Research Report

The effect of pre- and posttreatment with diazoxide on the early phase of chronic cerebral hypoperfusion in the rat

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Abbreviations:
2VO, permanent, bilateral common carotid artery occlusion (two-vessel occlusion)
ANOVA, analysis of variance
DAB, diaminobenzidine
DIAZ, diazoxide
GFAP, glial fibrillary acidic protein
mKATP channel, mitochondrial, ATP-dependent, K+ channel
NCa-ATP channel, Ca2+-activated, ATP-sensitive, nonselective, cation channel
NFS, normal pig serum
PB, phosphate buffer
RT, room temperature
SHAM, sham-operated control
SUR, sulphonylurea

ABSTRACT

Diazoxide has been identified as a mitochondrial, ATP-dependent K+ channel opener, and a potentially neuroprotective compound under ischemic conditions. We set out to characterize the consequences of various treatment strategies with diazoxide in a rat model of chronic cerebral hypoperfusion. Cerebral hypoperfusion was induced by permanent, bilateral occlusion of the common carotid arteries (2VO, n = 36), sham-operated rats serving as controls (SHAM, n = 29). Diazoxide or its vehicle was administered i. p. daily (5 × 0.5 mg/kg/0.25 ml) or as a bolus injection (5 mg/kg/0.25 ml) before surgery or daily after surgery (5 × 0.5 mg/kg/0.25 ml). Spatial learning performance was assessed 1 week after 2VO in the Morris maze. Hippocampal pyramidal cell loss was assessed on cresyl violet-stained sections, while glial reactivity was labeled immunocytochemically. Daily or bolus pretreatment with diazoxide significantly improved 2VO-related learning impairment, whereas posttreatment was ineffective. The number of CA1 pyramidal neurons was reduced by 2VO, which was prevented by repeated or bolus pretreatment with diazoxide. Astrocyte proliferation and microglial activation were enhanced by posttreatment with diazoxide in the hippocampus CA1 area of 2VO animals compared with SHAM. These data demonstrate that the neuroprotective effect exerted by diazoxide depends on the time of administration with respect to the onset of ischemia; pretreatment but not posttreatment with the compound has proved to be neuroprotective in chronic cerebral hypoperfusion. Thus, pretreatment with diazoxide offers therapeutic prospects for the treatment of cerebral ischemia.

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Diazoxide, a mitochondrial, ATP-dependent K⁺ (mK<sub>ATP</sub>) channel opener, has originally been used in clinical practice in hypertensive emergencies (Goodman Gilman et al., 1990). By now, an accumulating body of experimental data has demonstrated that diazoxide can be used for pharmacological preconditioning, attenuating ischemic heart damage (O’Rourke, 2004). In view of its preventive action in cardiac ischemia, diazoxide has become the focus of recent investigation as a potential anti-ischemic agent in the brain. The growing interest in the pharmacological action of diazoxide on the central nervous system was encouraged by the finding that the brain contains a considerably higher concentration of mK<sub>ATP</sub> channels (the target of diazoxide) than other peripheral organs (Bajgar et al., 2001).

Compelling evidence of the neuroprotective effect of diazoxide has emerged from experiments on brain slices or neuronal cell cultures, where diazoxide applied before oxygen and glucose deprivation enhanced the cell viability and preserved the electrophysiological properties of the hippocampal neurons (Zawar and Neumcke, 2000; Kis et al., 2003). Although neuroprotection on cell cultures and the intracellular pathways initiated by diazoxide have been documented in detail (Busija et al., 2004), little is known about the neuroprotective potential of diazoxide at the level of the brain. To date, only a few studies have administered diazoxide to experimental animals with the aim of examining the effect of the drug on the ischemic brain. In this way, diazoxide has been shown to limit the infarct size after middle cerebral artery occlusion (Busija et al., 2004). Despite these preliminary data, no clear evidence has been gathered as to whether diazoxide can actually prevent the cognitive dysfunction after an ischemic insult, and whether the cognitive status correlates with the histologic neural markers. Furthermore, most studies have applied diazoxide as pretreatment because of its known effects of acute and delayed pharmacologic preconditioning (Busija et al., 2004). Although diazoxide pretreatment has thus appeared to be a neuroprotective strategy of promise in cerebral ischemia, it is of interest from a therapeutic point of view to establish whether postischemic administration of the drug can also exert beneficial effects on the central nervous system.

