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 (DIAZ), a benzothiadiazine derivative has long been used as an antihypertensive and antihyperglycemic 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]. Diazoxide (DIAZ), a benzothiadiazine derivative has long been used as an antihypertensive and antihyperglycemic 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 neuro-

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Keywords: Cerebral hypoperfusion; Diazoxide; Ischemia; Hippocampus; Microglia; Spatial learning

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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.

First, endogenous peroxidase activity was blocked with 4% H2O2. Non-specific binding sites were covered with 5% normal bovine serum (NBS) 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), 1:4000, 10% NPS, 5% normal rabbit serum and 0.03% merthiolate in 0.1 M Tris buffer. The color reaction was developed with DAB and H2O2.

Table 1

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Survival rate (%)</th>
<th>CNS lesions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM/DIAZ</td>
<td>81.81 (9/11)</td>
<td>0.00 (0/9)</td>
</tr>
<tr>
<td>2VO/DIAZ</td>
<td>60.23 (9/15)</td>
<td>11.11 (1/9)</td>
</tr>
<tr>
<td>SHAM/C</td>
<td>72.72 (8/11)</td>
<td>0.00 (0/8)</td>
</tr>
<tr>
<td>2VO/DIAZ</td>
<td>40.00 (9/23)</td>
<td>22.22 (2/9)</td>
</tr>
</tbody>
</table>

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% H2O2 in 0.01 M PBS, followed by preincubation in 20% normal NBS at RT for 1 h. The sections were then incubated overnight in the 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 STAIN-96PER (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 H2O2.

A second set of sections was immunocytochemically stained for glial fibrillary acidic protein (GFAP) to visualize astrocytic proliferation. Brieﬂy, sections were treated with 3% H2O2 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. Finally, the sections were incubated in STAIN-96PER (Jackson), 1% NPS and 0.03% merthiolate in 0.1 M Tris buffer, and the color reaction was developed conventionally with DAB and H2O2.

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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 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...
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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References