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MRI study on delayed ancrod therapy of focal cerebral ischaemia in rats

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Abstract

The therapeutic window for efficient post-treatment of focal cerebral ischaemia with the fibrinogen lowering agent ancrod was studied by magnetic resonance imaging (MRI) in spontaneously hypertensive rats (SHR). Ancrod or vehicle solution (0.9% NaCl) were i.v. infused (0.12 IU/kg per min) via implanted mini pumps starting 0.5, 1.5, 3 or 6 h after permanent proximal middle cerebral artery occlusion and lasting until brain mapping by multislice T2-weighted magnetic resonance imaging in vivo 24 h after middle cerebral artery occlusion. Plasma fibrinogen concentrations were measured before middle cerebral artery occlusion, before pump implantation and after magnetic resonance imaging. Total brain lesion volumes as determined by magnetic resonance imaging 24 h after middle cerebral artery occlusion were 131 ± 36 (188 ± 28)*, 151 ± 39 (194 ± 39)*, 147 ± 44 (207 ± 33)* and 209 ± 60 (214 ± 42) mm^3 in rats with 0.5, 1.5, 3 and 6 h, respectively, delay of ancrod treatment (means $\pm$ S.D., 8–11 animals/group, corresponding control groups in parentheses, * $P < 0.05$). Continuous i.v. ancrod infusions reduced plasma fibrinogen levels significantly ($P < 0.05$) in all ancrod-treated groups as compared to vehicle-treated controls until the end of the experiments 24 h after middle cerebral artery occlusion. In conclusion, significant cerebroprotection was achieved even when the onset of ancrod therapy for lowering of the plasma fibrinogen level was delayed for up to 3 h. To the best of our knowledge no drug efficacy has been reported so far with a therapeutic window of 3 h after permanent middle cerebral artery occlusion in spontaneously hypertensive rats suggesting that ancrod may provide an efficient therapy of acute human stroke. © 1997 Elsevier Science B.V.

Keywords: MRI (magnetic resonance imaging); Ancrod; Cerebral ischemia; Hypertensive rat; Fibrinogen; Middle cerebral artery occlusion; (Delayed therapy); Therapeutic window

1. Introduction

Ancrod is a thrombin-like enzyme, isolated from the venom of the Malayan pit viper, *Calloselasma rhodostoma* (formerly *Agkistrodon rhodostoma*). The compound cleaves fibrinopeptide A from fibrinogen A α chains (Ewart et al., 1970) but in contrast to thrombin it does not liberate fibrinopeptide B from the B β chain of fibrinogen (Holleman and Coen, 1970). In consequence, intravenous infusion of ancrod leads to a rapid decrease in the plasma fibrinogen concentration. Hypofibrinogenaemia may improve blood flow by reduced blood viscosity. Furthermore, reduced blood concentrations of plasminogen activator inhibitor (PAI-1), plasminogen and α_2-antiplasmin indicate enhanced endogenous fibrinolysis after ancrod treatment (Pollak et al., 1990; Prentice et al., 1993).

Several experimental investigations have shown pronounced brain lesion reductions by ancrod in various cerebrovascular disorders. A study on thrombotic cortical brain infarction revealed that the volume of brain lesion was dose-dependently diminished by ancrod in rats (Elger et al., 1997). In supplement to these findings reductions of brain lesions by ancrod have been demonstrated in middle cerebral artery occluded rats (Elger et al., 1997). An investigation on intracerebral haemorrhage has shown that ancrod does not aggravate bleeding but reduces the size of the lesion suggesting that ancrod may provide a safe therapy of stroke (Elger et al., 1995). All of these studies have methodologically in common that ancrod was administered by an intravenous infusion starting 30 min after induction of the cerebrovascular insult and lasting for 30 min only. Since most stroke patients arrive at the hospital with substantial time delays (Herderscheê et al., 1991; Harper et al., 1992; Jorgensen et al., 1996; Azzimondi et al., 1997) and since cerebroprotection by a number of drugs has been observed only by treatment before or early after experimental brain ischaemia (Chen et al., 1993;

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Simon et al., 1993; Margaill et al., 1996; Aronowski et al., 1996a), the aim of the present study was to evaluate the therapeutic window for efficient post-treatment of focal cerebral ischaemia by using various delays of ancrod infusion after permanent middle cerebral artery occlusion in rats.