In the present study, we followed previously devised methods (Farkas et al., 2004; 2005a,b) to identify the potentially neuroprotective effects of diazoxide in a rat model of chronic cerebral hypoperfusion. Since our previous work had focused on the effect of diazoxide at a late phase of chronic cerebral ischemia, our present objective was to investigate the action of diazoxide at an early phase of cerebral hypoperfusion. Further, as diazoxide had been administered only as posttreatment in our previous experiments (while others had used it only as pretreatment), in the present study, we also set out to compare the neural effects of pretreatment and posttreatment with diazoxide.

### Results

The spatial learning curves obtained with the Morris maze test demonstrated that 2 weeks of experimental cerebral hypoperfusion induced a marked decrease in learning performance in the non-treated 2VO group as compared with the non-treated SHAM group (Fig. 1A). Both repeated and bolus pretreatment with diazoxide improved the spatial memory. The 2VO animals that received either repeated or bolus diazoxide pretreatment exhibited the same performance as concerns the average swimming distance as compared with their respective SHAM controls, specifically on days 2, 3, and 5 (Figs. 1B and C). Conversely, the 2VO animals that received diazoxide posttreatment did not succeed in learning the Morris maze paradigm. On days 2 and 5, the 2VO rats posttreated with diazoxide achieved significantly worse results as compared with the corresponding SHAM posttreated group (Fig. 1D).

Corresponding with the results of the learning test, cerebral hypoperfusion induced a moderate but constant loss of hippocampal CA1 pyramidal neurons (Fig. 2). Although massive necrotic cell death was not detected, the CA1 str. pyramidale in the 2VO animals displayed a loose cellular arrangement as compared with the compact structure of the pyramidal layer in the SHAM animals (Figs. 2A and B). Cell counting revealed that 2VO decreased pyramidal cell number with 6%. Both the repeated and the bolus pretreatment with diazoxide preserved the compact structure of the CA1 str. pyramidale (Fig. 2C) and prevented pyramidal cell loss (Fig. 2D).

GFAP-immunoreactive astrocytes were present in all the hippocampal regions and in all the experimental groups. Neither 2VO nor pretreatment with diazoxide exerted a detectable influence on the GFAP immunoreactivity in the investigated hippocampal areas. However, posttreatment with diazoxide doubled the GFAP signal in the CA1 str. radiatum and str. oriens of the 2VO rats (Fig. 3).

Activated microglia labeled with OX-42 were scarce not only in the SHAM animals but also in the 2VO non-treated rats in all regions of the hippocampus. In contrast, dense staining was detected in the CA1 str. oriens and str. radiatum of the 2VO animals posttreated with diazoxide (Figs. 4A–C). Quantitative analysis demonstrated that the application of diazoxide after 2VO increased the OX-42 signal approximately 2.5-fold in the CA1 str. oriens and 7-fold in the CA1 str. radiatum as compared with the SHAM groups (Figs. 4D and E).

### Discussion

The present study has revealed that pretreatment with diazoxide at the dose of 5 × 0.5 mg/kg or 1 × 5 mg/kg, given i.p. is neuroprotective as regards chronic cerebral hypoperfusion in rats. The neuroprotective action of the pretreatment with diazoxide was reflected in an improvement in spatial learning performance and the inhibition of pyramidal cell loss in the hippocampus CA1 region.

