MitoK$_{ATP}$-Channel Opener Protects against Neuronal Death in Rat Venous Ischemia

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Received, September 14, 2004.
Accepted, December 2, 2004.

OBJECTIVE: Mitochondrial adenosine triphosphate-dependent potassium (mitoK$_{ATP}$) channels are present in the brain, and several reports have shown their neuroprotective, preconditioning effect against an ischemic insult. The role of mitoK$_{ATP}$ channels in the penumbra area has not been studied thoroughly. In a model of venous ischemia, widespread penumbra-like low flow areas are created, which are susceptible to cortical spreading depression. Thus, we studied effects of mitoK$_{ATP}$ channels on infarct size in this model.

METHODS: Male Wistar rats were subjected to two-vein occlusion by photochemical thrombosis of two adjacent cortical veins combined with KCl-induced cortical spreading depression. The rats were assigned to four experimental groups pretreated intraventricularly 15 minutes before two-vein occlusion with 1) vehicle, 2) the mitoK$_{ATP}$ channel opener diazoxide (2 mmol/L), 3) diazoxide (2 mmol/L) plus the selective mitoK$_{ATP}$ channel blocker 5-hydroxydecanoate (5-HD; 100 mmol/L), or 4) 5-HD alone (100 mmol/L). Regional cerebral blood flow (laser Doppler scanning) and brain cell swelling (impedance) were monitored acutely. Infarct volume was assessed 7 days after ischemia.

RESULTS: Pretreatment with diazoxide significantly reduced the infarct volume from 6.2 $\pm$ 0.7 mm^3 to 3.8 $\pm$ 0.4 mm^3, whereas regional cerebral blood flow in the vicinity of the two veins was comparable in both groups 70 minutes after two-vein occlusion. Effects of diazoxide were abolished by 5-HD, whereas 5-HD alone even increased infarct volume.

CONCLUSION: These results suggest that the opening of mitoK$_{ATP}$ channels plays a major role in brain protection under penumbra-like conditions, as shown in this venous occlusion model.

KEY WORDS: Cortical spreading depression, Diazoxide, Mitochondria, Penumbra, Potassium channels, Preconditioning, Tolerance induction, Venous ischemia

Neurosurgery 57:334-340, 2005 DOI: 10.1227/01.NEU.0000166681.88736.86 www.neurosurgery-online.com

It is well documented that activation of adenosine triphosphate-dependent potassium (K$_{ATP}$) channels prevents ischemic neuronal cell death and is involved in ischemic tolerance (12, 19, 20). K$_{ATP}$ channels are found in many locations within cells, including the surface membrane and the inner mitochondrial membrane (mitoK$_{ATP}$), and they have different pharmacological and functional properties. Bajgar et al. (3) recently found that brain mitochondria contain 7 times more mitoK$_{ATP}$ channels than heart mitochondria, which indicates an important role for these channels in neurons. Studies of cardiac myocytes have provided convincing evidence that the opening of mitoK$_{ATP}$ channels plays a major role in ischemic tolerance (16, 17). The application of diazoxide, a selective mitoK$_{ATP}$ channel opener, induces protective effects similar to ischemic preconditioning, whereas the application of 5-hydroxydecanoate (5-HD), a selective mitoK$_{ATP}$ channel blocker, abolishes ischemic tolerance in the heart (7, 17). In the brain, comparable results have been obtained in the middle cerebral artery occlusion model (13, 28). Recent findings suggest that diazoxide prevents glutamate release from presynaptic terminals (4), and reactive oxygen species release (6) and inhibit apoptosis (16, 30). However, the mechanisms leading

to ischemic tolerance by opening of these channels have not been investigated thoroughly.

We have developed a venous infarct model with occlusion of two adjacent cortical veins (21–24). At this location, cerebral blood flow (CBF) in the vicinity of occluded veins is reduced rather homogeneously, with a large penumbra, a slowly growing infarct, and delayed cell death (11, 18). This two-vein occlusion (2VO) model therefore seems suited to the study of pathophysiological mechanisms, including cortical spreading depression (CSD) threatening nervous tissue in the penumbra. In the present study, we evaluated whether pharmacological opening of mitoK$_{ATP}$ channels by diazoxide in a concentration effective for middle cerebral artery occlusion (28) suppresses infarct growth into the penumbra and whether effects can be prevented by a mitoK$_{ATP}$ channel blocker (28).