2. Materials and methods

2.1. Animals

The study has been carried out in accordance to the requirements of the German laws for the protection of animals (Deutsches Tierschutzgesetz) and in compliance with the EC directive 86/609 (off J. of the E.C. No. L358/1 dated 18 Dec 1986). Male spontaneously hypertensive rats (SHR) weighing 180–300 g were obtained from Iffa Credo (Brussels). The animals were maintained under standard conditions (12 h day/night cycle, 22°C, 50% humidity) with free access to food and tap water. Before use for the experiments, the animals were allowed to recover from transportation for at least one week. The animals were fasted over night before the day of surgery for middle cerebral artery occlusion.

The left proximal middle cerebral artery was occluded by a slight modification of the technique of Tamura et al. (1981) in anaesthetised rats (sodium pentobarbital, 60 mg/kg i.p.). Briefly, a vertical 2 cm skin incision was made between the left eye and the left ear. The temporalis muscle was divided and retracted in order to expose the zygoma and the squamosal bone. Parts of the temporalis muscle were removed. The zygomatic bone was left intact. The left middle cerebral artery was exposed through a burr hole craniectomy (2 mm diameter) drilled in close proximity to the foramen ovale. The proximal portion of the middle cerebral artery was electrocauterised with bipolar forceps (Erbotom T130, Erbe, Elektromedizin, Tübingen, Germany). The burr hole was closed using tissue glue (Histoacryl®, Braun, Melsungen, Germany). Retracted parts of the temporalis muscle were put back into position and the skin was sutured. During surgery for middle cerebral artery occlusion, the rectal temperature of the anaesthetised animals was maintained at 37 ± 0.5°C by a feedback regulated heating device.

2.2. Drug treatment

Ancrod was prepared and purified from the venom of the Malayan pit viper, *Calloselasma rhodostoma*, at Knoll (Ludwigshafen, Germany).

Animals were randomly assigned to the different experimental groups before anaesthesia for surgical middle cerebral artery occlusion. Groups of this study (8–11 animals per group) were different with respect to the time delay of the start of treatment after middle cerebral artery occlusion: 1. control (0.5 h), 2. ancrod (0.5 h), 3. control (1.5 h), 4. ancrod (1.5 h), 5. control (3 h), 6. ancrod (3 h), 7. control (6 h), 8. ancrod (6 h). Ancrod was diluted in 0.9% NaCl and continuously infused by implanted osmotic mini pumps (Alzet®, Alza Pharmaceuticals, Palo Alto, CA, USA, pump rate 8 μl/h) via the femoral vein (0.12 IU/kg per min) starting with various delays after middle cerebral artery occlusion as described above. Corresponding control groups received the vehicle solution by implanted pumps. All infusions lasted until the animals were killed after the MRI measurements. To secure drug delivery into the circulation immediately after pump implantation and constant pump rate, the prefilled pumps were placed in 0.9% saline for 4 h at 37°C before implantation as suggested by the manufacturer.

2.3. Fibrinogen measurements

Blood samples (0.5 ml) were drawn by catheter from the femoral artery before middle cerebral artery occlusion, before pump implantation for ancrod infusion and by cardiac puncture 24 h after middle cerebral artery occlusion. The blood was immediately mixed (8.5 parts + 1.5 parts) with sodium citrate solution (0.11 mol/l) and centrifuged for 5 min at $5000 \times g$. Fibrinogen content of samples was determined by a slight modification of the method of Clauss (1957). Briefly, the clotting time was measured by an automated ball-type coagulometer (CL 8, Bender and Hobein, Munich) after addition of an excess of thrombin to citrated plasma. Under these conditions the clotting time depends mainly on the fibrinogen content of the plasma sample. The evaluation of the clotting time was carried out using a double logarithmic reference curve established using standards of rat fibrinogen (Sigma, Deisenhofen, Germany).