Our line of investigation is novel in the sense that the action of diazoxide on learning and memory has not previously been
characterized in cerebral hypoperfusion or ischemia. The present results demonstrate that diazoxide prevents the cerebral hypoperfusion-related learning dysfunction when administered as pretreatment. However, posttreatment with diazoxide exerted no clear effect on the learning performance, similarly as we earlier observed at a later time point in cerebral hypoperfusion (Farkas et al., 2005a). The finding that pretreatment with diazoxide has a beneficial effect on learning, and the integrity of the hippocampal pyramidal cells is in accord with the pharmacological action of diazoxide revealed in studies on cultured neurons. The pretreatment of neurons with diazoxide before oxygen-glucose deprivation increased their viability as compared with that of non-treated cultures (Nagy et al., 2004). Diazoxide is known to depolarize the mitochondria and to simultaneously elevate the production of reactive oxygen species, which is assumed to play a significant role in the preconditioning effect of diazoxide (Busija et al., 2004). The transient, moderate elevation of the concentration of reactive oxygen species by diazoxide is suggested to condition the cells to be able to tolerate a later, robust ischemic event better. Such preconditioning may account for the preserved learning performance and enhanced hippocampal neuronal viability of 2VO rats following pretreatment with diazoxide.

The immunocytochemical signals for both astrocytes and microglia were markedly enhanced by posttreatment with diazoxide in the 2VO animals, while 2VO or diazoxide alone had no effect on the glial cells. This suggests that, given the sequence of 2VO and treatment, diazoxide was able to stimulate the glial cells, provided that the glia were already made sensitive by ischemia.

Although the lack of evidence means that it is difficult to establish a causal link between treatment with diazoxide and a glial reaction, the intriguing observation was made that a subpopulation of reactive astrocytes expressed a Ca²⁺-activated, ATP-sensitive nonselective cation channel (NCa-ATP) under hypoxic conditions. Interestingly, these channels are controlled by a sulfonylurea regulatory subunit, SUR1, which is the target of diazoxide. It has been shown that the treatment of such astrocytes with diazoxide results in activation of the NCa-ATP channels, which are suggested to control the swelling and migration of astrocytes in hypoxic nervous tissue (Chen et al., 2003). Even though a strict correlation cannot be drawn with our data, a similar mechanism may play a role in the diazoxide-induced astrocytic proliferation shown here.

The background of diazoxide-related microglial activation is even more obscure. Although the activity of the microglia is dependent on the opening state of the surface ion channels (Eder, 1998), regulatory SUR subunits (the potential sites of the action of diazoxide) have not been associated with these channels. Further, microglial activation is accompanied by an increased number and elongated shape of mitochondria (Banati et al., 2004), but a potential contribution of mitochondrial K⁺ currents to these processes has not been identified, yet. Finally, the existing data on the effect of diazoxide on microglial activation in cerebral hypoperfusion are inconsistent: posttreatment with diazoxide prevents microglial activation at a late time point in cerebral hypoperfusion (Farkas et al., 2005a).
al., 2004, 2005a,b), while the same treatment enhances microglial activation at an early time point, as shown here. This may suggest that the effect of diazoxide on ischemia-related microglial activation is defined by the time of sampling with respect to the treatment and the onset of cerebral hypoperfusion.

The concentration of diazoxide was chosen to be low enough to exclude potentially harmful side effects such as hypotension, reduced cerebral blood flow or hyperglycemia, which are known to potentiate ischemic injury. Recent results have demonstrated that the plasma glucose level and mean arterial pressure in rats are not significantly affected by even 6 mg/kg diazoxide i.p. (Lenzsér et al., 2005). A selective effect of a low dose of diazoxide on the brain is probably due to the distribution and molecular composition of the mKATP channels. The brain contains a 6–7 times higher concentration of mKATP channels than the liver or the heart (Bajgar et al., 2001). Our present observation that a low concentration of diazoxide attenuates ischemic hypoperfusion-induced brain damage, yet does not alter the vascular and metabolic parameters, may be of therapeutic potential in cerebral ischemia.

