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Effects of cerestat and NBQX on functional and morphological outcomes in rat focal cerebral ischemia

N. Pitsikas*, A. Brambilla, C. Besozzi, P. Bonali, F. Fodritto, N. Grippa, A. Scandroglio, F. Borsini

Department of Biology, Boehringer Ingelheim Italia, S.p.A., Via Lorenzini 8, 20129 Milan, Italy

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Abstract

This study investigated the ability of NBQX, an AMPA receptor antagonist, and cerestat, a NMDA receptor antagonist, to counteract neurological deficits and morphological damage induced by permanent occlusion of the left middle cerebral artery (MCAO model) in the rat. NBQX (3, 10, and 30 mg/kg, ip) injected at 10, 60, and 120 min postocclusion did not reduce the volume of infarct in the MCAO model of cerebral ischemia and had marginal effects on sensory dysfunctions (vibrissae stimulation and body proprioception) and no effects on motor dysfunctions (forelimb flexion and footfault test). Conversely, cerestat (0.3, 1, and 3 mg/kg, sc) injected at 10 and 120 min postocclusion significantly reduced the ischemic volume at the dose of 1 mg/kg, and, at the same dose, significantly attenuated behavioural deficits in the body proprioception and in the forelimb flexion tests.

Keywords: Brain ischemia; MCA occlusion; Rat; Cerestat; NBQX; Neurological outcome; Morphological damage

1. Introduction

Permanent occlusion of the middle cerebral artery (MCA) in rats provides an animal model to investigate the pathophysiology of focal cerebral ischemia, to assess ischemia-induced behavioural and neurological deficits and to evaluate putative neuroprotective compounds (Ginsberg and Busto, 1989).

Glutamate is an important excitatory neurotransmitter in the mammalian central nervous system. It has been observed that during and after ischemia, glutamate is extensively released and activation of various subtypes of its receptor causes direct and indirect Ca^{2+} influx into the cell, leading to neuronal damage (Benveniste et al., 1984; Bederson et al., 1986b; Choi, 1987). At present, metabotropic and ionotropic glutamate receptors have been characterized (Choi, 1990). The ionotropic receptors are subdivided into NMDA, AMPA, and kainate receptors.

Several lines of evidence suggest that antagonism of NMDA and AMPA receptors may represent an important mechanism, though not the only one, for blocking ischemia-induced neuronal damage (Meldrum, 1990). In fact, different investigations suggest that both cerestat, an NMDA antagonist, and the AMPA antagonist NBQX significantly reduced the volume of infarct in a variety of animal models in different species (Gill, 1994). At present, however, there is lack of evidence whether or not these compounds exert a neuroprotective action on the functional deficits, which are commonly displayed by ischemic animal models. It is interesting to note that in stroke patients, functional (primarily motor) outcome rather than the volume of infarct is the most important event (Aranowski et al., 1996).

Thus, the aim of the present study was to investigate and compare the neuroprotective effects of cerestat and NBQX on sensorimotor impairments produced by permanent occlusion of the medial cerebral artery in the rat. To this aim, a battery of different neurological paradigms and then a morphological evaluation of the brain were utilized.

2. Method

Procedures involving animals and their care were conducted in conformity with the institutional guidelines, in

* Corresponding author. Tel.: +39-2-5355315; fax: +39-2-5355368.
E-mail address: nikpit@yahoo.it (N. Pitsikas).

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compliance with national and international laws and policies (EEC Council Directive 86/609, = J L 358, 1, Dec. 12, 1987; NIH Guide for the Care and Use of Laboratory Animals. NIH publication no. 85-23, 1985).

2.1. Animals

One hundred forty four 2-month-old male Fischer 344 rats (Charles River, Calco, Italy), weighing 220–250 g, were used in our study. The animals were kept in a regulated environment (21 ± 1°C, before surgery, 24 ± 1°C, 4 h afterwards; 50–55% relative humidity, 12 h light–dark cycle, lights on at 7:00 AM) for 11–12 days before surgery. The rats had access to food (Mucedola, Italy) and water ad libitum.