2.4. Magnetic resonance imaging in *vivo*

Animals were reanaesthetised for MRI 24 h after middle cerebral artery occlusion. Spin echo MRI was carried out on a General Electric CSI-II 2.0-T-MR-spectrometer (85.542 MHz for proton) equipped with Acustar™ self-shielded gradient coils (15 cm inner bore diameter). The animals were fixed in a home-built low pass birdcage proton imaging coil (inner diameter: 36 mm) consisting of eight segments separated by ATC-22 pF-chip capacitors. Body temperature of the animals was maintained constant by a recirculation system of warm water into the animal support. T2-weighted spin-echo images (TR = 2 s; TE = 70 ms) were obtained with a field of view of 35 mm in which four scans (NA = 4) were averaged for each one of the 128 phase encoding steps. Multislice images were acquired in 8 adjacent coronal planes, each with a slice thickness of 1.5 mm. The stereotactic positions of planes 1–8 were 13, 11.5, 10, 8.5, 7, 5.5, 4 and 2.5 mm anterior to the interaural line (Paxinos and Watson, 1986).

After processing the MRI data using the standard GEMCSI® software on a Nicolet 1280 computer the images were quantified by a computerised image analysis system (Cardio 200, Kontron, Eching/Munich, Germany). The volume of brain lesion was calculated in each animal from the hyperintense brain areas on the 8 images multiplied by the slice thickness. After cessation of the MRI measurements the animals were killed by i.v. injection (0.4 ml) of T61 (Hoechst, Unterschleissheim, Germany).

2.5. Statistical analysis

All results are presented as mean ± S.D. unless indicated otherwise. Differences between means were analysed using Student's *t*-test. Student's *t*-test for paired observations was used were appropriate. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Magnetic resonance imaging

Brain lesions were manifested as regions with increased signal intensities on multislice standard T2-weighted images taken 24 h after permanent middle cerebral artery occlusion (Figs. 1 and 2). All the animals of both the ancrod-treated groups and the corresponding vehicle-treated groups had pronounced brain lesions not only in cortical regions but also in subcortical regions of the brain hemisphere ipsilateral to the occluded middle cerebral artery. The results of the lesion volume measurements by MRI are summarised in Table 1 and Fig. 3. Lesion volume was significantly ($P < 0.05$) reduced by 30% from 188 ± 28 mm^3 in control animals ($n = 11$) to 131 ± 36 mm^3 in rats ($n = 9$) administered ancrod by continuous intravenous infusion starting 0.5 h after middle cerebral artery occlusion. Similar effects were observed even when the infusion was delayed for 3 h. Under these experimental conditions lesion volume was significantly ($P < 0.05$) reduced from 207 ± 33 mm^3 in control animals ($n = 10$) to 147 ± 44 mm^3 in ancrod-treated animals ($n = 11$). No cerebroprotection was, however, obtained in animals which had pump implantations for intravenous ancrod infusion 6 h after middle cerebral artery occlusion. Regional analysis of the magnetic resonance images revealed that brain lesion reductions were most pronounced in posterior brain regions of animals given ancrod with delays up to 3 h after permanent middle cerebral artery occlusion (Fig. 2). No significant regional differences in lesion sizes were observed in the 6 h delay group (data not shown).

3.2. Fibrinogen

The results of the repeated plasma fibrinogen measurements are summarised in Table 2. Before middle cerebral artery occlusion the baseline level of plasma fibrinogen was on average 2.3 ± 0.09 g/l (mean ± S.E.M.) in all groups. Slight reductions of plasma fibrinogen concentrations were observed 0.5 h to 6 h after middle cerebral

