Calcium Antagonists Reduce the Extent of Infarction in Rat Middle Cerebral Artery Occlusion Model as Determined by Quantitative Magnetic Resonance Imaging

AndrŠ Sauter, Ph.D., and Markus Rudin, Ph.D.

SUMMARY The appearance and evolution of brain infarcts over 3 days following proximal occlusion of the left middle cerebral artery (MCA) in SHR rats were measured non-invasively by magnetic resonance imaging (MRI). Infarcts were clearly visible in coronal, T2 weighted brain sections, 24, 48 and 72 h after MCA occlusion in the left hemisphere, as areas of increased NMR signals. The infarcts were quantified by pixel counting in each section, the sum of 4 sections representing an accurate estimate of the total infarct size. The location and extent of infarction, determined by MRI, were found to be highly reproducible and correlated well with post-mortem histological and biochemical data. A neurological score, made every 24 h, paralleled the evolution of the infarct size, which culminated after 48 h. Pre- or post-treatment of MCA occluded rats with the dihydropyridine calcium antagonist PN 200-110 resulted in a substantial reduction of infarct size, determined by MRI 24, 48 and 72 h after infarction, compared to vehicle treated controls. These findings were corroborated by corresponding improvements of the neurological scores as well as histological and biochemical data. Post-treatment with nimodipine showed qualitatively similar effects. These results support the notion that calcium antagonists, through vascular and/or metabolic mechanisms, are effective in treating acute stroke. Since they were obtained in a chronic, relevant model of stroke with a method directly applicable also to humans, they should encourage further clinical studies with calcium antagonists.

FEW CONCLUSIVE CLINICAL STUDIES on drug therapies of acute stroke have been published to date, probably because clinical studies in stroke are inherently complex and difficult to perform with current methodology. In addition, most of them unfortunately turned out to be quite disappointing. Nevertheless, great interest in stroke persists, as evidenced by a wealth of knowledge, mainly based on preclinical studies, concerning the pathophysiology of stroke. As a consequence, a variety of strategies for pharmacological interventions has been proposed, such as e.g. prevention of cellular calcium overload by calcium antagonists, depression of cerebral metabolism by barbiturates, scavenging of free radicals etc. 1-4 However, few studies on drug treatments in clinically relevant experimental animal models have been published, experiments that seem to be essential to convince and encourage clinicians to initiate the appropriate multicenter studies mandatory for progress in stroke therapy.

The aim of this study was therefore to investigate in a clinically relevant animal model of stroke, with methods readily available also to clinicians, the therapeutic potential of a class of drugs which, based on preclinical and clinical data, seems to be most promising for treatment of acute stroke. The surgical occlusion of an intracranial artery, such as the middle cerebral artery (MCA), is a method widely used in a variety of animal species to produce focal ischemia with...
symptoms closely resembling embolic stroke in humans. We have chosen the rat for MCA occlusion because, among other obvious reasons, of the similarity to man regarding the anatomy of cerebral circulation, particularly when contrasted with other species such as the gerbil, cat or dog. Magnetic resonance imaging (MRI) has proven a sensitive, clinically valuable diagnostic method for a number of CNS diseases, in particular also stroke because it is a very sensitive probe for tissue water content. In addition, it is possible to quantitatively determine the extent of infarcts from three dimensional data sets. Since MRI is noninvasive such quantifications can be made repeatedly and therefore seems to be a method of choice to follow-up the evolution of infarcts with and without drug treatment. Among the proposed therapies for acute stroke calcium antagonists have recently gained wide interest. Calcium has been proposed to be the trigger of ischemic cell death and therefore calcium antagonists might show, besides vascular, also beneficial neuronal, metabolic effects.

Materials and Methods

Middle Cerebral Artery Occlusion

Adult male SHR/KYO rats (250–300 g) which had been anaesthetised with Evipan (150 mg/kg i.p.). During surgery anaesthesia was maintained and controlled with halothane (0.5–1%). A vertical 2 cm skin incision was permitted free access to food and water were avoided. The zygoma was partially removed and the temporalis muscle was exposed and an incision made around its superior margin in order to scrape it from the lateral aspect of the skull. The temporalis muscle was then divided by a vertical incision and reflected backwards and forwards. The zygoma was partially removed and the infero-temporal fossa exposed. A craniectomy of 1 mm diameter was made using a dental drill, approximately 1 mm superior to the foramen ovale. The dura was opened with a sharp needle and the exposed MCA electrocauterised with a small bipolar. The soft tissues were put back into place and the skin sutured. Sham operated animals were treated exactly the same way except for closing the MCA.

