight of the rat, so as to occlude the origin of the middle cerebral artery. After suture placement, the neck incision was closed and the animal was allowed to regain consciousness. Two hours following occlusion of the artery, the rats were tested on a standard neurological battery to confirm the presence of a neurological deficit. Animals that did not exhibit a forelimb flexion were excluded from further study. At this time, the rats were reanaesthetised and the intraluminal suture was completely withdrawn to restore the blood supply.

2.4. Behavioural testing (neurological battery)

2.4.1. Postural reflex test

Two hours following the onset of ischemia, all rats were subjected to the postural reflex test (which is designed to examine upper body posture), in which they were suspended by the tail above a flat surface (Bederson et al., 1986). A normal rat would extend the entire body and both forelimbs towards the surface. Rats with an infarction would consistently flex the contralateral limb and show signs of body rotation.

Prior to surgery and 24 h postischemia, a battery of neurological tests was performed to ascertain neurological function. These tests included the forelimb flexion, as described above.

2.4.2. The grip test

The grip test, which determines the forepaw grip capacity, was assessed. The animal was held by the body and placed with forepaws on the apparatus (Columbus Instruments, Columbus, OH 43204, USA). It was then gently—and in an even fashion—pulled away from the apparatus and the grip strength noted.

2.4.3. The hindlimb placement test

On removal of a hindlimb from a surface, a normal rat immediately tries to replace it. A delayed placement or no replacement is seen with injured rats.

2.4.4. The vertical screen test

The vertical screen test is a test in which a rat is placed facing downward. Normally, a rat can make the 180° turn and proceed to the top of a screen. Injured animals cannot make this turn to proceed up the screen.

Each test received a score from 1 to 3 reflecting the severity of the deficit, with 3 being the worst score. These individual scores were then added to give a cumulative score, which gave an indication of the neurological deficit of each animal.

2.4.5. The prehensile test

In the prehensile test, a rope 70 cm long and 1 cm in diameter was suspended vertically. Each rat was allowed to cling to the rope by its forepaws and the length of time it remained suspended from the rope was recorded in seconds.

2.5. Histology

Twenty-four hours post-middle cerebral artery occlusion, the animals were anaesthesised and sacrificed, and the brains were removed and cut into 2-mm coronal sections. The coronal sections were incubated with (2%) triphenyl tetrazolium chloride to demonstrate the infarcted brain tissue, the extent and location of which were verified and quantified by means of image analysis using an MCID system. The infarct volume was expressed as cubic millimeters and as a percentage after correction for oedema formation in the ipsilateral hemisphere, using the "indirect" method of Swanson et al. (1990).

2.6. Magnetic resonance imaging (MRI) measurements

Measurements were carried out on Sprague–Dawley rats subjected to the transient model of middle cerebral artery occlusion as described earlier (Section 2.3).

Measurements were performed 30 min, 3 h, and 24 h after the onset of ischemia on a 7-T Bruker BIOSPEC experimental scanner (DBX; Bruker Medical, Ettlingen, Germany) with a 30-cm bore magnet and actively shielded gradient coils (200 mT/m; rise time <80 μs).

A 72-mm resonator was used for *rf* transmission; signals were detected with a 35-mm inductively coupled surface coil placed over the skull of the animal. The *rf* coils were decoupled from each other—the transmitter coil actively and the receiver coil passively.

Using gradient-echo imaging, sagittal pilot scans were performed to assure accurate positioning of the animal in the magnet. For this purpose, the coronal plane 5.9 mm posterior to the rhinal fissure was placed in the isocenter of the magnet, thus focusing on the center of the ischemic territory resulting from the middle cerebral artery occlusion.

For the determination of the temporal evolution of the ischemic lesion, two NMR imaging modalities were used. A field of view of 3.2 cm, a slice thickness of 1.5 mm, and an interslice distance of 2 mm were used for both sequences. Multislice packages were recorded by placing the center of the multislice imaging packet 5.9 mm posterior to the rhinal fissure.