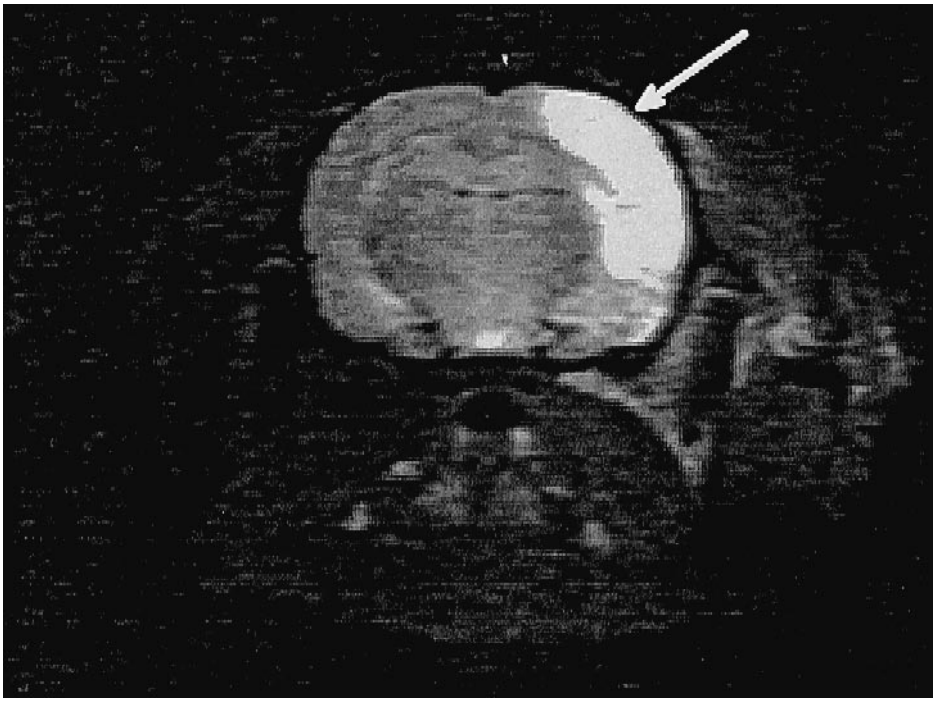

Fig. 1. Representative T2-weighted magnetic resonance image of a control rat showing a coronal brain slice measured 4 mm anterior to the interaural line 24 h after middle cerebral artery occlusion. Note the high signal intensities which indicate massive brain lesion in cortical (arrow) brain regions.

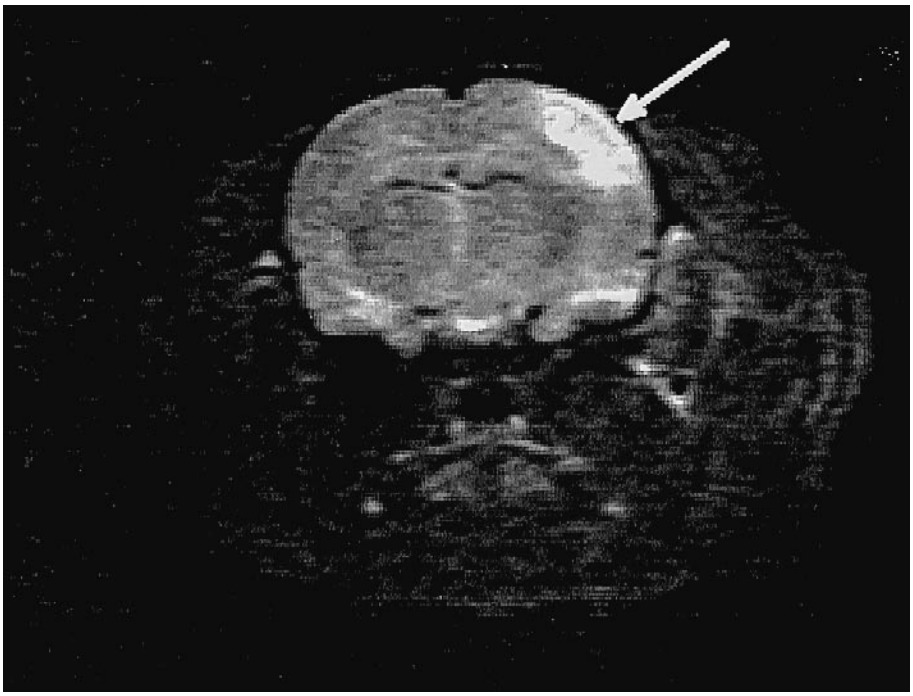

Fig. 2. Representative magnetic resonance image of an ancrod-treated rat measured corresponding to the control rat shown in Fig. 1. Ancrod infusion was started 3 h post middle cerebral artery occlusion in this rat. Note the reduced cortical brain area of increased signal intensities (arrow) in the ancrod-treated rat as compared to the control rat shown in Fig. 1.