In conclusion, we have found that a low dose of diazoxide that is ineffective on the mean arterial pressure or blood glucose concentration (Lenzsér et al., 2005) is potent on the central nervous system. Further, diazoxide prevents an ischemia-induced learning impairment when the drug is administered as pretreatment, but not as posttreatment. Finally, diazoxide potentiates glial reactivity when given as posttreatment, but not as pretreatment. The present findings on the glial reaction call for further research on the mechanisms of action of diazoxide on glia cells. Our results may additionally provoke investigations on the neuroprotective properties of diazoxide with therapeutical prospects in ischemia.

4. Experimental procedures

Sixty-five male Wistar rats (302 ± 18 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 induced in half of the animals by permanent bilateral occlusion of the common carotid arteries (2VO), the other half serving as sham-operated controls (SHAM) (Farkas et al., 2004). Prior to surgery, the animals were anesthetized with 400 mg/kg chloralhydrate i.p., followed by 0.05 ml 0.1% atropine i.m. The common carotid arteries were exposed via a ventral cervical incision, carefully separated from their sheaths and vagal nerves, and permanently ligated with surgical sutures. Lidocaine (1%) was applied as local anesthetic. The same procedure was performed on the SHAM group but without the actual ligation.

The animals were treated with diazoxide or its vehicle (0.25 ml 0.1 N NaOH), i.p. In the first group, diazoxide (0.5 mg/kg/0.25 ml) or its vehicle was injected on 5 consecutive days immediately before 2VO surgery. In the second group, diazoxide (5 mg/kg/0.25 ml) was administered once (bolus) the day before 2VO surgery. In the third group, diazoxide (0.5 mg/kg/0.25 ml) or its vehicle was injected on 5 consecutive days after 2VO surgery, the first injection given within 1 h following surgery. The final compositions of the experimental groups and the survival rates are presented in Table 1.

One week following 2VO surgery, the animals were trained in the Morris water maze (Farkas et al., 2004), which consisted of a circular pool (diameter: 160 cm, depth: 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. Invariant visual cues were placed on the wall of the testing room, and an auditory source with a fixed location was switched on throughout the testing. All the rats performed two trials per day, with a constant inter-trial interval of 4 h, for 5 consecutive days. The starting positions were selected at random from four standard entry points. 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 allowed to stay on it for 15 s. Swimming paths were recorded with a computerized video imaging analysis system (EthoVision, Noldus Information Technology BV, Wageningen,

Fig. 2 – Cresyl violet staining and cell counting in the hippocampus CA1 str. pyramidale. (A–C) Representative photomicrographs of the hippocampal CA1 area from pretreated animals. (D) Quantitative data on pyramidal cell viability in the hippocampus CA1 str. pyramidale; area unit is 0.024 mm² (8 x 10 grid holes at 40× magnification). Data are presented as means ± SEM; *P < 0.05. The P value indicated in the graph was obtained with a Student’s t test. Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); C: control for treatment; DIAZ: diazoxide; pre: pretreatment; SHAM: sham-operated control.
The Netherlands). In each trial, the escape latency and the swimming distance traveled before the rats reached the platform were analyzed.

Seven days after the beginning of the Morris water maze training, and 14 days after the performance of 2VO, the animals were reanesthetized with an overdose of chloralhydrate (i.p.)

Fig. 3 – GFAP immunocytochemistry for astrocyte proliferation. (A–C) Representative photomicrographs of the hippocampal CA1 area from posttreated animals. (D) Quantitative data on GFAP immunocytochemistry in the hippocampal CA1 str. oriens of the posttreated animals. (E) Quantitative data on GFAP immunocytochemistry in the hippocampal CA1 str. radiatum of the posttreated animals. Data are presented as means ± SEM; *P < 0.05, **P < 0.01. Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); C: control for treatment; DIAZ: diazoxide; post: posttreatment; SHAM: sham-operated control.