Diffusion-weighted imaging was performed using a Stejskal–Tanner spin-echo sequence [echo time (TE) = 37.2 ms, repetition time (TR) = 2325 ms, eight slices] in six rats per group. To enable quantification of the apparent diffusion coefficient (ADC) of brain water, three *b* values were used ($b = 50$, 825, and 1600 s/mm^2). ADC maps were calculated pixelwise using the monoexponential model (Le Bihan et al., 1986).

Perfusion-weighted imaging was performed with an ultrafast version of the arterial spin tagging technique (Kerskens et al., 1996; Franke et al., 2000) in four rats per group. In independent experiments, three coronal slices were recorded, thus covering the central part of the ischemic lesion. Measurement parameters were: TE = 3.5 ms, TR = 7.4 ms, matrix = 128 × 64, average = 8. Each experiment consisted of two image acquisitions separated by a recovery period. During the first acquisition, blood flowing through the neck was adiabatically inverted (preparation time = 3 s; *z*-gradient = 5 mT/m; B1 field = 150 mG; off-resonance frequency = 6000 Hz; mean preparation distance = 2.8 cm upstream from the imaging plane); in the second acquisition, inflowing spins were left undisturbed. Both phases were separated by a recovery period of 10 s. In each perfusion experiment, the two images suffered the same signal loss due to magnetization transfer effects but differed in the magnetization of the inflowing blood. Perfusion-weighted images were obtained by subtraction of the acquisitions with and without prior adiabatic spin inversion. In the second acquisition, inflowing spins were left undisturbed. Perfusion-weighted images were obtained by subtraction of the acquisitions with and without prior adiabatic spin inversion.

Data were transferred to a PC and image analysis was carried out using the image processing software Scion Image for Windows (Scion Corporation, Frederick, MD, USA). Lesion volumes were calculated using ADC maps, as the ischemic lesion area was estimated by summing up all pixels with a relative ADC $<80\%$ compared to the healthy, contralateral hemisphere (Hoehn-Berlage et al., 1995). Perfusion signal intensities are referred to the homologous contralateral regions and given as ratios of ipsilateral to contralateral values.

2.7. Drug treatment

ACEA 1021 treatment was initiated 0.25, 1, 2, and 3 h after the onset of permanent middle cerebral artery occlusion. The rats received a single intravenous bolus injection of ACEA 1021 (5 or 10 mg/kg) followed by an infusion (3.5 or 7.0 mg/kg/h) for the following 6 h. These doses were selected on the basis of preliminary experiments, when it was shown that they induced a decrease in infarct volume in a transient model of middle cerebral artery occlusion and reperfusion. Rats subjected to transient middle cerebral artery occlusion received a bolus of 10 mg/kg ACEA 1021 15 min after the onset of ischemia, followed by an infusion of 7 mg/kg/h, 30 min after the onset of ischemia, which was infused for the following 6 h. Mice were injected intravenously 5 min after permanent middle cerebral artery occlusion with ACEA 1021 (5 mg/kg) followed by subcutaneous injections (30 mg/kg) 1 and 4 h post-artery occlusion. Control animals received an equivalent volume of a mixture 1:1 50 mM Tris [TRIZM, Tris(hydroxymethyl)aminomethane] and 50 mM Tris, pH 7.8. ACEA 1021 was initially dissolved in 50 mM Tris, using half the required final volume in plastic vials. Solutions were ready for use after the addition of the second half of the volume, 50 mM Tris, pH 7.8.

2.8. Statistical analysis

Results are expressed as the mean ± S.E.M. of 4–12 animals in each group. An ACEA 1021-treated group was compared to a vehicle-treated control group by means of a Student's *t* test for unpaired data. $P<0.05$ was considered statistically significant. In cases where more than one ACEA-treated group was involved, a one-way ANOVA with application of Dunnett's multiple comparison test was used to determine the level of significance.

3. Results

3.1. General physiological parameters

Rectal body temperature, blood gases, pH, and cardiovascular parameters were not affected by the operative procedures associated with middle cerebral artery occlusion, be it transient or permanent. In Sprague–Dawley rats, for example, the pO_2 15 min prior to and 15 min postsurgery

was 167.5 ± 9 and 161.3 ± 9 mm Hg, respectively, and pCO_2 was 38.7 ± 1 and 36.4 ± 5 mm Hg, respectively.