MATERIALS AND METHODS

This study was conducted according to German animal protection legislation and has been reviewed by the regional ethics committee.

Animal Preparation

The experiments were performed using 49 male Wistar rats (weight 351 3.27 g, range 305–400 g; Charles River Laboratories, Sulzfeld, Germany). The animals were housed in individual cages and allowed free access to food and water. Anesthesia was induced by an intraperitoneal injection of chloral hydrate (36 mg/100 g body weight), and the animals were premedicated with 1 mg of subcutaneously administered atropine. Anesthesia was maintained with chloral hydrate (12 mg/100 g body weight/h) administered continuously through a peritoneal catheter. All animals were intubated with silicon tubing (outer diameter, 2.5 mm) and mechanically ventilated using a rodent ventilator (Model 683; Harvard Apparatus, South Natick, MA) with 30% inspired oxygen and controlled end expiratory PCO_2 (Artema MM206C; Heyer, Sundbyberg, Sweden). Rectal temperature was kept at 37.0°C by using a feedback-controlled heating pad (Harvard Apparatus), and the left temporal muscle temperature was monitored throughout the experiment. A polyethylene catheter (outer diameter, 0.96 mm; Portex; Smiths Industries Medical Systems Co., London, England) was inserted into the tail artery to monitor mean arterial blood pressure and arterial blood gases, pH, electrolytes, and glucose levels. Another polyethylene catheter was inserted into the left femoral vein. After rats were mounted in a stereotactic frame (Stoelting, Wood Dale, IL), a midline skin incision was prepared and a left frontoparietal cranial window was made to access the brain surface by using a high-speed drill under an operating microscope (OP-Microscope; Zeiss, Wetzlar, Germany). During the craniectomy, the drill tip was cooled continuously with physiological saline to avoid thermal injury to the cortex. The dura was left intact.

Cortical Vein Occlusion by Photochemical Thrombosis

The occlusion of two adjacent cortical veins was induced with the use of rose bengal dye (Sigma Chemical Co., St. Louis, MO) and fiberoptic illumination using a 50 W mercury lamp (6500–7500 lx, 540 nm) and a 100-μm fiber (21, 22). Only animals with similar venous anatomy (i.e., with two prominent adjacent veins connecting into the superior sagittal sinus) were used (*Fig. 1B*). The diameter of the occluded veins was approximately 100 μm. Rose bengal dye (50 mg/kg body weight) was injected slowly, and care was taken not to illuminate tissue and other vessels near the target vein. After photochemical thrombosis of the first vein (10–15 min), half of the initial rose bengal dose was injected intravenously and the second selected vein was illuminated.

Measurement of CBF and Tissue Impedance

As described previously (21, 22), local CBF was measured by using laser Doppler (LD) scanning (Model BPM 403a; Vasomedics, St. Paul, MN) with a 0.8-mm needle probe. Flow is expressed in LD units. Local CBF was measured at 25 locations with the occluded veins lateral to the scanning field using a stepping-motor-driven and computer-controlled micromanipulator (14, 21, 23, 29). Thus, one scanning procedure yielded information from 25 different locations each at a distance of 300 μm. Scanning was performed before and 0, 15, 30,