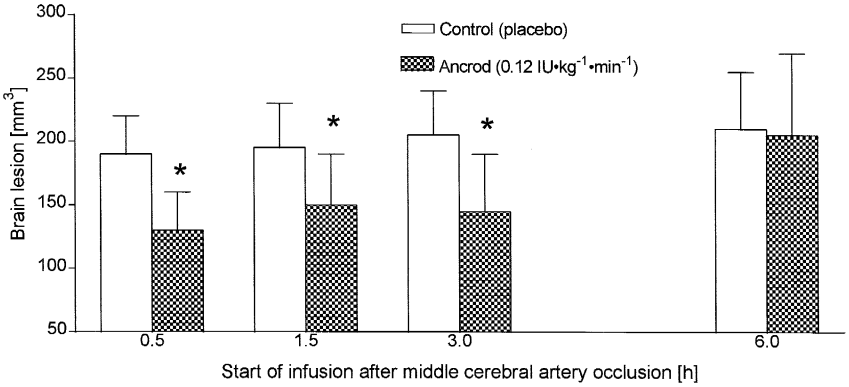

Fig. 3. Effect of increased delays in ancrod post treatment on lesion volume measured non-invasively by MRI 24 h after permanent middle cerebral artery occlusion in SHR. Osmotic mini pumps were implanted 0.5, 1.5, 3 or 6 h after middle cerebral artery occlusion for continuous intravenous infusion of either ancrod or vehicle solution. * $P < 0.05$ versus corresponding controls (8–11 animals/group).

Table 1
Effect of delayed continuous ancrod infusion (0.12 IU kg^{-1} min^{-1}) on total volume of brain lesion in SHR 24 h after middle cerebral artery occlusion

Start of infusion after cerebral vessel occlusion (h)	Total lesion volumes in control groups [a] (mm^3)	Total lesion volumes in ancrod groups (mm^3)	Decrease in lesion volumes (% vs. controls)
0.5	188 ± 28 (11)	131 ± 36 (9) [b]	30
1.5	194 ± 39 (9)	151 ± 39 (10) [b]	22
3	207 ± 33 (10)	147 ± 44 (11) [b]	29
6	214 ± 42 (8)	209 ± 60 (9)	2

Means ± S.D. Numbers of animals in parentheses.
[a] Control animals received i.v. infusions of the vehicle solution at times and in amounts corresponding to the ancrod-treated groups.
[b] $P < 0.05$ vs. control (Student's *t*-test).

Table 2
EEffect of delayed continuous ancrod infusion (0.12 IU/kg per min) on plasma fibrinogen in middle cerebral artery occluded SHR

Groups of treatment	Start of infusion (h after middle cerebral artery occlusion)	Plasma fibrinogen (g l^{-1})		
		before middle cerebral artery occlusion	before start of infusion	24 h after middle cerebral artery occlusion
Control	0.5	2.1 ± 0.6	1.7 ± 0.8	7.0 ± 1.9 [a]
Ancrod	0.5	2.7 ± 0.7	1.8 ± 0.7 [b]	0.6 ± 0.2 [ac]
Control	1.5	2.2 ± 0.7	2.0 ± 0.6	6.7 ± 2.1 [a]
Ancrod	1.5	1.9 ± 0.8	1.7 ± 0.7	4.1 ± 2.0 [ac]
Control	3	2.2 ± 0.2	1.9 ± 0.4	5.7 ± 1.3 [a]
Ancrod	3	2.4 ± 0.4	1.9 ± 0.4 [b]	1.1 ± 0.3 [ac]
Control	6	2.4 ± 0.6	2.2 ± 0.4	6.3 ± 2.3 [a]
Ancrod	6	2.5 ± 0.6	2.1 ± 0.8	0.8 ± 0.3 [ac]

Mean ± S.D. Control groups received i.v. infusions of the vehicle solution at times and in amounts equal to the corresponding ancrod-treated groups.
[a] $P < 0.05$ vs. before infusion (paired Student's *t*-test).
[b] $P < 0.05$ vs. before middle cerebral artery occlusion (paired Student's *t*-test).
[c] $P < 0.05$ vs. control.

artery occlusion in all groups of rats. However, significant 3-fold increases of plasma fibrinogen levels were measured in control groups 24 h after middle cerebral artery occlusion. All ancrod-treated groups had significantly ($P < 0.05$) lower plasma fibrinogen levels after 24 h than the corresponding control groups. This reduction in plasma fibrinogen level was comparable in all ancrod-treated groups except for the group with pump implantations 1.5 h after middle cerebral artery occlusion. Accordingly, the decrease in lesion volume was only 22% in the group with 1.5 h delayed start of ancrod infusion (Table 1) whereas the decrease in lesion volume comprised on the average 29–30% in the groups with 0.5 and 3 h delayed start of ancrod infusion. It cannot be excluded that methodological reasons (e.g., error in the dilution of ancrod) might account for the discrepant findings in the 1.5 h treatment group. In any way these results show that the percent lesion volume reduction corresponds to the extent of plasma fibrinogen lowering in rats which had pump implantations up to 3 h after middle cerebral artery occlusion.