3.2. Permanent model of focal ischemia

3.2.1. Fisher 344 rats

This model of distal middle cerebral artery occlusion produced an infarct of 68.4 ± 13.3 mm^3 with mainly cortical involvement. ACEA (10 mg/kg), administered as a bolus 15 min after the onset of ischemia followed by an infusion of 7 mg/kg/h, beginning 30 min after ischemia and continuing for the following 6 h, produced a significant 68% decrease in infarct volume (Fig. 1A).

The anti-ischemic activity of ACEA 1021 observed in the permanent middle cerebral artery occlusion rat model was dose-related (Fig. 1B). A lower dose of 5 mg/kg bolus followed by an infusion of 3.5 mg/kg/h decreased infarct volume by 52%, whereas a twofold higher dose (10 mg/kg bolus and an infusion of 7 mg/kg/h) decreased infarct volume by 74%. This reduction in infarct volume was associated with an improvement in neurological outcome as assessed by the rope suspension test (Fig. 2), although effects on other neurological procedures did not attain statistical significance (Table 1).

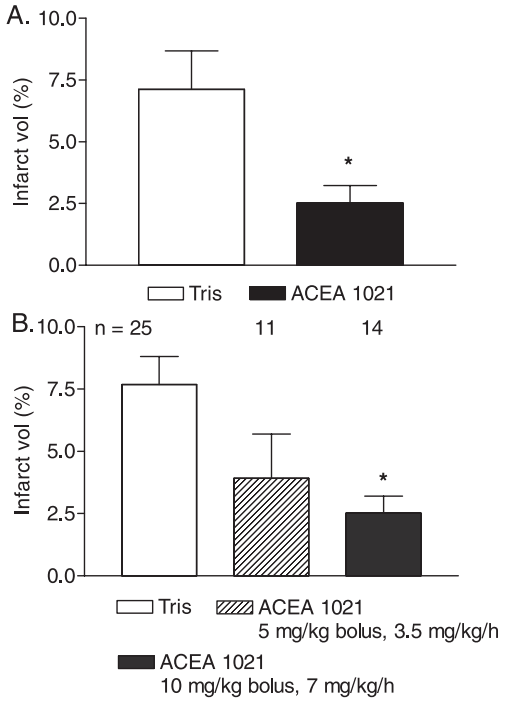

Fig. 1. (A) The effects of ACEA 1021 in Fisher 344 rats subjected to permanent middle cerebral artery occlusion. ACEA 1021 (10 mg/kg bolus) administered as a bolus 15 min after the onset of ischemia followed by an infusion of 7 mg/kg/h, beginning 30 min after ischemia and continuing for 6 h, decreased infarct volume, measured 24 h postinsult. Infarct volume is expressed as a percentage after correction for oedema formation in the ipsilateral hemisphere, using the "indirect" method of Swanson et al. (1990). Results are expressed as mean ± S.E.M. of 17 and 14 rats in the Tris- and ACEA-treated groups, respectively, and were compared by means of a Student's t test for unpaired data (*$P < 0.05$). (B) The anti-ischemic activity of ACEA 1021 observed in the permanent middle cerebral artery occlusion model was dose-related, as shown by the effects of two doses of ACEA 1021 on infarct volume, measured 24 h postinsult in Fisher 344 rats. The data are expressed as a percentage after correction for oedema formation in the ipsilateral hemisphere, using the "indirect" method of Swanson et al. (1990). Results are expressed as mean ± S.E.M. and were compared by means of ANOVA with application of Dunnett's multiple comparison test (*$P < 0.05$).

