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Post-ischemic administration of diazoxide attenuates long-term microglial activation in the rat brain after permanent carotid artery occlusion

Eszter Farkas [a,*], Nienke M. Timmer [b], Ferenc Domoki [c], András Mihály [a], Paul G.M. Luiten [b], Ferenc Bari [c]

[a] *Department of Anatomy, School of Medicine, University of Szeged, H-6701 Szeged, P.O. Box 427, Hungary*
[b] *Department of Molecular Neurobiology, University of Groningen, The Netherlands*
[c] *Department of Physiology, School of Medicine, University of Szeged, Hungary*

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Abstract

Diazoxide is a putative mitochondrial, ATP-sensitive potassium channel opener that has been implicated in neuroprotection in cerebral ischemia. Administered as pretreatment, diazoxide can attenuate ischemia-related neuronal injury, but little is known about the potential neuroprotective properties of the drug when it is given after the onset of an ischemic insult. In a previous study, we applied diazoxide after imposing chronic cerebral hypoperfusion by means of permanent, bilateral occlusion of the common carotid arteries (2VO) in rats. We observed that ischemia-induced learning impairment assessed in the Morris water maze, and microglial activation visualized by immunocytochemistry, were prevented by diazoxide as determined at 13 weeks after 2VO. However, dimethyl sulfoxide, the organic solvent of diazoxide also prevented memory deficits, without any effect on microglial activity. Therefore, we have repeated our experiments with the use of an inorganic solvent, aqueous NaOH solution in order to clarify the effect of diazoxide independent of dimethyl sulfoxide. The present results demonstrated that diazoxide alone did not improve learning performance, but it prevented microglial activation in the hippocampus 13 weeks after the onset of 2VO. These data provide evidence that post-treatment with diazoxide is not effective in impeding a long-term memory deficiency, but it can attenuate ischemia-induced microglial activation, independently of the solvent used.
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Keywords: Cerebral hypoperfusion; Diazoxide; Ischemia; Hippocampus; Microglia; Spatial learning

Diazoxide (DIAZ), a benzothiadiazine derivative has long been used as an antihypertensive and antihypoglycemic drug [8]. DIAZ recently emerged as a selective, mitochondrial, ATP-dependent potassium channel opener that can protect cardiac myocytes and neurons against ischemia [1,2,11].

DIAZ has mostly been applied as pretreatment in various in vivo cerebral ischemia models and in neuronal cell cultures exposed to oxygen–glucose deprivation [1,4,9,11,15,16]. The experimental data unequivocally demonstrate the neuroprotective effect of the drug. For instance, pretreatment with DIAZ restricts the infarct size in experimental animals after middle cerebral artery occlusion [10,15], and preserves neuronal viability, probably via the induction of mitochondrial depolarization, free radical production and protein kinase C activation in neuronal cell cultures [1,9,16]. Although pretreatment with DIAZ has thus been proven to be a potent neuroprotective drug in experimental ischemia, it is of interest from a therapeutic point of view to learn whether a post-ischemic administration of the drug can also exert beneficial effects on the nervous tissue.

In order to investigate this possibility, in a previous study, we imposed chronic cerebral ischemia by permanently occluding the common carotid arteries of rats. Directly after surgery, DIAZ dissolved in dimethyl sulfoxide (DMSO) was applied, in a post-operative manner. Thirteen weeks later, we observed that DIAZ dissolved in DMSO successfully prevented a hypoperfusion-induced spatial learning impairment, and restored the microglial activation in the hippocampus to

* Corresponding author. Tel.: +36 62 545666; fax: +36 62 545707.
E-mail address: farkase@anat-fm.szote.u-szeged.hu (E. Farkas).

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Table 1
Survival rate and the incidence of CNS lesions

Experimental group	Survival rate (%)	CNS lesions (%)	
		Hippocampus	Cerebral cortex
SHAM/C	81.81 (9/11)	00.00 (0/9)	00.00 (0/9)
2VO/C	69.23 (9/13)	00.00 (0/9)	11.11 (1/9)
SHAM/DIAZ	72.72 (8/11)	00.00 (0/8)	00.00 (0/8)
2VO/DIAZ	60.00 (9/15)	11.11 (1/9)	22.22 (2/9)

the baseline. However, the organic solvent DMSO given alone also improved the spatial learning of animals with cerebral hypoperfusion, but it did not alter the microglial activation [7]. In order to determine the specific effects of the post-treatment with DIAZ independently of the biologically active DMSO, we have repeated the experiments with the use of an inorganic solvent, an aqueous solution of NaOH.