Fig. 4 – OX-42 immunocytochemistry relating to microglia activation. (A–C) Representative photomicrographs of the hippocampal CA1 area from posttreated animals. (D) Quantitative data on OX-42 immunocytochemistry in the hippocampal CA1 str. oriens of the posttreated animals. (E) Quantitative data on OX-42 immunocytochemistry in the hippocampal CA1 str. radiatum of the posttreated animals. Data are presented as means ± SEM; *P < 0.05, **P < 0.01. Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion); C: control for treatment; DIAZ: diazoxide; post: posttreatment; SHAM: sham-operated control.
Table 1 – Experimental groups and survival rate

<table>
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<th>Type of treatment</th>
<th>Experimental group</th>
<th>No. of operated animals</th>
<th>No. of survived animals</th>
<th>Survival rate (%)</th>
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<td>5</td>
<td>83</td>
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<tr>
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<td>89</td>
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<tr>
<td></td>
<td>SHAM-DIAZ</td>
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<td>75</td>
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<td>100</td>
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<tr>
<td></td>
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<td>5</td>
<td>83</td>
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<tr>
<td>Posttreatment</td>
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<td>6</td>
<td>86</td>
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<tr>
<td></td>
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</table>

Abbreviations: 2VO: permanent, bilateral common carotid artery occlusion (two-vessel occlusion), C: control for treatment, DIAZ: diazoxide, SHAM: sham-operated control.

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 M PB containing 0.1% sodium azide.

Free-floating coronal sections at the level of the dorsal hippocampus were cut at 20-μm thickness on a cryostat. Three sections per animal that contained the dorsal hippocampus (Bregma –3.60, Paxinos and Watson, 1986) were mounted on gelatine-coated microscopic slides and stained with cresyl violet.

A second set of sections was stained immunocytochemically for glial fibrillary acidic protein (GFAP) to visualize the astrocytic proliferation. Briefly, sections were treated with 3% H2O2 and 0.5 Triton X-100 in 0.01 M PBS and preincubated in 20% normal pig serum (NPS). The samples were then incubated overnight at RT in a primary antibody solution containing mouse anti-GFAP antibody (Sigma), 1:4000, 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 conventionally developed with diaminobenzidine (DAB) and H2O2.

To detect and analyze the microglial activation over the hippocampal areas, OX-42 antibody was used on a third set of sections. The procedure started with rinsing and pretreating the sections with 0.5% Triton X-100 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 then 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 H2O2. All the sections were mounted on gelatin-coated microscopic slides, air-dried, dehydrated, and coverslipped with DPX.

The number of pyramidal neurons in the hippocampus CA1 region was counted with the help of an ocular grid at 40× magnification on a surface of 0.024 mm² (3 × 10 grid holes) that covered the entire diameter of the CA1 str. pyramidal. Cell counting was performed bilaterally on three consecutive brain sections. The six values were averaged, and the average was used for further statistical analysis.

The percentage surface areas of GFAP-positive astrocytes and OX-42-immunoreactive microglia in the dorsal hippocampus were quantified by using a computerized image analysis system (Olympus BX50, DP50, software: ImagePro Plus, Media Cybernetics, U.S.A.). Briefly, three consecutive coronal sections at Br. –3.60 mm (Paxinos and Watson, 1986) were selected for the analysis. Hippocampal regions of interest were delineated manually at 10x magnification, after background subtraction and gray scale threshold determination. The area covered by immunoreactive material was computed as a percentage of the total area delineated. Measurements were carried out on both sides of the hippocampus. Six values per animal per area were averaged, and the average was used for further statistical analysis. The following areas were analyzed separately: the CA1 str. radiatum, the CA1 str. oriens, the CA3 str. radiatum, the CA3 str. oriens, the inner and outer molecular layers of the dentate gyrus, and the hilus.

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

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