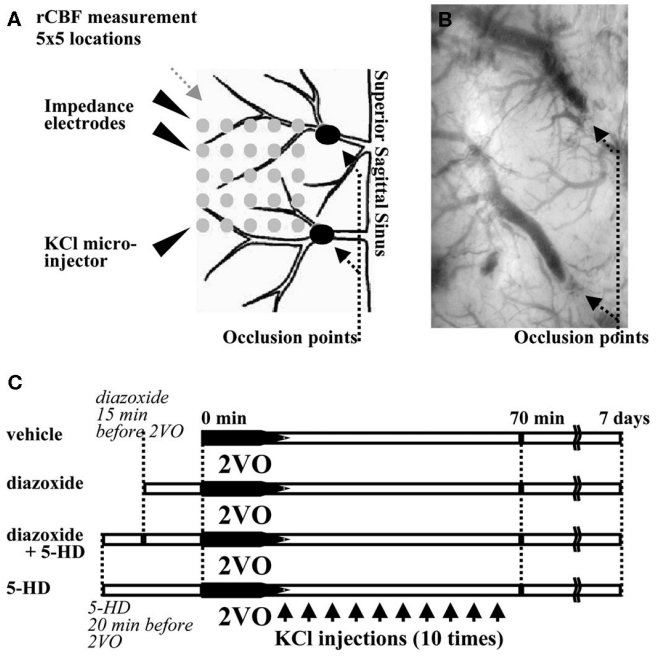

FIGURE 1. A, *schematic drawing of the experimental setup. The location of impedance electrodes, scanning field (25 locations), and occlusion sites of two adjacent cortical veins of the parietal cortex are shown. Impedance electrodes, KCl microinjector, and scanning field were placed to ensure that each distance was comparable.* B, *brain surface as observed through the closed dura 70 minutes after vein occlusion.* C, *timing of drug injections, vein occlusions, and KCl injections for all groups.*

50, and 70 minutes after 2VO. The median of observation frequency histograms correlates with absolute blood flow as determined by hydrogen clearance (31). Probes were positioned close to the dura surface.

To measure cell swelling that occurred during cortical spreading depression and during ischemia, two impedance electrodes were introduced into the cortex (depth, 0.4–0.5 mm; distance, 3 mm) (*Fig. 1A*). The impedance electrodes were made from two stainless steel wires (outer diameter, 0.5 mm) covered by polyvinyl chloride for electrical insulation except for the 0.3-mm sharp-pointed tip. Impedance was measured at 1 kHz (10 mV, bias-free) throughout the experiment using a precision LCR monitor (4284A; Hewlett-Packard, Avondale, PA). At that frequency, the alternating current travels through the extracellular space and impedance increases if extracellular space shrinks, i.e., cells swell (26).

Induction of Cortical Spreading Depression

Ten minutes after impedance electrode insertion, a glass micropipette for KCl injection was placed into the lateral parietal cortex (*Fig. 1A*). The micropipette was filled with 150 mmol/L KCl solution, and rats received 10 5.0-μl KCl injections at 7-minute intervals (*Fig. 1C*). KCl administration using a microinjection pump (CMA/100; Carnegie Medicine, Stockholm, Sweden) started after completion of the 2VO and ended 70 minutes thereafter.

Experimental Design and Treatment Groups

The rats were randomly assigned to one of four groups (*Fig. 1C*): 1) a vehicle-treated group receiving either saline (n = 5) or 0.1 mol/L NaOH (n = 12); 2) a diazoxide-treated group (n = 12); 3) a diazoxide plus 5-HD-treated group (n = 13); and 4) a 5-HD-treated group (n = 7). In all groups, drugs were injected into the right cerebral ventricle (anteroposterior −0.8 mm, lateral 1.5 mm, and dorsoventral 4.0 mm) (27).

Diazoxide and 5-HD were purchased from Sigma Chemical Co. Diazoxide was dissolved in 0.1 mol/L NaOH, and a total of 15 μl diazoxide (2 mmol/L) was administered 15 minutes before induction of venous ischemia. 5-HD was dissolved in saline, and 10 μl of a 100 mmol/L solution was injected intracerebroventricularly 20 minutes before vein occlusion. We also studied vehicle-treated animals that received either saline or 0.1 mol/L NaOH administered before ischemia. Because the groups that received vehicle did not differ in infarct volume, we combined these rats into one vehicle group.

Histological Preparation

After surgery, the skin wounds were closed with 4-0 silk. The rats were returned to individual cages and killed 7 days after surgery. Each rat was perfusion-fixed with 4% paraformaldehyde (pH 7.4) under deep anesthesia, and the brain was carefully removed from the skull. Brains were embedded in paraffin, and coronal sections (3 μm thick) were cut throughout the parietal region containing the infarct. Sections were stained with hematoxylin and eosin. The histological evaluation of the infarct size was performed by using light microscopy connected to a calibrated image-analyzing system consisting of a Sony charge-coupled device camera (Sony, Tokyo, Japan) and Optimas 6.51 software (Optimas Corp., Seattle, WA). The infarction area was evaluated in serial sections of 200-μm steps. The infarction volume was calculated and expressed in cubic millimeters.

Statistical Analysis

Data are expressed as means ± standard error of the mean. A one-way analysis of variance test was used to compare data. Statistical analyses were performed by using SigmaStat software (Jandel Scientific, SPSS, Erkrath, Germany). Statistical significance was assumed at an error probability of $P < 0.05$.