Thirty-five male Wistar rats (290 ± 45 g) were used for the study. All animal experiments were approved by the ethical committee of the University of Szeged. Chronic, experimental cerebral hypoperfusion was imposed on half of the animals by permanent bilateral occlusion of the common carotid arteries (2VO); the other half served as sham-operated controls (SHAM) [5]. Prior to surgery, the animals were anesthetized with 400 mg/kg chloralhydrate given i.p., followed by 0.05 ml atropine (1 mg/ml) i.m. The common carotid arteries were exposed via a ventral cervical incision, separated from their sheaths and vagal nerves, and permanently ligated with surgical sutures. The same procedure was performed on the SHAM group, but without the actual ligation. The survival rates for the groups are presented in Table 1.

Half of the animals in each surgical group underwent post-operative treatment with 0.5 mg/kg diazoxide (DIAZ) dissolved in 0.25 ml 0.1N NaOH as vehicle. The other half of the animals received 0.25 ml vehicle alone. The animals were injected i.p. on five consecutive days. The first injection was applied directly after surgery. The final compositions of the experimental groups are presented in Table 1.

Twelve weeks after surgery, the animals were trained in the Morris water maze [3,7]. This consisted of a circular pool (diameter: 160 cm, height: 35 cm) filled with water (22 °C), made opaque with milk so that the rats were unable to see an underwater platform 2 cm below the water surface. Visual cues were placed on the wall of the testing room, and a constant source of auditory stimulus with a fixed location was switched on throughout the testing. All rats performed two trials per day, with a constant intertrial interval of 4 h, for five consecutive days. The animals were placed in the water at one of four starting quadrant points, which was varied randomly over the trials. The rats were given 2 min to find the platform and sit on it for 15 s. Rats that failed to find the location within the given time were gently guided to the platform and were allowed to stay on it for 15 s. Swimming paths were recorded by a computerized video imaging analysis system (EthoVision, Noldus Information Technology BV, Wageningen, The Netherlands). In each trial, the escape latency, and the swimming distance traveled before reaching the platform were analyzed.

Seven days after the beginning of the Morris water maze training, the animals were anesthetized with an overdose of chloralhydrate (i.p.), and perfused transcardially with 100 ml saline followed by 400 ml 3.5% paraformaldehyde and 0.5% picric acid in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed and postfixed in the same solution for up to 1 h, and then stored in 0.1 PB containing 0.1% sodium azide.

Free-floating coronal sections at the level of the dorsal hippocampus were cut at a thickness of 20 μm on a cryostat microtome. Synaptophysin (a synaptic vesicle protein) labeling was performed on the first set of sections as follows. First, endogenous peroxidase activity was blocked with 3% H_2O_2. Nonspecific binding sites were covered with 5% normal porcine serum (NPS) and membrane permeability was enhanced with 0.5% Triton X-100. The sections were incubated overnight at room temperature (RT) in primary antibody solution containing rabbit anti-synaptophysin antibody (DAKO), 1:2000, 20% NPS and 0.3% merthiolate in 0.01 M PBS (pH 7.4). Next, incubation was performed in a solution of goat anti-rabbit biotinylated IgG (Jackson) 1:400, 10% NPS, 5% normal rabbit serum and 0.03% merthiolate in 0.1 M Tris buffer for 1 h at RT. Finally, the signal was amplified by STA-PER (Jackson), 1% NPS, and 0.03% merthiolate in 0.1 M Tris buffer for 1 h at RT. The color reaction was developed with nickel-diaminobenzidine (Ni-DAB) and H_2O_2.

A second set of sections was immunocytochemically stained for glial fibrillary acidic protein (GFAP) to visualize astrocytic proliferation. Briefly, sections were treated with 3% H_2O_2 and 0.5% Triton X-100 in 0.01 M PBS, and preincubated in 20% NPS. The samples were then incubated overnight at RT in a primary antibody solution containing mouse anti-GFAP antibody (Sigma), 1:40,000, 20% NPS, and 0.03% merthiolate in 0.01 M PBS. The secondary antibody solution consisted of goat anti-mouse biotinylated IgG (Jackson), 1:400, 10% NPS, 5% normal rabbit serum and 0.03% merthiolate in 0.01 M PBS. Finally, the sections were incubated in STA-PER (Jackson), 1% NPS and 0.03% merthiolate in 0.1 M Tris buffer, and the color reaction was developed conventionally with DAB and H_2O_2.