Drug Application

The doses of PN 200-110 and nimodipine were chosen based on their cardiovascular activities and effects on high energy phosphates in the CNS. They were dissolved in ethanol-polyethylene glycol 400 (1 + 1 by vol) at a concentration of 0.3 mg/ml. All injections were done subcutaneously, the daily dose being 3 × 0.3 mg/kg. The amount of drug for the first 8 hours was subdivided into 3 doses: Pretreated rats received 0.1 mg/kg 45 min before and 45 and 135 min after MCA occlusion. Posttreated rats received 3 × 0.1 mg/kg 15, 105 and 195 min after MCA occlusion. Subsequently the first 0.3 mg/kg dose was given 8 hours after the first injection. Control animals were injected accordingly with solvent alone.

Neurological Score

A simple neurological score was made every day before injection of Evipan (for subsequent imaging). The rats were judged according to their posture (bent, inclined), movement (walking, turning) and paralysis of the hindpaws by an observer who was unaware of the treatment. For each observable the scale ranged from normal (= 0 points) to severe (= 3 points), the sum of all points being the neurological score.

Magnetic Resonance Imaging

The experiments were carried out on a Bruker CXP-200 NMR spectrometer equipped with a 4.7 T/15 cm horizontal bore magnet and an imaging accessory kit. The NMR probe was a home made resonator of the type described by Alderman and Grant and adapted to MRI by several groups. The inner diameter of the probe was 70 mm and the length of the resonating structure 100 mm. Pulse length for a 90° radio frequency pulse of 25 to 30 µs at 500 W incident power have been achieved for the resonator loaded with a rat. The magnetic field gradient strengths were 5 kHz/cm, 4 kHz/cm and 2 kHz/cm for the slice selection, read-out and maximum phase encoding gradient, respectively. The slice thickness was 2 mm, the spatial resolution in the imaging plane 0.3 mm × 0.3 mm. The anaesthetized animals (Evipan 120 mg/kg i.p.) were positioned in a stereotactic holder and images of four coronal sections, 3, 6, 9 and 12 mm anterior to the intraural line, have been taken in multislice spin-echo acquisition mode. This allows to cover the rat forebrain accurately in a single 8 min experiment. The echo delay of 25 ms was found to be optimal with respect to image contrast and signal-to-noise ratio, resulting in pronounced T2 weighting. The repetition time was set to 2 seconds. With this SE (2000/25) sequence the signal intensity differed by approximately 50% between infarcted and intact tissue (see fig. 1) allowing to discriminate the lesioned area with good reproducibility. The images have been quantified using region of interest analysis by a person who was unaware of the treatment. For each coronal section the area of the infarct has been determined by pixel counting. The sum of the values obtained from four sections yielded the total infarct size. In order to quantify the prolongation of T2 due to the infarction, a multi-echo experiment (8 echoes) has been carried out. The T2 relaxation times were 250 to 300 ms for edematous and 60 to 80 ms for the corresponding intact tissue.

Biochemical Analysis

Exactly two hours after the last Evipan injection (for imaging) each rat was injected i.v. with 9 mg neutral red dissolved in 0.5 ml Krebs-Ringer-Henseleit buffer. Thirty seconds later they were killed by decapitation and the brains rapidly removed and dissected on ice into the left and right hemisphere, discarding the cerebellum and brain stem. Each hemisphere was immediately frozen on dry ice, weighed and afterwards homogenised in 7 ml 0.1 N perchloric acid (PCA) containing as an internal standard dihydroxybenzylamine as an internal standard dihydroxybenzylamine.
FIGURE 1. Coronal MR image through rat head, 9 mm anterior to intraaural line, 24 h after unilateral MCA occlusion, showing the intensity profile at the indicated line. The intensity increase due to the infarction (edema) is 40 to 50%.

(DHBA, 100 ng/ml). The homogenate was kept at -20°C until analysed. Relative CBF measurement: 1 ml homogenate was thoroughly mixed with 0.2 ml polyethylene glycol 400 and centrifuged. The absorbance, read at 533 nm, of the left hemisphere was compared to that of the right hemisphere. Sodium and potassium ions were determined in the PCA supernatant by flame photometry, protein was determined in the PCA precipitate after dissolving it in 1N NaOH, according to Lowry. Biogenic amines and metabolites were determined in the PCA supernatant by HPLC with electrochemical detection using DHBA as internal standard, according to published methods.

Statistical Analysis
The results have been statistically analyzed using the non-parametric Wilcoxon-Mann-Whitney U-test or the t-test, as indicated in the text and figures.