2.2. MCA occlusion

Rats fasted 18 h before surgery, were anesthetized with fluothane (2% in air); during surgery, their body temperature was monitored by a rectal probe and maintained at 37 ± 1°C with a heating lamp. After left retroorbital skin incision and partial cutting and retracting of the temporalis muscle, the zygoma and the squamosal bone were exposed. The left MCA was approached by subtemporal craniotomy beside the foramen ovale and performed by means of a dental drill (Micromot 2000, Unitronic, Germany), leaving the zygoma intact (Tamura et al., 1981). The dura was then pierced with a little bent needle, the artery was occluded by microbipolar coagulation (BET 2001, LASER, Italy) starting before it crosses the olfactory tract, and for a 2–3 mm tract distally, and then subsequently cut (Tamura et al., 1981).

To avoid drug-induced hypothermia, rats were maintained in a thermostatic chamber at 30°C for 4 h starting immediately after surgery, and were then housed under the aforementioned conditions.

2.3. Neurological assessment

Forty-eight hours after surgery, rats were submitted to a battery of neurological tasks. The tests were carried out by an observer who was unaware of the pharmacological treatment, and a grading scale of 0–2 was used to assess the effects of the occlusion, as described below. Rats that presented with severe motor deficits (unable to move) were excluded from behavioural testing.

2.3.1. Response to vibrissae stimulation

A blunt stick was brushed against the vibrissae on each side; to avoid entering the visual fields, the stick was moved toward the whiskers from the rear of the animal. The used scores indicated the following: 2 = rat reacting by turning head or equally startled by the stimulus on both sides; 1 = rat reacting slowly to stimulus on right (contralateral) side; and 0 = rat not responding to stimulus on the right (contralateral) side (Garcia et al., 1995).

2.3.2. Body proprioception

The rat was touched with a blunt stick on each side of the body, and the reaction to the stimulus was recorded. Scores indicated the following: 2 = rat reacting by turning head and equally startled by the stimulus on both sides; 1 = rat reacting slowly to stimulus on right (contralateral) side; and 0 = rat not responding to the stimulus placed on the right (contralateral) side (Garcia et al., 1995).

2.3.3. Forelimb flexion

The rat was gently held by the tail, suspended 20 cm above the grid floor of its cage, and the position of the forelimbs was observed for 5 s. Scores indicated the following: 2 = both forelimbs extended symmetrically; 1 = right (contralateral) forelimb extended less and more slowly than the left (ipsilateral); 0 = right (contralateral) forelimb flexed (Garcia et al., 1995).

2.3.4. Footfault test

The rat was placed in a cage, on a grid floor (45 × 30 cm with grid openings of 3 cm^2) for 2 min. The rat placed its paws upon the wire frame while moving about the grid; occasionally a foot would be misplaced and fall through a grid opening (a 'foot fault'). Footfaults are typically symmetrical in intact animals. The number of footfaults for each forelimb was recorded and the total number of forelimb steps was counted for each animal. A measure of interest was the asymmetry index, that is, the number of contralateral minus ipsilateral footfaults (Barth et al., 1990).

2.4. Morphological evaluation of infarct volume

Two days after MCAO and after neurological examination, the rats were reanesthetized with fluothane, the

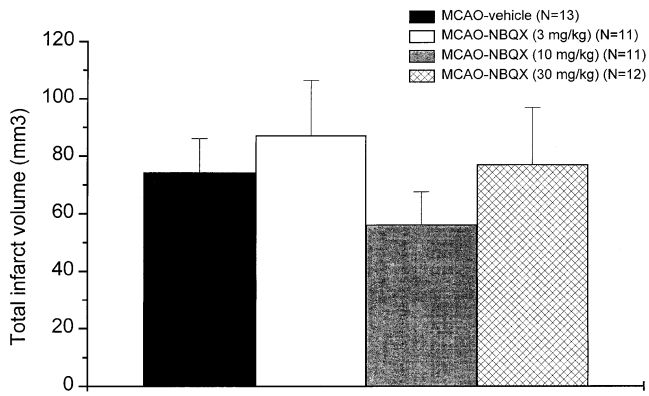

Fig. 1. Effect of NBQX treatment on total volume of the infarct determined from 2,3,5-triphenyltetrazolium (TTC)-stained sections at 48 h after occlusion of the MCA of rats. Results are expressed as means ± S.E.M. (NBQX was injected intraperitoneally at 10, 60, and 120 min postocclusion at doses of 3, 10, and 30 mg/kg.] See text for details.