To detect and analyze microglial activation over the hippocampal areas, OX-42 antibody was used on a third set of sections. The procedure started with rinsing and pretreatment of the sections with 0.5% Triton X-100 and 3% H_2O_2 in 0.01 M PBS, followed by preincubation in 20% normal NPS and 0.5% Triton X-100 in 0.01 M PBS for 1 h. The sections were incubated overnight in a primary antibody solution containing biotinylated mouse anti-CD11b antibody (OX-42, Serotec), 1:500, 20% NPS and 0.03% merthiolate in 0.01 M PBS at RT. Next, the sections were rinsed, and incubated in a solution of STA-PER (Jackson), 1% NPS and 0.03% merthiolate in 0.1 M Tris buffer for 1 h at RT. Finally, the color reaction was developed with Ni-DAB and H_2O_2. All the sections were mounted on gelatin-coated microscopic slides, air-dried, dehydrated and coverslipped with DPX.

The percentage surface areas of synaptophysin-labeled terminals, GFAP-positive astrocytes and OX-42 immunoreactive microglia in the dorsal hippocampus were quantified by using an image analysis system (Olympus BX50, DP50, software: ImagePro Plus, Media Cybernetics). Briefly, three consecutive coronal sections at Bregma −3.60 mm [13] were selected for the analysis. Hippocampal regions of interest were manually delineated at 10× magnification, after background subtraction and gray-scale threshold determination. The area covered by immunoreactive material was calculated as a percentage of the total area delineated. Measurements were carried out on the hippocampus in both hemispheres. Six values per area per animal were averaged for use in further statistical analysis. Synaptophysin labeling was measured in the hippocampal CA3 str. lucidum. GFAP and OX-42 signals were measured in the CA1 str. radiatum, CA1 str. oriens, CA3 str. radiatum, CA3 str. oriens, the inner and outer molecular layers of the dentate gyrus, and the hilus.

The Morris maze test results were statistically analyzed by repeated measures of the general linear model of the software SPSS. Individual day comparisons were performed by analysis of variance (ANOVA). The immunocytochemical results were analyzed statistically with two-way ANOVA, followed by the LSD post hoc test.

As in our previous study [7], tendencies to a decreased survival rate and a higher prevalence of macroscopic cerebrocortical and hippocampal lesions were observed in the 2VO groups (Table 1).

The Morris water maze test confirmed the previous data in that the learning performance of the control 2VO animals was significantly worse than that of their SHAM controls throughout the entire training period. While the SHAM animals gradually learned the platform's location, the 2VO animals showed hardly any day-to-day improvement. The post-operative administration of DIAZ did not improve the learning capacity in the 2VO group except on day 4, when the 2VO group treated with DIAZ performed similarly to the SHAM groups (Fig. 1).

Synaptophysin labeling quantified in the CA3 str. lucidum demonstrated an insignificant, small increase in synaptic density in the nontreated 2VO group as compared with all the other experimental groups. GFAP immunocytochemistry revealed no astrocytic proliferation due to either cerebral hypoperfusion or treatment with DIAZ in any of the seven hippocampal areas investigated (Fig. 2A–E). In contrast, OX-42 immunoreactivity reflecting microglial activation showed a moderate but consistent, 15–25% increase in the nontreated 2VO group as compared with its respective SHAM control, specifically in the CA1 area and the dentate gyrus (Fig. 2F–J). Treatment with DIAZ restored the microglial activation completely to the baseline level.

