Diazoxide preserves hypercapnia-induced arteriolar vasodilation after global cerebral ischemia in piglets

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Mitochondria have recently been shown to play an important role in the mechanism of brain injury after I/R. In addition to being the major site of ATP synthesis and numerous metabolic processes, mitochondria are involved in intracellular Ca\(^{2+}\) homeostasis, produce large amounts of reactive oxygen species (ROS), and are the source of apoptogenic proteins (7). Because these mechanisms have been identified as important factors leading to cell death after I/R, preservation of the mitochondrial structure and function can lead to cellular survival. Mitochondria can therefore be considered important intracellular targets of experimental neuroprotective strategies. The mitochondria can be affected by diazoxide (Diaz), because Diaz can activate the mitochondrial ATP-sensitive K\(^{+}\) (mitoK\(_{ATP}\)) channels (17). We recently showed that Diaz pretreatment limits Ca\(^{2+}\) influx into brain mitochondria and prevents mitochondrial swelling and that these beneficial effects are reversed by coapplication of the mitoK\(_{ATP}\) channel antagonist 5-hydroxydecanoate (5-HD) (9).

Diaz has been shown to protect neurons from ischemic cell death in vivo and in vitro (13, 15, 22, 31, 33, 39). However, very limited evidence is available concerning possible protective effects of Diaz on arteries. In addition to its direct effects on brain cells (37), we hypothesized that Diaz might also preserve the function of cerebral arteries, thereby contributing indirectly to the neuroprotective effect. In the newborn piglet, these ischemia-sensitive vascular responses include the pial arteriolar dilation response to hypercapnia, which is dependent on intact endothelial cell function (24, 29). The loss of CO\(_{2}/\text{pH}\) sensitivity of cerebral resistance vessels after I/R reflects serious impairment of cerebral blood flow (CBF) regulation and could lead to the uncoupling of CBF and the metabolic rate. Among the endothelium-derived vasodilator substances, prostacyclin is one of the most important in the newborn pig; it is also involved in hypercapnia-induced vasodilation (26, 28). Indeed, arteriolar vasodilation induced by iloprost (Ilo), the stable prostacyclin analog, is also attenuated by I/R (4), indicating vulnerability of the arteriolar vascular smooth muscle (VSM) to I/R.

We investigated whether Diaz could preserve hypercapnia- and/or Ilo-induced pial arteriolar vasodilation after I/R in piglets. Our positive results with Diaz-induced preservation of posts ischemic responses to hypercapnia impelled us to further test whether 5-HD, a mitoK\(_{ATP}\) channel inhibitor (19), can diminish this preserving effect of Diaz, whether Diaz or 5-HD per se affects hypercapnia-induced vasodilation, and whether Diaz has a direct effect on mitochondria in piglet cerebrovascular endothelial cell cultures. We also tested the action of another neuroprotective drug, the cyclooxygenase (COX)-2 blocker NS-398, on the preservation of hypercapnia-induced vasodilation.

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MATERIALS AND METHODS

Animals. Newborn piglets of either gender (<1 day old, 1–2 kg body wt, n = 48) were used. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Szeged and Wake Forest University Health Sciences. The animals were anesthetized with thiopental sodium (30–40 mg/kg ip; Biochemic, Vienna, Austria) followed by an injection of a-chloralose (40 mg/kg iv; Sigma, St. Louis, MO). Supplemental doses of a-chloralose were given to maintain a stable level of anesthesia. The right femoral artery and vein were catheterized to record blood pressure and to administer drugs (Diaz, 5-HD, and NS-398) and fluids, respectively. The piglets were intubated via tracheotomy and artificially ventilated with room air. The ventilation rate (~20/min) and tidal volume (~20 ml) were adjusted to maintain arterial blood gases and pH in the physiological range. Body temperature was maintained with a water-circulating heating pad. Body temperature, arterial pH, and blood gases were kept in the normal ranges and did not vary significantly between the different groups: 37.8 ± 0.3°C, pH 7.47 ± 0.02, 31.4 ± 1.4 mmHg Pco2, and 80 ± 3 mmHg Po2 for group 1.