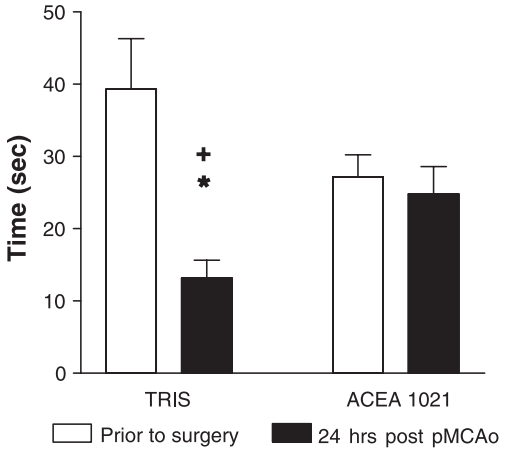

Fig. 2. The ability of rats to remain suspended from a rope, measured in seconds, before permanent middle cerebral artery occlusion and 24 h after surgery and treatment with ACEA 1021 (10 mg/kg bolus followed by a 6-h infusion of 7 mg/kg/h), or Tris vehicle. Results are expressed as the mean time spent suspended from a rope ± S.E.M. of 12 and 8 rats in the ACEA 1021- and Tris-treated groups, respectively. A Student's paired t test was used to compare data obtained in the same animal before and after surgery ($^{+}P < 0.05$) and a Student's unpaired t test to compare results obtained in ACEA 1021- and Tris-treated animals (*$P < 0.05$).

In Fig. 3, the therapeutic window of opportunity can be seen from data generated in Fisher 344 rats subjected to the permanent model of middle cerebral artery occlusion. ACEA 1021 induced a significant reduction in infarct volume if the onset of treatment was delayed for 0.25, 1.0, or 2.0 h postocclusion. After a delay of 3.0 h, treatment with ACEA 1021 did not significantly decrease infarct volume.

Hourly measurement of body temperature demonstrated that ACEA 1021, at a dose of 5 mg/kg bolus followed by an infusion of 3.5 mg/kg/h for 6 h, significantly decreased body temperature to a mean of 36.3 °C after 1 h and then gradually returned to control values. The higher dose of 10 mg/kg bolus followed by an infusion of 7.0 mg/kg/h markedly decreased body temperature to 35.1 °C, 4 h after the start of infusion (Fig. 4).

Table 1
The cumulative behavioural score measured prior to permanent middle cerebral artery occlusion and 24 h post-ischemia onset in Fisher 344 rats

Cumulative score	Tris vehicle (2 ml/kg bolus, 0.9 ml/h, $n = 8$)	ACEA 1021 (5.0 mg/kg bolus, 3.5 mg/kg/h, $n = 12$)	ACEA 1021 (10.0 mg/kg bolus, 7.0 mg/kg/h, $n = 8$)
Presurgery	0	0.1 ± 0.1	0
24 h post-pMCAo	3.8 ± 0.4	2.3 ± 0.4	3.8 ± 0.9

pMCAo = permanent middle cerebral artery occlusion.

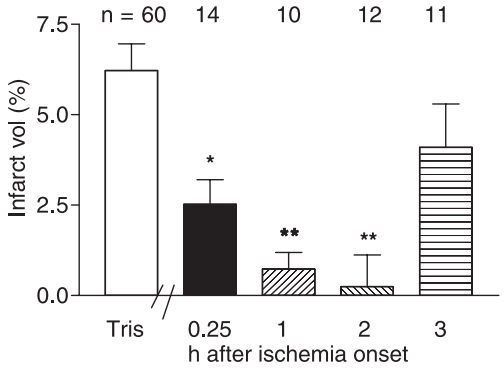

Fig. 3. The window-of-opportunity after permanent middle cerebral artery occlusion during which time ACEA 1021 can be administered and still produce a significant decrease in infarct volume, measured 24 h postinsult. ACEA 1021 (10 mg/kg bolus followed by a 6-h infusion of 7 mg/kg/h) or Tris was administered to rats subjected to permanent middle cerebral artery occlusion at the time intervals described in the graph. Results are expressed as mean ± S.E.M. and were compared by means of ANOVA with application of a Dunnett's multiple comparison test (*$P<0.05$, **$P<0.01$).