Our present experiments were aimed at resolving the question of whether the administration of DIAZ after the onset of chronic cerebral ischemia can really cause improvements in spatial learning and the histological parameters, or whether the beneficial effect observed in our previous study

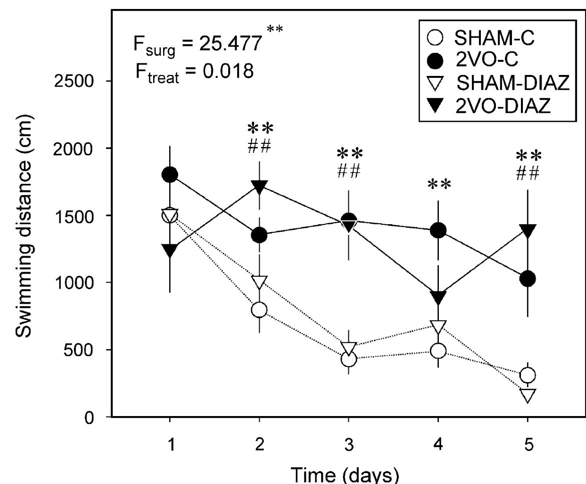

Fig. 1. Learning curves of the Morris water maze spatial learning test. Values are given as mean ± S.E.M.; statistical *F* values are based on a two-way repeated measurement ANOVA model ($^{*}p < 0.05$, $^{**}p < 0.01$). Individual days were analyzed in ANOVA and LSD post hoc tests ($^{*}p < 0.05$, $^{**}p < 0.01$). Asterisk (*) indicates a significant difference between SHAM-C and 2VO-C, hash (#) indicates a significant difference between SHAM-DIAZ and 2VO-DIAZ. Abbreviations: 2VO: bilateral carotid artery occlusion, DIAZ: diazoxide, SHAM: sham operation.

was attained in concert with the organic solvent, DMSO [7].

The present experimental data obtained with the Morris water maze test revealed no definite protective effect of the post-treatment with DIAZ on the learning impairment, which suggests that the neuroprotective action of DIAZ recorded in our previous study was a synergistic effect of DIAZ and DMSO. This conclusion is supported by the finding that DIAZ dissolved in aqueous NaOH solution did not prevent the development of macroscopic lesions in the hippocampus and cerebral cortex after 2VO. The result that the treated 2VO group performed as well as the SHAM group on day 4 in the Morris maze cannot be taken as sufficient evidence of the protective properties of the post-operative administration of the drug. The present results raise two possible explanations. First, it may be assumed that DIAZ could not prevent the deterioration of the spatial learning because it was given after (and not before) the onset of ischemia. Secondly, the possibility may be considered that DIAZ appeared to be ineffective on the learning performance because memory capacity was assessed at a rather late time point in chronic cerebral hypoperfusion. The first suggestion stands in line with the previously identified pharmacological action of DIAZ, i.e. the fact that the neuroprotective properties of DIAZ lie in its preconditioning effect. At a neuronal level, pretreatment with DIAZ can increase neuronal viability and moderate the deleterious outcome of an ischemic attack through an increased production of reactive oxygen species, the inhibition of succinate dehydrogenase and the activation of protein kinases [1,9,12]. However, direct evidence has not yet been acquired that pretreatment with DIAZ can actually prevent an ischemia-induced learning dysfunction. In fact, our previous