RESULTS

Physiological Data

Physiological parameters were within normal range in all groups throughout the entire experiment. Mean arterial blood pressure and brain temperature were not significantly changed before and after the 2VO (*Table 1*).

Sequential Changes of Regional CBF

The calculation of median regional CBF (rCBF) values from the 25 locations in each animal demonstrated no significant difference in all groups before drug application and venous ischemia (*Fig. 2*). Before the intraventricular injection, rCBF values were 41.0 ± 4.0, 42.0 ± 3.5, 39.6 ± 3.3, and 40.7 ± 4.4 LD units in the vehicle, diazoxide, 5-HD plus diazoxide, and 5-HD groups, respectively. There were no CBF changes after drug administration (rCBF after intracerebroventricular injection: 41.2 ± 3.8, 41.1 ± 3.4, 39.1 ± 3.7, and 39.1 ± 3.7 LD units, respectively) 70 minutes after 2VO. rCBF values in the vicinity of the two occluded veins were reduced by approximately 50% in all groups (18.4 ± 1.5, 21.0 ± 2.3, 22.0 ± 2.8, and 21.3 ± 3.3 LD units, respectively) 70 minutes after 2VO. There was no statistical difference between these rCBF reductions owing to 2VO between groups (*Fig. 2*).

Impedance Change after CSDs

Under baseline conditions, cortical impedance values showed no significant difference among groups (vehicle, 6.5 ± 0.6 kΩ; diazoxide, 5.1 ± 0.5 kΩ; diazoxide plus 5-HD, 6.1 ± 0.5 kΩ; and 5-HD, 5.4 ± 0.6 kΩ). Within 60 to 120 seconds after KCl injection into the cortex, a solitary episode of CSD was always observed as a sudden temporal increase of tissue impedance (*Fig. 3*). Impedance values normalized after each CSD wave, and terminal depolarization-like alterations of impedance were never seen.

Infarct Volume

All rats studied had a cortical infarct. Quantitative assessment of infarct volume showed that after diazoxide, i.e., in the

TABLE 1. Physiological variables before and after two-vein occlusion[a]

	MABP (mm Hg)	Brain temp. (°C)	Glucose (mg/dL)	pH	PO_2 (mm Hg)	PCO_2 (mm Hg)
Vehicle						
Before 2VO	99.1 ± 0.4	36.8 ± 0.02		7.424 ± 0.01	130.6 ± 2.2	41.7 ± 0.8
After 2VO	98.9 ± 0.4	36.7 ± 0.04	170.6 ± 5.5	7.397 ± 0.01	126.4 ± 2.9	40.4 ± 0.5
Diazoxide						
Before 2VO	98.0 ± 0.5	36.8 ± 0.04		7.413 ± 0.01	128.3 ± 3.5	41.9 ± 0.6
After 2VO	98.6 ± 0.3	36.8 ± 0.04	173.9 ± 6.1	7.417 ± 0.01	123.4 ± 2.6	40.2 ± 0.7
Diazoxide + 5-HD						
Before 2VO	98.6 ± 0.4	36.9 ± 0.02		7.448 ± 0.01	123.4 ± 3.0	39.9 ± 0.4
After 2VO	100.3 ± 0.4	36.8 ± 0.02	162.4 ± 6.1	7.415 ± 0.01	126.4 ± 3.6	40.2 ± 0.5
5-HD						
Before 2VO	99.5 ± 0.6	36.8 ± 0.03		7.423 ± 0.01	127.5 ± 5.0	41.7 ± 1.0
After 2VO	99.3 ± 0.6	36.8 ± 0.02	170.9 ± 5.3	7.423 ± 0.02	117.6 ± 5.3	39.7 ± 0.5

[a] MABP, mean arterial blood pressure; temp, temperature; PO_2, partial pressure of oxygen; PCO_2, partial pressure of carbon dioxide; 2VO, two-vein occlusion; 5-HD, 5-hydroxydecanoate. The data are expressed as the means ± standard error of the mean. There were no statistically significant differences in any physiological variable among the four groups.