3.2.2. CD-1 mice

ACEA 1021 (60 mg/kg) injected subcutaneously 60 min prior to permanent occlusion in mice induced a significant decrease in infarct size; mean infarct volumes were 40.7 ± 4.0 mm^3 ($n=10$) in the Tris control group and 25.8 ± 5.3 mm^3 ($n=11$) in the ACEA 1021-treated group ($P<0.05$). However, when the same dose was administered subcutaneously 5 min after the onset of permanent middle cerebral artery occlusion, ACEA 1021 had no effect on infarct size. The Tris-treated animals had an infarct volume of 37.6 ± 4.6 mm^3 ($n=10$) and the ACEA-treated group had a mean value of 40.1 ± 4.9 mm^3 ($n=10$). A significant reduction in infarct size occurred, post-artery occlusion, when the treatment parameters were altered. ACEA 1021 treatment, initiated 5 min after the induction of permanent focal ischemia (5 mg/kg, i.v.) followed by 30 mg/kg, s.c. injected at 1 and 4 h, decreased infarct volume to 22.02 ± 3.1 mm^3 ($n=8$) from a control value of 39.0 ± 2.5 mm^3 ($n=11$; $P<0.005$).

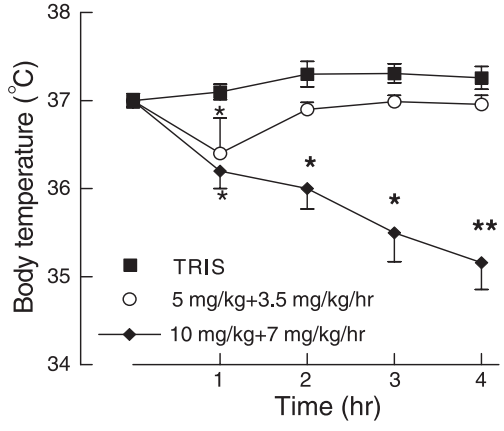

Fig. 4. The decrease in body temperature induced by ACEA 1021 in Fisher 344 rats subjected to permanent middle cerebral artery occlusion. Results are expressed as mean ± S.E.M. of eight rats in each group and were compared by ANOVA with application of Dunnett's multiple comparison test (*$P<0.05$, **$P<0.01$) when compared to Tris treatment.

3.3. Transient model of focal ischemia in Wistar and Sprague–Dawley rats

Two hours of middle cerebral artery occlusion followed by 22 h of reperfusion induced an infarct of 338.4 ± 52.5 and 379.5 ± 41.4 mm^3 in Sprague–Dawley and Wistar rats, respectively, representing $31.2 \pm 6.0\%$ and $34.4 \pm 3.0\%$ when correction is made for oedema. Administration of an intravenous bolus injection of ACEA 1021 (10 mg/kg), 15 min after the onset of ischemia followed by an infusion of 7 mg/kg/h, beginning 30 min after the onset of ischemia and continuing for 6 h, reduced the infarct volume to 206 ± 60 and 258 ± 27.0 mm^3 in Sprague–Dawley and Wistar rats, respectively. The decrease in the Wistar rats attained significance at the $P<0.05$ compared to vehicle-treated controls.

3.4. MRI

The development of the cerebral infarct volume in the tMCAo model over the 24-h period following ischemia onset in ACEA 1021- and Tris-treated rats can be seen in Fig. 6A.