The piglets were equipped with a closed cranial window as previously described (13, 14). The pial circulation was visualized with an operating microscope (Wild) equipped with a charge-coupled device camera (A. Krüss) connected to a television monitor (Panasonic). In each experiment, a ~100-μm-diameter pial arteriole was selected. Pial arteriolar diameters were then determined with a video microcaler. After surgery, the cranial window was repeatedly flushed with artificial cerebrospinal fluid (aCSF) until a stable arteriolar baseline diameter was obtained. At the end of the experiments, the anesthetized animals were killed with an intravenous bolus of saturated KCl solution.

Global cerebral I/R. To induce global cerebral ischemia, a 3-mm hole was made with an electric drill with a toothless bit, and the dura was exposed. A hollow brass bolt was inserted into the left frontal cranial rostral to the cranial window and secured in place with cyanoacrylate ester and dental acrylic. Cerebral ischemia was produced by infusion of aCSF to raise the intracranial pressure above the arterial pressure. Ischemia was verified by cessation of blood flow in the vessels observed through the cranial window. By using microscopes, we have shown repeatedly that the CBF in all examined brain areas is virtually zero during the ischemic period (5, 8, 25). Venous blood was withdrawn as necessary to maintain mean arterial blood pressure (MABP) near normal values. At the end of the ischemic period, the infusion tube was clamped and the intracranial pressure returned to the preischemic levels. The withdrawn and heparinized blood was reinused.

Assessment of cerebrovascular reactivity. Subsequent to determining the stable baseline arteriolar diameters, we examined the responses of the pial arterioles to graded hypercapnia or Ilo. Hypercapnia was elicited by ventilating the animals with a gas mixture containing 5–10% CO2-21% O2-balance N2. Ilo was dissolved in aCSF (10–100 μM) and applied to the pial surface. Arteriolar diameters were measured continuously for 5–7 min. After each stimulus, the baseline diameter was obtained. At the end of the experiments, the withdrawn and heparinized blood was centrifuged at 150 g for 5 min. The supernatant was removed, and ≥20% bovine serum albumin (BSA, 2 ml/brain; Sigma) was added to the pellet. The digested brain tissue was completely redistributed in the BSA solution by repeated aspiration through sterile Pasteur pipettes. The homogenate was centrifuged at 350 g for 5 min. The supernatant was removed, and ≥20% bovine serum albumin (BSA, 2 ml/brain; Sigma) was added to the pellet. The digested brain tissue was completely redistributed in the BSA solution by repeated aspiration through sterile Pasteur pipettes and then centrifuged at 1,000 g for 20 min. The separated myelin plug and the BSA solution were discarded, and the pelleted microvessels were washed once in DMEM and further digested with the same enzymes for 1.5 h at 37°C. After digestion, the cell suspensions were centrifuged at 500 g for 5 min. The cells were layered on a continuous 33% Percoll (Amersham, Uppsala, Sweden) gradient and centrifuged at 1,000 g for 10 min. The band of the endothelial cell clusters was aspirated and washed twice in DMEM. The cells were seeded onto collagen type IV- and fibronectin-coated 12-mm glass coverslips. Culture medium consisted of DMEM supplemented with 20% fetal bovine plasma-derived serum (Animal Technologies, Tyler, TX), 2 mM glutamine, 1 ng/ml basic fibroblast growth factor (Sigma), 50 μg/ml endothelial cell growth supplement (BD Biosciences, Bedford, MA), 100 μg/ml heparin, 5 μg/ml vitamin C, and antibiotics. Confluent cultures (days 4–5 in vitro) consisted of ≥95% cerebral endothelial cells, as verified by positive immunohistochemistry for von Willebrand factor and negative immunohistochemistry for glial fibrillary acidic protein and α-smooth muscle actin. The cultured pig endothelial cells displayed prominent blood-brain barrier characteristics; culturing the cells on collagen type IV- and fibronectin-coated Transwell inserts (diameter 24 mm, pore size 3 μm; Corning, Corning, NY), we measured (EVOM resistance meter, World Precision Instruments, Sarasota, FL) high transendothelial electrical resistance (495 ± 9 Ω·cm2, n = 12).