Results
In order to determine the range wherein beneficial drug effects might be expected, untreated sham operated rats were compared to untreated left MCA occluded animals. Coronal sections through the forebrain were obtained by MRI 6, 24, 48 and 72 h after operation. No differences between the left and right hemispheres existed in sham operated rats at any time after surgery. In contrast, well defined areas of enhanced NMR signal intensity were visible in the left hemispheres of MCA occluded animals, 24, 48 and 72 h after operation, while images taken 6 h after MCA occlusion did not yet show any left/right asymmetry. Representative 24 h-pictures for both groups are shown in figure 2. Post-mortem histological examination revealed that the increased NMR signal was mainly due to edema and necrotic cell damage. The demarcation of the injured tissue, determined by MRI, corresponded exactly to the damage seen in cryostatic sections post-mor-
As shown in figure 2, the main structures affected were: cortex (frontal, fronto-parietal, temporal), caudate-putamen, globus pallidus, hippocampus, lateral thalamus, amygdala. The location and size of the infarcts were quite reproducible: the average total infarct size (i.e. sum from the 4 coronal sections) was, 24 h after MCA occlusion, 17800 ± 800 voxels (mean ± SEM, N = 8). As depicted in figure 3, upper panel, it slightly increased to 21200 ± 1200 voxels over the next 24 h and thereafter began to slowly decrease to 20700 ± 1500. The corresponding neurological scores are shown in figure 3, lower panel. Sham operated rats had an average score of 1 point, while MCA occlusion resulted in an average score of 3–4 points. The time course of the neurological score over the 3 days of observation paralleled the evolution of infarct size measured by MRI.

To further characterize the impact of MCA occlusion, a series of biochemical post-mortem determinations was made in the left and right hemispheres of sham operated and MCA occluded animals immediately after the final MRI examination, i.e. 72 h after operation. The results are summarized in table 1. Identical relative blood flow values for the left and right hemisphere were observed in sham operated rats, as reflected by the same amount of neutral red per mg protein present in each hemisphere, resulting in a left/right ratio close to 1. The left/right ratio in MCA occluded rats was 0.62, indicating that the average blood flow to the left hemisphere was reduced by approximately 40%. The wet weights of the left and right hemispheres in sham operated rats were identical and not statistically different from the right control hemisphere of left MCA occluded rats, which showed increased wet weights (+14%) of the left hemisphere. The presence of edema in the left hemisphere of MCA occluded rats was also evidenced by a reduced concentration of protein (−20%), decreased potassium (−43%) and increased sodium concentration (+83%), while no left/right differences existed in sham operated animals. Left/right differences in catecholamines and metabolites were also observed and were interpreted as a sign of neuronal necrosis. In sham operated rats the levels of dopamine and noradrenaline as well as those of the metabolites DOPAC, HVA and 5-HIAA were approximately 10% higher in the right than in the left hemisphere. This difference is most likely due to sham operation, in particular opening of the dura and subsequent efflux of CSF, since non-operated control rats showed no difference between the left and right side. MCA occlusion resulted in a further reduction of dopamine (−53%) and noradrenaline (−26%) and increase of DOPAC (+55%), HVA (+84%) and 5-HIAA (+50%) in the left hemisphere compared to the right, confirming the findings obtained by MRI, that cortical and striatal structures are affected.

Treatment of MCA occluded rats with calcium antagonists resulted in improvements of most parameters measured, as compared to vehicle treated controls. The data and statistical significances are summarized in figures 3 and 4. Posttreatment with PN (3 × 0.3 mg/kg/day s.c.) was in general less effective than pretreatment with the same dose, whereas posttreatment with nimodipine (3 × 0.6 mg/kg/day s.c.) was clearly less effective and statistically not significantly different from controls (p > 0.05, Mann-Whitney-U-test), except for the infarct size after 24 h, with a significance of p < 0.05 (t-test). An obvious effect (see fig. 2) of calcium antagonist treatment consisted in a pronounced reduction of infarct size, measured by MRI. As shown in figure 3, pretreatment with PN nearly halved the infarct size at any time after MCA occlusion, a finding corroborated by a parallel reduction of the neurological score. Effects of similar magnitude have also been found post-mortem, 72 h after MCA occlusion (see fig. 4), such as reduced changes of left hemispheric wet weight, protein, potassium and sodium content as compared to the vehicle group. The decreased blood flow to the left hemisphere relative to...
the right was, however, only slightly improved, reaching statistical significance only in the PN pretreatment group. Post-mortem histological examinations as well as data showing normalization of dopamine, noradrenaline, DOPAC, HVA and 5-HIAA levels is compatible with the notion that treatment with calcium antagonists in fact reduces the necrotic neuronal damage rather than only the extent of perifocal edema caused by MCA occlusion. As illustrated by figure 4, all these biochemical post-mortem data are in agreement with the findings obtained in vivo by MRI demonstrating a substantial reduction of infarct size after calcium antagonist treatment.