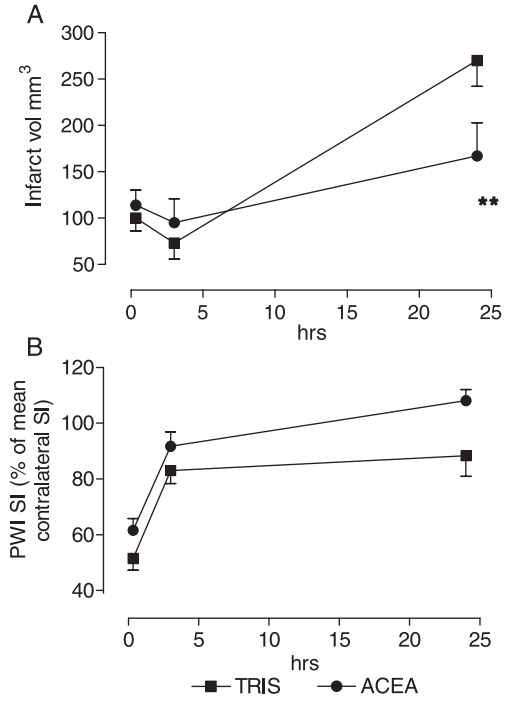

Fig. 5. (A). The decrease in infarct volume and (B) the increase in perfusion induced by ACEA 1021 (10 mg/kg bolus followed by a 6-h infusion of 7 mg/kg/h) in Sprague–Dawley rats subjected to transient middle cerebral artery occlusion and reperfusion, as assessed by MRI. The infarct volumes are expressed as mean ± S.E.M. of six rats in each group. A Student's *t* test for unpaired data was used to compare data obtained 22 h after reperfusion in the Tris- and ACEA 1021-treated groups (**$P<0.015$). The perfusion data are expressed as mean ± S.E.M. of four rats in each group.

Using ADC maps 30 min after the onset of ischemia, the detectable infarct volume was 110 ± 10 and 114 ± 15 mm^3 in the Tris- and ACEA-treated groups, respectively. By 3 h after the onset of ischemia, although there was no difference between the groups, the infarct volume decreased due to the early reperfusion time point in both groups (67 ± 17 and 95 ± 23 mm^3 for the untreated and treated group, respectively). Twenty-four hours after reperfusion, infarct volume increased again in all animals. However, it was significantly larger in the Tris-treated group, reflecting a volume of 284 ± 27 mm^3. In the ACEA 1021-treated group, an infarct volume of 167 ± 32 mm^3 was detected ($P < 0.01$; Fig. 5A).

In Fig. 5B, the development of the relative PWI SI, which reflects the ratio between the mean signal intensity of the ischemic hemisphere and that of the contralateral hemisphere, over 24 h can be seen. Again, there was no statistically significant difference in PWI SI between both groups after the onset of ischemia (51 ± 4% and 62 ± 4% in Tris- and ACEA-treated animals, respectively). After reperfusion, in the Tris-treated control group, the signal intensity had reached a value of 83 ± 5.0 at 3 h compared to 88 ± 7 at 24 h post-ischemia onset. In the ACEA-treated group, however, signal intensity increased from 92 ± 5 at 3 h to 108 ± 4 at 24 h ($P = 0.058$).