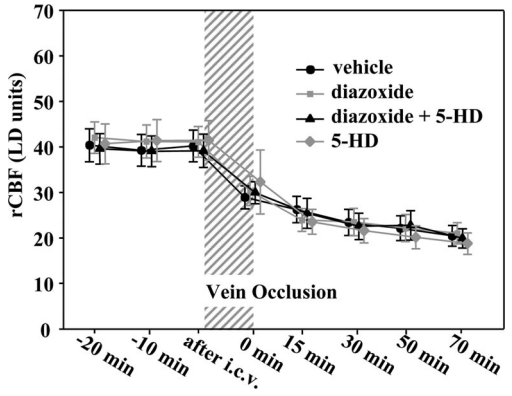

FIGURE 2. *Sequential changes of regional CBF (obtained from median local CBF data of individual rats). A typical 50% reduction of rCBF 70 minutes after 2VO can be seen in all groups.* i.c.v., *intracerebroventrical administration.*

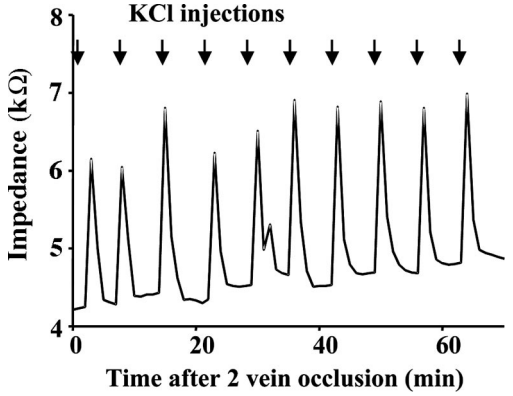

FIGURE 3. *Typical example of KCl-induced CSDs. Graph shows sequential changes of cerebral tissue impedance.* Arrows *indicate KCl injections (10 times every 7 min after 2VO).*

mitoK$_{ATP}$ channel opener-treated group, infarct volume was significantly reduced to 3.7 ± 0.4 mm^3 compared with that of the vehicle-treated group (6.2 ± 0.7 mm^3). The reduction of infarct volume was abolished by co-treatment with 5-HD, the selective mitoK$_{ATP}$ channel blocker (6.1 ± 0.5 mm^3). In the experimental group treated with 5-HD alone, the infarcts were significantly larger (8.8 ± 1.0 mm^3) than after vehicle treatment (*Figs. 4* and *5*).

DISCUSSION

In the current study, we present evidence that pretreatment with diazoxide reduces infarct growth in a venous focal cerebral ischemia model. In previous studies, we have shown that infarct size increases after vein occlusion as a result of spreading depression (26), that this increase involves caspase-dependent mechanisms (9), and that it can be reduced by measures that improve the microcirculation (10). The effect of diazoxide in decreasing infarct size was not correlated with a restoration of local CBF before, during, or after 2VO. This neuroprotective effect was abrogated by the selective mitoK$_{ATP}$ channel blocker 5-HD (5, 16, 28). Therefore, our present study indicates that the opening of mitoK$_{ATP}$ channels plays a pivotal role in triggering the development of pharmacologically induced ischemic tolerance by diazoxide. A previous report suggests that diazoxide acts on presynaptic terminals, where many mitochondria are located, to prevent the increase in glutamate release during ischemia (20). Similar

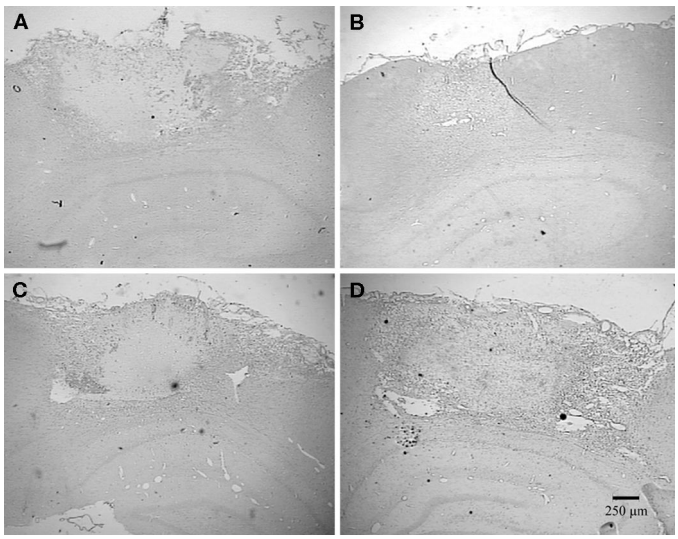

FIGURE 4. *Representative light micrographs of coronal sections 7 days after vein occlusion. Infarct volumes were smaller in the diazoxide-treated group compared with those in the three other experimental groups (hematoxylin and eosin; original magnification, ×20).* A, *vehicle;* B, *diazoxide;* C, *diazoxide plus 5-HD;* D, *5-HD.*