4. Discussion

The main finding of the present magnetic resonance imaging study is significant reduction of brain lesion by ancrod in middle cerebral artery occluded SHR even when ancrod infusion is started with a delay of 3 h post occlusion. In general, a delay of several hours occurs before patients with acute stroke are amenable to therapy by an experienced clinician (Herderscheê et al., 1991; Harper et al., 1992; Jorgensen et al., 1996; Azzimondi et al., 1997). The observation that brain lesions propagate after focal cerebral ischaemia and reach a maximal size after about 48 h (Seega and Elger, 1993) may offer an opportunity for pharmacological intervention by delayed posttreatment. In the present study a substantial reduction of brain lesion size was manifested in spontaneously hypertensive rats, although intravenous infusion of ancrod was started with half an hour delay after permanent occlusion of the proximal middle cerebral artery. This result corroborates data of two previous ancrod studies on focal cerebral ischaemia in rats using also half an hour post-treatment schedules (Elger et al., 1997). Furthermore, significant brain lesion reductions were still achieved when ancrod infusions were started 1.5 or 3 h after vessel occlusion. A 6 h delay in the start of ancrod infusion, however, turned out to be too late in order to provide cerebroprotective effects under the experimental conditions of the present study. In a survey of the literature Touzani et al. (1994) concluded that the window of therapeutic opportunity is rapidly closed in SHR because e.g. post-treatment regimens with the nitric oxide donor 3-morpholinosydnonimine (SIN-1) reduced infarct size only when the drug was administered within 60 min but not 2 h after permanent middle cerebral artery occlusion in SHR (Zhang and Iadecola, 1994). To the best of our knowledge the present investigation showed for the first time significant brain lesion reductions by infusion of a drug 3 h after permanent middle cerebral artery occlusion in SHR. In other species such as primates, the delay of ancrod therapy for more than 3 h might still have beneficial effects because reduction of infarct volume by middle cerebral artery reopening at 6 h in the baboon (Young et al., 1997) suggests a larger therapeutic window than has generally been assumed.

Brain lesions were quantified by standard T2-weighted MRI 24 h after middle cerebral artery occlusion. Meanwhile, MRI in vivo has become a valuable tool for routine evaluation of drug effects in preclinical studies on focal cerebral ischaemia (Elger et al., 1994, 1996, 1997; Sauter and Rudin, 1995). MRI is more reproducible, faster and

simpler than histological methods for quantification of brain lesions (Allegrini and Sauer, 1992). Moreover, non-invasive imaging techniques such as MRI have been recommended as ideal outcome measures in drug testing for stroke (Hsu, 1993). Total volume of cerebral lesion was decreased by about 30% in the present and also in a previous study (Elger et al., 1997) when ancrod infusions were started 30 min after permanent middle cerebral artery occlusion in SHR. As concerns the magnitude of the lesion reduction by ancrod two fundamental aspects need to be considered. First, the total volume of brain lesion has been measured which consists of infarction in the cerebral cortex plus the striatum after occlusion of the proximal portion of the middle cerebral artery by the method of Tamura et al. (1981). Since most drugs exert only little or no effect on striatal lesions (Roussel et al., 1992; Chen et al., 1993; Sauter and Rudin, 1995) the calculation of per cent reduction of total lesion volume yields values which are less impressive than reports on more than 50% lesion reduction which have been achieved using a method of middle cerebral artery occlusion that affects only the cerebral cortex (Aronowski et al., 1996b). Secondly, it is commonly known that it is more difficult to demonstrate cerebroprotection in rats with chronic hypertension than in normotensive rat strains (Ginsberg and Busto, 1989; Touzani et al., 1994). Comparative studies indicated that for example the noncompetitive NMDA receptor antagonist dizocilpine (MK-801) significantly reduced infarct volume in normotensive Fischer-344 rats but not in SHR after permanent middle cerebral artery occlusion (Roussel et al., 1992; Sauter and Rudin, 1995). Likewise, opposing effects have been reported on the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor antagonist 2,3-dihydroxy-b-nitro-7-sulfamoylbenzo(F)-quinoxaline (NBQX) after permanent focal cerebral ischaemia in normotensive and hypertensive rats (De Graba et al., 1994; Graham et al., 1996). Intravenous injection of the sodium channel blocker 4-amino-2-(4-methyl-1-piperazinyl)-5-(2,3,5-trichlorophenyl) pyrimidine (BW 619C89) a few minutes after permanent middle cerebral artery occlusion elicits 63% reduction of total infarct volume in normotensive rats and only 32% in SHR (Swan and Leach, 1995). The maximal lesion reductions that we have observed in SHR with drugs acting via different mechanisms after middle cerebral artery occlusion have also been in the range of 30% (Elger et al., 1996, 1997). In view of the published restrictions in the magnitude and the therapeutic window of drug effects after permanent occlusion of the proximal middle cerebral artery in SHR, a 29% reduction of total brain lesion in rats with 3 h delayed ancrod infusion suggests that this compound may offer a potential approach for therapy of acute human stroke.