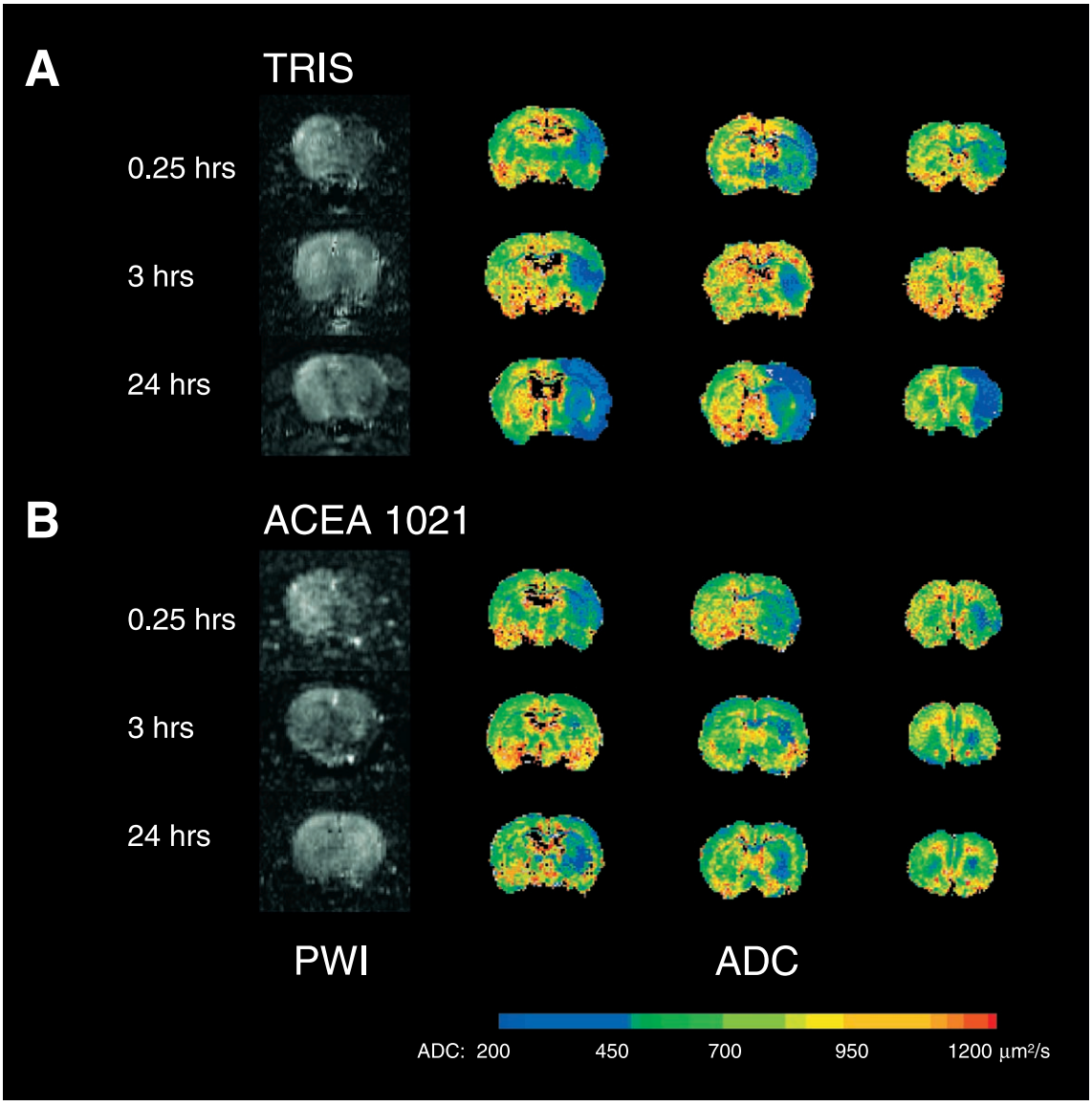

Fig. 6. Comparison of the central MRI between Tris-treated (A) and ACEA 1021-treated animals (B). In both animals, a clear perfusion deficit as well as an ADC lesion are shown under ischemic conditions on the right hemisphere. Reperfusion resulted in a strong reperfusion and reduction of the ADC lesion volume 3 h after ischemia again in the treated and untreated groups. However, 24 h after reperfusion, the ACEA-treated animal showed a hyperperfusion in the ischemic hemisphere resulting in a significant smaller infarct volume compared to the Tris-treated rat. In untreated animals, the reperfusion signal remained less than normal.

In Fig. 6, typical MR slices of an untreated and an ACAE 1021-treated animal are shown. Under ischemic conditions, a clear perfusion deficit as well as an ADC lesion are shown in both animals on the right hemisphere. Reperfusion resulted in a reduction of the ADC lesion volume 3 h after ischemia. Twenty four hours after reperfusion, the ACEA-treated animal showed a hyperperfusion in the ischemic hemisphere, resulting in a significant smaller infarct volume compared to the Tris-treated rat.

4. Discussion

Intravenous administration of ACEA 1021, an antagonist at the strychnine-insensitive glycine site associated with the NMDA receptor complex, either as a combination of an intravenous bolus and infusion or an intravenous bolus plus multiple subcutaneous injections, induced a decrease in infarct volume in both transient and permanent models of middle cerebral artery occlusion in rats and mice. These studies indicate that ACEA 1021 is able to penetrate ischemic brain tissues and reduce pathological activation of NMDA receptors. They also show that ACEA 1021 is affective against a large infarct, as in the transient model of middle cerebral artery occlusion, and also against a small infarct, which occurs on permanent distal middle cerebral artery occlusion.

ACEA 1021 induced a dose-related reduction in infarct volume with a 2-h therapeutic window-of-opportunity: ACEA 1021 was equally effective at reducing infarct volume when administered intravenously either 15 min or 2 h after the onset of permanent middle cerebral artery occlusion in rats. In mice, however, a single subcutaneous injection 5 min after the onset of middle cerebral artery occlusion was without effect, suggesting that absorption from this site may not be rapid enough. In addition to decreasing infarct volume, ACEA 1021 improved neurological outcome as assessed by a rope suspension procedure, although effects in other behavioural tests were not as robust.