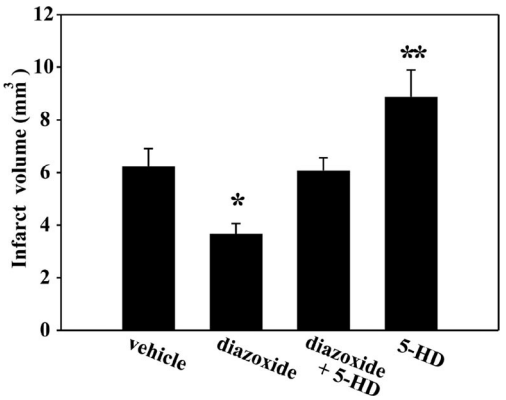

FIGURE 5. *Infarct volumes 7 days after ischemia. Values are means ± standard error of the mean. Diazoxide pretreatment reduced infarction size compared with that in the vehicle-treated group. Coadministration of 5-HD with diazoxide prevented this reduction, and 5-HD administration alone increased infarct size. *, P < 0.05 versus vehicle, diazoxide plus 5-HD, and 5-HD; **, P < 0.05 versus vehicle, diazoxide, and diazoxide plus 5-HD.*

results have recently been published for rat cerebellar granule neurons (30). In experiments using microdialysis, we also have seen increased extracellular glutamate concentrations around the occluded veins (data not shown; Nakagawa et al., in preparation). This suggests that reduced glutamate release may be one mechanism of neuroprotection by diazoxide, although the precise mechanism by which activation of mitoK_{ATP} channels prevents glutamate release remains obscure.

It could be suggested that mitoK_{ATP} channels are at least partly activated during venous ischemia without administration of diazoxide, because the infarct volume of the group treated with 5-HD was larger than that of the vehicle-treated group (*Fig. 5*). This partial activation of mitoK_{ATP} channels may be explained by a decrease in presynaptic adenosine triphosphate concentration in the venous penumbra. Pretreatment with 5-HD alone did not aggravate ischemic damage in the middle cerebral artery occlusion model (13); however, the effect of mitoK_{ATP} blockers may have been underestimated because of the larger infarct volumes and the comparably smaller penumbra seen in arterial occlusion models.

The occlusion of two cortical veins goes along with a rather widespread reduction of CBF (21, 26) (*Fig. 2*) and the development of a small infarct that is approximately one-thirtieth the size of infarcts that develop after middle cerebral artery occlusion. Furthermore, the progress of flow reduction and infarct development is slower than that in arterial ischemia (23). CBF mapping in the drainage area of the two occluded veins shows comparably large low flow areas, whereas no-flow areas are hardly detectable in the acute phase (26). Thus, this model has been proposed as a penumbra model (10, 26). The ischemic penumbra has been described as tissue with critically low perfusion and a loss of electrical activity but a maintained membrane function, which therefore can potentially survive (2). In an additional ongoing study, the infarct growth in venous ischemia has been shown to include caspase-dependent mechanisms (9). Recent reports suggest that the prevention of apoptosis may be an important mechanism of mitoK_{ATP}-related ischemic tolerance in the heart (1) and brain (30). Activation of mitoK_{ATP} channels may prevent inner mitochondria membrane opening by reducing matrix Ca^{2+} overload (18), preclude a subsequent large increase of reactive oxygen species production (6), or suppress the cytochrome *c* release and the translocation of Bax and the increase of Bcl-2 protein (15). Recently, K_{ATP} channel-independent targets of diazoxide have been reported in the heart (8). Partial inhibition of respiratory chain complexes by diazoxide may be an additional mechanism for pharmacological preconditioning similar to proposed mechanisms of preconditioning by mitochondrial toxins such as 3-nitropropionic acid (4). Thus, the prevention of caspase-dependent cell death may be an additional mechanism of tolerance induction by diazoxide.