Analysis of mitochondrial membrane potential. Mitochondrial membrane potential was monitored using Mitotracker Red [chloromethyl-X-rosamine (CMXRos); Molecular Probes, Eugene, OR]. Confluent cultures were loaded in the dark at 37°C in a 5% CO2 incubator with 0.5 μM CMXRos in DMEM for 10 min. After the cultures were loaded, the cells were washed three times with PBS. Experiments were carried out at 22°C in PBS. Confocal images of cellular CMXRos fluorescence were acquired on a laser scanning microscope (model LSM 510) using a ×63 water immersion objective (Zeiss, Jena, Germany). Fields of cells were randomly selected. The cells were treated with vehicle or Diaz (100 μM), and fluorescent images were recorded every 30 s for 15 min (excitation wavelength = 543 nm, emission wavelength > 560 nm). The average pixel intensity in individual cell bodies was determined using software supplied by the manufacturer (Zeiss).

Drugs. Ilo (Sigma) was dissolved in aCSF. NS-398 (Sigma) was dissolved in dimethyl sulfoxide (10 mg/ml). Diaz (Sigma) was dis-
solved in 1 N NaOH (30 mg/ml) and then diluted with saline to 3 mg/ml. 5-HD (Sigma) was dissolved in saline.

**Statistics.** Values are means ± SE. Pial arteriolar diameter data were analyzed by using one-way repeated-measures ANOVA, and CMXRos fluorescence pixel intensity data were evaluated with 2-way repeated-measures ANOVA. For post hoc analysis, we used the Student-Newman-Keuls test where appropriate. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

The MABP was in the normal range throughout the experiments and was not affected significantly by induction of hypercapnia. The MABP was only slightly, and not significantly, decreased 1 h after I/R; in *group 3*, the MABP changed from 72 ± 4 to 60 ± 2 mmHg. The administration of Diaz transiently decreased the MABP; in *group 7*, the MABP was 71 ± 4 mmHg before and 53 ± 6, 63 ± 7, and 64 ± 6 mmHg at 5, 10, and 15 min, respectively, after Diaz. I/R did not alter the baseline arteriolar diameters significantly; the values before vs. after I/R were as follows: 103 ± 5 vs. 105 ± 15 \( \mu \text{m} \) (*group 1*), 99 ± 8 vs. 98 ± 7 \( \mu \text{m} \) (*group 2*), 105 ± 4 vs. 96 ± 8 \( \mu \text{m} \) (*group 3*), 97 ± 5 vs. 105 ± 4 \( \mu \text{m} \) (*group 4*), 115 ± 12 vs. 116 ± 9 \( \mu \text{m} \) (*group 6*), and 124 ± 14 vs. 121 ± 15 \( \mu \text{m} \) (*group 7*). Neither Diaz nor 5-HD affected the baseline diameters; in *group 5*, the values were 107 ± 7, 98 ± 6, and 105 ± 9 \( \mu \text{m} \) before Diaz, after Diaz, and after 5-HD, respectively.