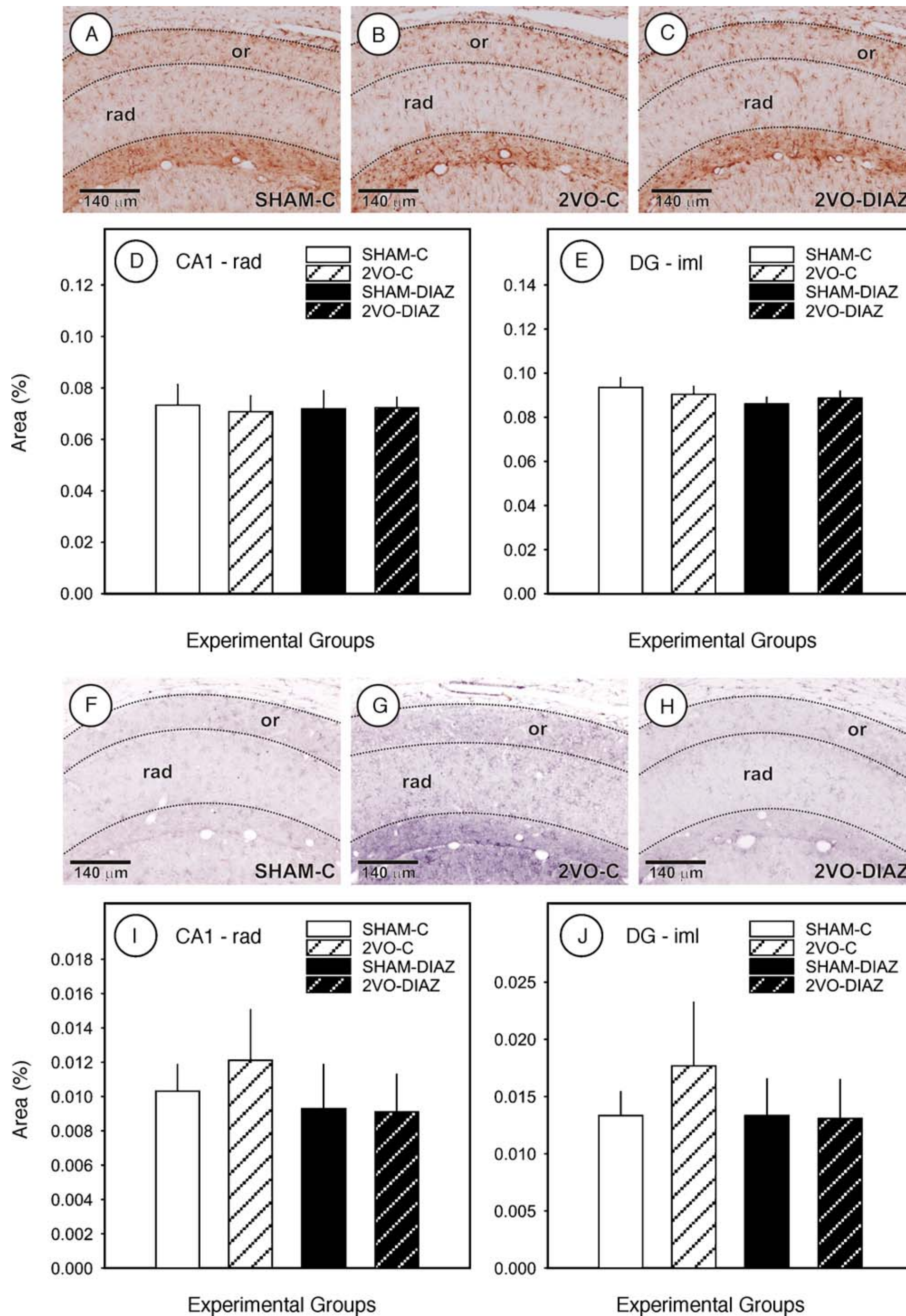

Fig. 2. GFAP and OX-42 immunocytochemistry. Panels A–C: representative photomicrographs of GFAP-labeled astrocytes in the dorsal hippocampus. Panels D and E: quantitative analysis of astrocytic proliferation. Panels F–H: representative photomicrographs of OX-42-labeled microglia in the dorsal hippocampus. Panels I and J: quantitative analysis of microglial activation. Data are shown as mean ± S.E.M. Abbreviations: 2VO: bilateral carotid artery occlusion, DIAZ: diazoxide, or: str. oriens, rad: str. radiatum, SHAM: sham operation.

study is the only one to have tackled the question of whether the effect of DIAZ can be retrieved at a behavioral level [7]. This is also the reason why it cannot be debated whether the time point for the testing (which we did not alter for our present study) is most appropriate. Therefore, our ongoing experiments have the goal of testing the animals at an earlier time point following the onset of 2VO and the administration of DIAZ, and to compare the test results obtained after pre and post-treatment with DIAZ.

Similarly as in our earlier study, the present data demonstrated an increased level of microglial activation in the hippocampus due to cerebral hypoperfusion, which could be prevented by the post-operative administration of DIAZ [7]. Besides the hippocampus, the same pattern of microglial

reaction was observed in the corpus callosum [6]. In this respect, DIAZ emerges as a potent drug for the attenuation of microglial activation in chronic ischemia, irrespective of the solvent used.

Although the action of DIAZ on cultured neurons and astrocytes has been repeatedly tested and comprehensively described [1,9,12,14], there are virtually no data on the potential mechanisms to account for the effects of DIAZ on microglia. Further, the in vivo nature of our experiments may raise the possibility that, even though microglia are most probably a primary target of DIAZ, reduced microglial activation may also be a secondary outcome of a protective effect of DIAZ on neurons. Nevertheless, this latter assumption appears to be unlikely, since the degree of microglial activation did not correlate with the spatial learning score, or the survival of labeled neurons in the hippocampus [7]. For the above reasons, the molecular and functional significance of decreased microglial activation due to DIAZ remains a subject for further investigation.

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