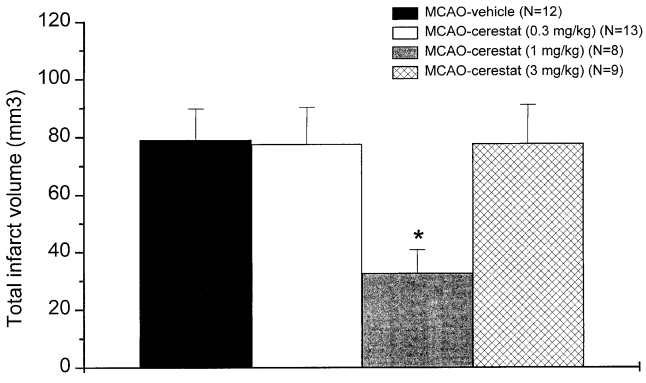

Fig. 2. Effect of cerestat treatment on total volume of the infarct determined from 2,3,5-triphenyltetrazolium (TTC)-stained sections at 48 h after occlusion of the MCA of rats. Results are expressed as means ± S.E.M. [Cerestat was injected subcutaneously at 10, 60, and 120 min postocclusion at doses of 0.3, 1, and 3 mg/kg. * $P < .05$ vs. MCAO–vehicle-treated rats; Dunnett's t test.] See text for details.

thorax was opened and 5 ml of 2% 2,3,5-triphenyltetrazolium chloride (TTC)/saline solution was injected transcardially (left ventricle), after cutting the right atrium. The animals were kept at 37°C for 1 h. The brains were then removed from the skull and stored in 10% formaldehyde for 2 days. Serial brain slices (1.5 mm thick) were cut (Vibroslice, Campden), and the infarcted area was measured by an imaging analysis system (MCDI, Imaging Research, Canada). To this purpose, the area of normal tissue in the infarcted hemisphere was subtracted from the total area of the non infarcted hemisphere, in order to minimize the influence of edema in the infarcted tissue (Swanson et al., 1990); this procedure was applied both to the total brain section and to the striata only, so that the total infarcted area could be separated into cortical and subcortical damage. The volume of infarction was then calculated by multiplying the areas for the thickness of slices (Bederson et al., 1986a; Swanson et al., 1990). The operator was unaware of the pharmacological treatment.

2.5. Drug administration

Rats were randomly divided into eight experimental groups ($n = 18$ rats/group) as follows: MCAO–vehicle of NBQX; MCAO–NBQX 3 mg/kg; MCAO–NBQX 10 mg/kg; MCAO–NBQX 30 mg/kg; MCAO–vehicle of cerestat; MCAO–cerestat 0.3 mg/kg; MCAO–cerestat 1 mg/kg; MCAO–cerestat 3 mg/kg.

2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) was synthesized at Boehringer Ingelheim Germany, dissolved in NaOH 0.2 ml + Tris–HCl buffer (pH = 9–10) and was delivered intraperitoneally. NBQX or vehicle was injected at 10, 60, and 120 min after the occlusion of the MCA in a volume of 2 ml/kg.

Cerestat (Cambridge Neuroscience) was dissolved in saline (0.9% NaCl) and given subcutaneously. Cerestat or vehicle was injected at 10 and 120 min after the occlusion of the MCA in a volume of 1 ml/kg.

2.6. Statistical analysis

Before performing any statistical analysis, neurological outcome data were reclassified from three to two categories (positive and negative response) according to the following procedures: (a) response to vibrissae stimulation and body proprioception: scores 0 and 1 = negative assessment; score 2 = positive assessment; (b) forelimb flexion: score 0 = negative assessment, scores 1 and 2 = positive assessment.

The above classification was used during the following statistical analysis, where a linear model for categorical data (i.e. positive vs. negative response) was applied in order to compare the drug-treated groups to the MCAO vehicle control groups.