Previous studies have reported that glycine site antagonists are neuroprotective in vitro and in vivo. HA-966 and 7-chlorokynurenic acid protected against hypoxia-induced increases in lactate dehydrogenase in rat cortical cell cultures (Priestley et al., 1990). ACEA 1021 and ACEA 1031 reduced cerebral infarct volumes and the incidence of hemiparesis after focal cerebral ischemia in rats (Warner et al., 1995). Moreover, ACEA 1021 reduced [^{125}I]MK-801 binding in vivo by 28% in the penumbral zone beneath experimentally induced acute subdural hematoma in rats (Di and Bullock, 1996). In addition, ACEA 1021 does not cause neuronal vacuolization or necrosis in the posterior cingulated/retrosplenial cortex of the rat as has been reported for noncompetitive and competitive NMDA receptor antagonists specific for the ion channel and glutamate recognition sites, respectively (Hawkinson et al., 1977).

The beneficial effects of ACEA 1021 were associated with a dose-related fall in body temperature, as has been reported previously in ACEA 1021 (Warner et al., 1995). These studies would suggest that the induced hypothermia could be responsible for the anti-ischemic activity of ACEA 1021, although it has been reported that the glycine site antagonist induced a significant neuroprotection even when brain temperature was controlled (Takaoka et al., 1997). In that particular study, ACEA 1021 was administered intravenously 10 min after the onset of transient middle cerebral artery occlusion (75 min occlusion) followed by 24 h of reperfusion. Such treatment resulted in a 60% reduction in mean cortical infarct volume as assessed 7 days later.

Infarct volume was decreased by about 35% in the transient model in both Sprague–Dawley and Wistar rats when assessed using the tetrazolium stain. In this instance, the Wistar rats had undergone permanent ligation of the common carotid artery as well transient middle cerebral artery occlusion. These results indicate that carotid artery occlusion does not significantly increase the infarct volume resulting from transient middle cerebral artery occlusion, although the use of two different rat strains must also be taken into consideration. Similarly, compared to Tris-treated rats, ACEA 1021 infusion resulted in a reduction of infarct volume by 41% 24 h after the onset of ischemia when infarct was assessed from the ADC maps. Furthermore, due to its noninvasiveness, MR measurements gave additional information concerning infarct growth after ischemia onset. The ischemic lesion volume improved within 1 h of reperfusion, but later decreased by the end of the experiment, which was not caused by hypoperfusion. The current findings confirmed the results presented in earlier studies showing a secondary deterioration of the lesion volume determined by histological (Li et al., 1999) and MRI investigations (van Lookeren Campagne et al., 1999; Li et al., 2000; Olah et al., 2000).

Furthermore, in the present study, the mean ipsilateral perfusion signal intensity had already reached a large value at 3 h (i.e., 1 h after the start of reperfusion), demonstrating reperfusion in both groups. However, in the ACEA-treated group, the PWI signal intensity increased further during the 22-h reperfusion period, suggesting improved perfusion compared to Tris-treated animals. Evidently, regional flow determinations could give more precise details of the contribution of perfusion-protective effects of ACEA 1021. However, absolute flow measured via autoradiography, for example, is invasive, the technique permitting determination of flow at one time point per animal alone. These results confirm the decreased infarct volume apparent in treated animals and again demonstrate the second reason for the beneficial effects of ACEA 1021—the improved reperfusion. Interestingly, in the normal conscious rat, ACEA 1021 has little effect on cerebral metabolic rate for glucose and cerebral blood flow, which is consistent with the absence of psychotomimetic effects, unlike the competitive and noncompetitive NMDA antagonists (Morimoto et al., 1998).

In conclusion, ACEA 1021 consistently decreased infarct volume in a variety of animal models. In addition, there was some correlation between infarct reduction and neurological outcome, as measured by the rope suspension procedure. The beneficial effects of ACEA 1021, however, were associated with a decrease in body temperature. Furthermore, a positive effect on reperfusion intensity could be detected using MRI. These anti-ischemic effects of ACEA 1021 make it a valuable reference compound for exploratory stroke research.

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