Graded hypercapnia significantly elevated the arterial PCO\(_2\) levels, with simultaneous reductions in arterial pH, during repeated challenges in all experimental groups. For instance, in *group 3*, 5% and 10% CO\(_2\) elevated PCO\(_2\) from 39.8 ± 2.2 to 48.3 ± 1.9 and 63.4 ± 2.8 mmHg and from 42.0 ± 3.3 to 50.8 ± 3.6 and 61.6 ± 3.9 mmHg before and after I/R, respectively. Simultaneously, the arterial pH was reduced from 7.40 ± 0.03 to 7.27 ± 0.03 and 7.14 ± 0.02 and from 7.37 ± 0.03 to 7.23 ± 0.03 and 7.09 ± 0.02 before and after I/R, respectively. Graded hypercapnia also resulted in large, concentration-dependent, reversible increases in pial arteriolar diameters (Fig. 1). I/R severely attenuated the hypercapnia-induced arteriolar vasodilation in the vehicle-treated animals (*group 1*, Fig. 1). Pretreatment with the COX-2 inhibitor NS-398 did not prevent the attenuation of vascular reactivity after I/R (*group 2*). In contrast, Diaz preserved the hypercapnia-induced vasodilation after I/R (*group 3*). The beneficial effect of Diaz was abolished by the application of 5-HD (*group 4*). In the absence of I/R (*group 5*), neither Diaz nor 5-HD altered the arteriolar dilation in response to graded hypercapnia (Fig. 2). Ilo elicited dose-dependent pial arteriolar vasodilation, which was also attenuated by I/R in the vehicle-treated animals (*group 6*, Fig. 3). In contrast with the hypercapnia-induced vasodilation, Diaz had no effect on the attenuation of vascular reactivity to Ilo (*group 7*, Fig. 3).

CMXRos fluorescence successfully labeled the mitochondria in cultured piglet cerebrovascular endothelial cells. Diaz gradually and significantly reduced the CMXRos signal (Fig. 4) compared with vehicle-treated controls, confirming a direct effect of Diaz on endothelial mitochondria.

**DISCUSSION**

The major findings of the present study are as follows: 1) A neuroprotective dose of Diaz, but not NS-398, preserved the endothelium-dependent hypercapnia-induced vasodilation after I/R. 2) 5-HD abolished the effect of Diaz, indicating a role for mitoK\(_{\text{ATP}}\) channels. 3) Diaz could directly stimulate mitochondria in cerebrovascular endothelial cells in vitro. 4) The effects of Diaz and 5-HD are not due to the direct facilitation or inhibition of the vascular reactivity to hypercapnia. However, Diaz effects were specific to cell type in the cerebral arteries, such that although the vasodilator response to hypercapnia was protected, Diaz treatment did not preserve Ilo-induced vasodilation damaged by I/R.

The beneficial effect of Diaz pretreatment on cerebrovascular reactivity/neuroprotection may be of clinical interest in the neonate, because incipient cerebral ischemia/asphyxia may be predicted in several clinical situations in the perinatal period. For example, asphyxiated babies often show subsequent seizure activity or arterial hypotension, and adequate time would be available for administration of a protective drug (Diaz) before these secondary insults. Additionally, babies often undergo cardiac surgeries that involve mechanical or surgical manipulations, which may compromise the blood flow to the brain. Pretreatment of these babies with Diaz may protect against additional neurological damage.

Hypercapnia-induced arteriolar vasodilation has been extensively studied in the piglet and in other species. In the pial circulation of the piglet, the vascular endothelium is clearly involved in the mechanism of vasodilation, because light/dye endothelial injury selectively eliminates the arteriolar responsiveness to hypercapnia, whereas vasodilation in response to...
Ilo or isoproterenol is retained (29). The role of the endothelium appears to be as a source of prostacyclin for the VSM to permit, rather than mediate, the vasodilation. Subvasodilator concentrations of Ilo have been shown to restore diminished vascular responsiveness to CO2 after light/dye endothelial injury (26) and indomethacin treatment (28, 40). Ex vivo findings also support this phenomenon, because hypercapnia/acidosis releases prostacyclin (and other prostanoids) from cultured piglet cerebrovascular endothelial cells (18), and Ilo augments the increases in cAMP levels of cultured piglet VSM cells in response to hypercapnia/acidosis (23). However, other endothelial mediators may play roles in the permissive response, because hypercapnia-induced vasodilation, which was abolished after indomethacin, could also be partially restored by treatment with sodium nitroprusside (40) or prostaglandin E2 (28, 40). I/R probably also inhibits hypercapnia-induced vasodilation through endothelial damage, because topical supplementation of arachidonic acid after I/R restored the vasodilation and the increases in aCSF 6-keto-PGF1α levels (the stable prostacyclin hydrolysis product) in response to hypercapnia (27).