Footfault test: The total number of forelimb steps data were analyzed by an analysis of variance followed by the two-tailed Dunnett's t test. By an analysis of covariance (covariate = total number of steps) followed by the two-

Table 1
Neurological outcome following MCAO in rats

	Neurological outcome (median and interquartile range)			
Treatment	No. of rats	Vibrissae touch	Body proprioception	Forelimb flexion
Vehicle–NBQX	16	0 (0–1)	0 (0–1)	0 (0–0)
NBQX 3 mg/kg	15	1 (0–2)	1 (0–2)	0 (0–0)
NBQX 10 mg/kg	13	2 (1–2)	2* (1–2)	0 (0–0)
NBQX 30 mg/kg	16	1 (0–2)	1 (1–2)	0 (0–0)
Vehicle–cerestat	14	0 (0–1)	0.5 (0–1)	0 (0–0)
Cerestat 0.3 mg/kg	14	1 (0–2)	1.5 (1–2)	0 (0–1)
Cerestat 1 mg/kg	12	2 (1–2)	2* (1–2)	1** (0–1)
Cerestat 3 mg/kg	16	1.5 (1–2)	1 (1–2)	0 (0–1)

NBQX and cerestat were administered intraperitoneally and subcutaneously, respectively. Observations were made 48 h after surgery.
* $P < .05$.
** $P < .01$ vs. vehicle-treated rats.

Table 2
Footfault test results observed after MCAO in rats

	Footfault test (mean ± S.E.)			
Treatment	No. of rats	Total no. of steps	No. of errors (right)	Asymmetry index
Vehicle–NBQX	15	42.5 ± 3.7	10.7 ± 1	7.9 ± 0.9
NBQX 3 mg/kg	15	37.9 ± 4.2	8.8 ± 1.1	6 ± 0.9
NBQX 10 mg/kg	13	39.9 ± 3.6	9.2 ± 1.4	6.3 ± 1.4
NBQX 30 mg/kg	16	42.6 ± 3.2	9.3 ± 0.8	6.5 ± 0.7
Vehicle–cerestat	14	42.7 ± 5.1	9.9 ± 1.1	7.2 ± 0.8
Cerestat 0.3 mg/kg	14	47.7 ± 3.4	10.4 ± 1.2	7.2 ± 1.1
Cerestat 1 mg/kg	12	37.4 ± 3.5	6.9 ± 1.2	4.2 ± 1
Cerestat 3 mg/kg	15	32.6 ± 2.7	6.3 ± 1.2	4.8 ± 1

NBQX and cerestat were administered intraperitoneally and subcutaneously, respectively. Observations were made 48 h after surgery.

tailed Dunnett's t test, the data relative to the number of contralateral forelimb errors and to the asymmetry index were analyzed.

Statistical evaluation of volumes of infarcted areas was performed by an analysis of variance followed by the two tailed Dunnett's t test.

Statistical analysis was performed using the SAS program, version 6.11 on a PC Compaq Deskpro 466, o.s. Windows 95.

3. Results

Of the 144 rats undergoing the MCAO, 23 died after surgery and three were excluded from testing because of incomplete occlusion. The number of excluded rats was seemingly independent from treatment (two to five rats in each group). Furthermore, the other 29 rats were excluded from histological evaluation, either because an edema has occurred (22 rats) or brain sections were poorly stained (seven rats).

Any amount of NBQX did not reduce the volumes of the infarct though the dose of 10 mg/kg exerted a slight protection, which, however, did not reach statistical significance. Conversely, cerestat at the dose of 1 mg/kg significantly reduced tissue damage; $P < .05$ vs. the MCAO–vehicle of cerestat-treated rats (Figs. 1 and 2, respectively).

Treatment with 10 mg/kg NBQX antagonized the MCAO-induced neurological disorder only in the body proprioception test; $P < .05$, vs. MCAO–vehicle of NBQX-treated rats (Table 1). Dosages of 3 and 30 mg/kg NBQX were completely ineffective.

Cerestat at the dose of 1 mg/kg (0.3 and 3 mg/kg were inactive) counteracted MCAO-induced impairments in the body proprioception and forelimb flexion procedure; $P < .05$ and $P < .01$, respectively, vs. the MCAO–vehicle of cerestat-treated rats (Table 1).

Footfault test data did not reveal any beneficial effect of treatment with NBQX in all the parameters recorded (Table 2). Although there was a reduction of errors in the groups of animals that received 1 or 3 mg/kg cerestat with respect to their control counterparts, this difference did not yield statistical significance (Table 2). No differences in the motor abilities of control and treated animals were revealed in this task (Table 2).