Elevated plasma fibrinogen levels have been repeatedly observed in stroke patients (Eisenberg, 1966; Coull et al., 1991; Satoh et al., 1993; Tanahashi et al., 1996). Similarly, an increase of plasma fibrinogen has been measured in control rats of the present study 24 h after middle cerebral artery occlusion, suggesting that this animal model may be clinically relevant with respect to haemorheological disturbances.

Continuous intravenous ancrod infusion decreased the plasma fibrinogen level significantly. Reduction of plasma fibrinogen leads to diminished blood viscosity and increased cerebral blood flow (Izumi et al., 1996). Thus, reduction of plasma fibrinogen levels may improve nutritive perfusion in the penumbra of a focal ischaemic brain lesion and prevent further spatial extension of evolving brain infarctions. This assumption is supported by the analysis of individual magnetic resonance images in this study revealing most pronounced lesion reduction in posterior cortical brain regions of ancrod-treated rats where collateral blood vessels are well developed and where substantial lesion development has been demonstrated by MRI between 3 and 24 h after middle cerebral artery occlusion (Seega and Elger, 1993). The regional distribution of the tissue salvaged from lesion was similar to that previously reported in this rat model (Elger et al., 1994, 1997) and involved the border zones between the arterial territories of the middle cerebral artery and those of the anterior and posterior cerebral arteries. Improved perfusion of these cerebral regions may not only result from decreased blood viscosity after reduction of the plasma fibrinogen concentration by ancrod. The generation of the vasodilator PGI_2 may also be enhanced by ancrod treatment (Kant et al., 1982). Moreover, evidence has accumulated that the specific enzymatic properties of ancrod (for a review see Eschenfelder, 1996) favour also activation of endogenous fibrinolysis and inhibition of further thrombosis.

Clinical stroke studies attempt to achieve plasma fibrinogen levels of about 0.7–1.0 g/l by infusion of ancrod (Pollak et al., 1990; The Ancrod Stroke Study Investigators, 1994). Similar plasma fibrinogen concentrations were obtained in the present study after infusion of a moderate ancrod dosage. In previous experiments much higher ancrod dosages have been infused after permanent middle cerebral artery occlusion in SHR causing plasma fibrinogen concentrations of about 0 g/l (Elger et al., 1997). No increased mortality or evidence of haemorrhagic transformation of the cerebral lesion was observed in these earlier studies despite the defibrinogenation by ancrod.

In conclusion, sustained reduction of plasma fibrinogen by continuous ancrod infusion in rats after middle cerebral artery occlusion and associated observation of brain lesion reductions give rise to the assumption of cerebroprotection in acute focal cerebral ischaemia by improvement of local cerebral blood flow. Significant brain lesion reduction is achieved in permanent focal cerebral ischaemia even when the onset of ancrod therapy is delayed for up to 3 h. Thus, this MRI study suggests that fibrinogen reduction by ancrod may provide an efficient therapy of acute ischaemic stroke.

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