Discussion

The majority of MRI applications today is directed towards non-invasive, qualitative diagnostic investigations. The technique has already proven invaluable for diagnosis of a variety of CNS diseases such as brain tumors, diseases of myelination and stroke, due to its soft-tissue contrasting capability. Regarding stroke, an excellent correlation between the findings obtained by MRI in vivo and post-mortem histological examinations has been found in different animal models. Using region of interest analysis we have been able to reliably determine the size of infarcts in the rat brain after MCA occlusion thereby showing that MRI is capable to measure quantitatively the evolution of infarcts with and without drug treatment. Our data suggest that the same methods should also be applicable to monitor the effects of a drug regimen in clinical stroke studies and be complementary or even superior to other methods currently in use such as CBF or glucose utilization measurements by positron-emission tomography. Spin echo T2 weighted pictures have been found optimal to demonstrate edema. With this pulse sequence clear signs of infarction are not yet present 6 h, however are clearly visible 24h after MCA occlusion, suggesting that the edema is of the cytotoxic rather than vasogenic type. An animal model with this type of...
edema is generally considered to be more relevant than models with vasogenic edema, such as e.g. the microsphere model. In addition, the MCA occlusion model turned out to be highly reproducible as revealed by the uniform, narrow distribution of infarct size and location. This is a prerequisite for finding drug effects with good sensitivity and reliability using a reasonable number of subjects, admittedly not as easily achievable in clinical studies. Another advantage of the MCA occlusion model is the large range wherein a drug effect can be expected. MCA occluded rats differed considerably in most parameters measured, particularly infarct size, from sham operated controls which in turn were not different from normal rats.

The main finding of this study is that either pre- or post-treatment with the dihydropyridine calcium antagonist PN 200-110 or nimodipine did reduce the pathological symptoms in the CNS caused by MCA occlusion. Both drugs are known to cross the blood-brain-barrier and showed qualitatively similar effects, although nimodipine was less effective than PN at the doses used, a finding which is not surprising in view of the fact that PN is a more potent calcium antagonist. PN not only decreased the infarct size, as determined by MRI, but also improved the function, as evidenced by a reduced neurological score, paralleling the infarct size. When comparing the evolution of infarct size in controls and PN treated rats over the 3 days of observation, it becomes evident (see fig. 2) that PN is not simply delaying inevitable damage but shows a genuine infarct reducing effect. This was further corroborated by improvements in a number of biochemical parameters, determined post-mortem. It is noteworthy that the various biochemical parameters were differently affected by these drugs. For instance, the relative CBF was less affected than the parameters reflecting edema, such as the brain wet weight, relative protein concentration, potassium and sodium levels. These observations suggest that calcium antagonists probably act, besides vascular, also by intraneuronal, metabolic mechanisms. A potential mechanism might be that calcium antagonists reduce the ATP demand of neurons required for the maintenance of intracellular calcium homeostasis, by reducing the excessive calcium influx occurring during ischemia. This notion is supported by recent NMR spectroscopic measurements in the CNS showing that calcium antagonists slow down the disappearance of ATP and concomitant increase of free phosphate following global ischemia, possibly reflecting a decreased ATP turnover. It is interesting to note that also in these experiments PN was approximately 3-5 times more potent than nimodipine. The decreased levels of neurotransmitters and increased levels of their metabolites found in the lesioned hemisphere may be considered as an indicator of neuronal necrosis, a well known observation made also after lesions with neurotoxins. The reduced concentrations of noradrenaline and dopamine in the left hemisphere confirm, on a biochemical basis, the findings obtained by MRI that the cortex and striatum are mainly affected. While the dopamine and noradrenaline levels are unchanged in the unlesioned, right hemisphere, decreased levels of metabolites were found. A reasonable explanation for this might be a compensatory decrease of neurotransmitter turnover in the intact hemisphere, correcting the functional imbalance caused by the occlusion of the left MCA.

A question of practical importance concerns the maximal time allowed between the onset of stroke and beginning of drug application. Post-treatment, starting with a subcutaneous injection, 15 min after MCA occlusion, was, as expected, less effective than pretreatment. It would indeed be of general interest when considering stroke therapy and important in view of future clinical stroke studies to determine the exact
time schedule of drug application for a successful treatment. In a chronic animal model in particular, the success of a cure depends much on the frequency, dosage and route of drug application. These aspects are important especially with dihydropyridine calcium antagonists, because they are rapidly metabolized in the rat and in addition lower blood pressure, which, in case of overdose, can become a serious problem for stroke therapy. Since we have not tried to optimize the administration of PN, even more spectacular effects might be possible with this class of drugs.

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A Sauter and M Rudin

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