4. Discussion

The results of our study suggest that treatment with NBQX did not provide protection of the neuronal damage occurring after the occlusion of the MCA. In all the behavioural and histological parameters recorded, NBQX (the only exception being at the dose of 10 mg/kg, which counteracted sensory deficits in the body proprioception test) did not antagonize the detrimental effects of MCAO.

The compound has been shown to be neuroprotective in some studies in different forebrain ischemia models (Diemer et al., 1992; Gill et al., 1992; Graham et al., 1996; Sheardown et al., 1990), but completely ineffective in other studies (DeGraba et al., 1994; McBurney, 1994). In all the aforementioned studies, evaluation of its efficacy was based on the morphometric assessment of the infarcted area, while, so far, the effects of NBQX on the neurological outcome have been evaluated only in one study (DeGraba et al., 1994). Our results are in agreement with those of the latter study, which also failed to detect any beneficial effect of NBQX on the neurological outcome of the MCAO rat (DeGraba et al., 1994).

Different reasons may underlie the inability of NBQX to provide neuroprotection in our experimental model. In principle, the hypothermic action of this compound might have been important. In most of the studies in which NBQX provided protection, temperature was not recorded during the postischemic period (Diemer et al., 1992; Sheardown et al., 1990). A recent experiment has shown that NBQX given at doses that are beneficial against ischemic injury had a mild hypothermic action that was linearly related to its neuroprotective efficacy (Nurse and Corbett, 1996). Therefore, in the present study, efforts were addressed to eliminate this nonspecific effect, which confounds NBQX's therapeutic efficacy. Thus, after surgery, animals were kept for 4 h in a thermostatic chamber

at 30°C. This procedure allowed the maintenance in rats of a postischemic body temperature of 36±1°C and did not permit to prove neuroprotection by NBQX suggesting that hypothermia and the alleged neuroprotective effects of NBQX were critically related.

Moreover, the well-known pharmacokinetic problems of this compound may perhaps have contributed to its inability to provide neuroprotection in our study (Gill, 1994). NBQX has a very short half-life (15–30 min) and at the doses required for neuroprotection, it is relatively insoluble in water, its administration resulting in precipitation into the renal tubules and nephrotoxicity (Gill, 1994).

Cerestat at the dose of 1 mg/kg significantly antagonized the neurological deficits exhibited by MCAO rats in the body proprioception and forelimb flexion tasks; it also significantly reduced volumes of infarct. It failed, however, to counteract the neurological and histopathological effects of the MCAO at 0.3 and 3 mg/kg. That only the intermediate dose of cerestat had a certain efficacy against the ischemic damage, whereas the extreme doses of the compound were ineffective, is in line with previous dose–response studies, where some NMDA receptor antagonists exhibited an inverted U type of dose–response (Meldrum, 1990). In this connection, the highest doses are seemingly responsible for the different side-effects displayed by noncompetitive NMDA receptor antagonists (Meldrum, 1990).

Cerestat has a high affinity for the NMDA ion-channel site. A tantalizing hypothesis for the mechanism of action of cerestat is that ion channel blockers, which inhibit NMDA receptor-mediated Ca^{2+} entry into neurons at any agonist concentration, are the preferred class of compounds to combat the Ca^{2+} overloading resulting from excessive stimulation of the NMDA receptor by the very high extracellular concentration of glutamate, which occurs in cerebral ischemia (McBurney, 1994).

The present results are in agreement with other findings in which some protectant effects of cerestat were evidenced (Aranowski et al., 1994, 1996). However, in our study, the beneficial action of cerestat was exerted on both the behavioural parameters and brain morphology, whereas in those studies, the efficacy of the compound was investigated separately (Aranowski et al., 1994, 1996).

Unfortunately, cerestat and all NMDA antagonists, if given in large-enough doses, cause severe side-effects in humans regardless of their pharmacology (Dorman et al., 1996).

In conclusion, this study shows that NBQX failed to antagonize the neurological and brain morphological deficits displayed by rats with focal ischemia, whereas cerestat, to some extent, did so. It is noteworthy that the results obtained in the present study with the use of glutamate receptor antagonists reflect to some extent the disappointing data obtained with these compounds in clinical trials on stroke (Dorman et al., 1996).

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