In conclusion, the present study provides direct evidence that the activation of mitoK_{ATP} provides neuroprotection against a penumbra-like ischemic insult in rat venous ischemia. The mitoK_{ATP} channel is a key target to study mechanisms of tolerance induction against infarct growth in the penumbra.

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Acknowledgments

This work was supported by Schwerpunkt Neurowissenschaften Mainz. We thank Fatemeh Kafai for excellent secretarial assistance and Annett Ehlert, Michael Malzahn, and Laszlo Kopacz for their technical help and support.

COMMENTS

The authors provide us with murine model data suggesting that diazoxide, a mitochondrial adenosine triphosphate-dependent potassium (mitoK$_{ATP}$) channel opener, is neuroprotective against ischemic insult. Before ischemic insult, 49 mice were randomly assigned to receive intraventicular placebo, diazoxide, diazoxide and 5-hydroxydecanate (a selective mitoK$_{ATP}$ channel blocker), or 5-hydroxydecanoate (5-HD) alone. The animals were then subjected to venous infarction by laser occlusion of adjacent cortical veins. Infarct volume, as defined by hematoxylin/eosin stain and light microscopy, was used as the study endpoint. Briefly, animals treated with diazoxide had a significant reduction in infarct volume when compared with placebo-treated animals. This reduction in infarct volume was negated by cotreatment with 5-HD. Moreover, treatment with 5-HD alone led to significantly larger infarcts.

This is important research that provides yet another potential therapeutic agent for acute stroke. MitoK$_{ATP}$ channels have been previously shown to have a role in cardioprotection after myocardial ischemia, and this study further confirms their role in cerebral infarction. The exact mechanism by which this agent exerts neuroprotection has yet to be elucidated, but the authors pose several interesting hypotheses. For mitoK$_{ATP}$ channels to become a realistic and clinically relevant target in acute stroke therapy, several factors must be addressed. Diazoxide is beneficial when administered intraventricularly before ischemic insult. However, is the drug efficacious when administered in the peripheral circulation after the ictus? Also, there are clear

physiologic differences between venous and arterial cerebral infarction. These issues have obvious implications for translation into clinical trials, and the authors have extended our current knowledge in this area.

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The authors test the hypothesis that mitoK_{ATP} channels are involved in mechanisms used by the brain for protection from ischemia. They used a rat venous occlusion model that caused decreased cerebral blood flow of a moderate degree and resulted in a large penumbra of ischemic, but not dead, tissue. They treated animals with a K_{ATP} opener, diazoxide, a K_{ATP} blocker alone, and diazoxide in combination with the blocker. Cell swelling related to spreading depression was measured using impedance electrodes, and infarct volume was measured as the primary endpoint. Cerebral blood flow was measured in all groups using laser Doppler flowmetry.

The results showed that the cerebral blood flow was similar in all control and treatment groups. Similarly, there was no change in any of the groups in response to KCl-induced spreading depression. The group with diazoxide showed significantly smaller infarct volumes than the vehicle only group or the group treated with the K channel blocker. Additionally, the channel blocker 5-HD reversed the effects of diazoxide. The authors conclude that ATP-dependent potassium channels are involved in protecting the neurons from the effects of ischemia and are an important target for further study.

This is an interesting study that extends previously known facts about the role of ATP-dependent potassium channels in cerebral protection. The model is clever and provides an excellent platform for evaluating penumbral effects and protective mechanisms. The results of the study are clear in demonstrating that the target channels have a significant effect on the response of neural tissue to ischemia. The work is well done and clearly presented, and the conclusions are justified by the data presented.

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Diazoxide, a drug with multiple effects including an effect to open mitochondrial mitoK_{ATP} channels, was found to reduce infarct size in a rat model of venous cerebral ischemia. The limitations include the use of laser Doppler blood flow measurements that, although potentially able to give an indication of changes in cerebral blood flow, are sensitive to numerous extraneous factors and do not give quantitative measures of blood flow. Second, there are no mechanistic data to show that the drugs administered into the ventricles had the desired, specific pharmacological effects. On the other hand, the results are potentially very important in view of the interest in this channel's role in ischemia in the heart, brain, and spinal cord, as seen in the references in this article. The authors could confirm that this channel mediates the effect in brain, maybe using genetic strategies to manipulate channel expression.

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Ulysses and the Sirens *(oil on canvas, 1891) by Waterhouse (courtesy of the National Gallery of Victoria, Melbourne).*