Ilo-induced (4), but not forskolin-induced (3), vasodilation is also severely attenuated after I/R, indicating I/R-induced damage of the prostacyclin receptor/signaling transduction pathway in the VSM. The present study confirmed this attenuation of Ilo-induced vasodilation. Diaz, however, did not prevent the attenuation of Ilo-induced vasodilation but preserved hypercapnia-induced vasodilation. We do not know why Diaz does not protect responses of VSM to I/R. However, in independent experiments with a different model of brain ischemia and the study of isolated middle cerebral arteries from rats, we obtained almost identical results (38).

COX inhibitors also differentially affect the attenuation of hypercapnia- and Ilo-mediated vasodilation after I/R. Inhibition of COX activity, the major source of extracellularly detectable superoxide anions in the piglet after I/R (2), by indomethacin before I/R resulted in preserved Ilo-induced dilation (4). In contrast, inhibition of COX-2 by NS-398 failed to exert any beneficial effect on hypercapnia-induced vasodilation after I/R in the present study. Unfortunately, we could not use indomethacin, because indomethacin, but not other COX inhibitors, uniquely abolishes hypercapnia-induced vasodilation (10, 40). Instead, we used the selective COX-2 inhibitor, because COX-2 is the major isoform expressed in the piglet brain and cerebrovascular endothelium (14, 35, 36). Furthermore, NS-398 and indomethacin were equally effective in preventing attenuation of N-methyl-D-aspartate-induced pial arterial vasodilation after I/R (11).

These data suggest that COX activity plays a prominent role in the impairment of the endothelium-independent neuronal-vascular (11, 12) or VSM (4, 32) function after I/R, but not in the attenuation of endothelium-dependent hypercapnia-induced vasodilation, in piglets. Our findings confirm that Ilo-induced vasodilation and the permissive effect of a subvasodilator concentration of prostacyclin/Ilo on hypercapnia-induced vasodilation are unrelated, and their vulnerabilities to I/R are apparently different.

Diaz pretreatment, however, may have resulted in decreased/shortened endothelial dysfunction after I/R, suggested by preserved hypercapnia-induced vasodilation in this study. The mechanism of this endothelial protection by Diaz is unclear, but a mitochondrial site of action is likely. Mitochondria are especially numerous in the cerebrovascular endothelium. Mitochondria make up 8–11% of the volume of the cytoplasm in the microvascular endothelial cells in the rat cerebral cortex and other brain areas compared with 2–5% in the endothelium of microvessels in cardiac muscle, skin, and lungs (34). In the present study, we cultured piglet cerebrovascular endothelial cells to confirm the direct effect of Diaz on piglet endothelial mitochondria. Using CMXRos, we demonstrated that Diaz had a major effect on the mitochondria in cerebrovascular endothelial cells in a concentration range and time frame (100 μM and 15 min) similar to those used in the in vivo studies. CMXRos is a mitochondrial potential-sensing dye that accumulates in active mitochondria with negative membrane potential. The decreasing CMXRos fluorescence signal in response to Diaz is mitochondrial depolarization elicited by mitoKATP channel opening and may be due to a change in the mitochondrial binding of the dye. Diaz likely targets the mitoKATP channel in the endothelial cells in vivo as well, because the vascular protective effect of Diaz was found to be sensitive to 5-HD, an inhibitor of this channel. Importantly, the applied dose of 5-HD did not inhibit vascular reactivity directly, and the diminished responsiveness after

**Fig. 2.** Effects of Diaz and 5-HD on hypercapnia-induced pial arteriolar vasodilation. Arteriolar responses to 5–10% CO2 ventilation were recorded 3 times: before treatment (1st stim), 15 min after intravenous administration of Diaz, and 15 min after intravenous administration of 5-HD. Graded hypercapnia resulted in reversible, concentration-dependent increases in pial arteriolar diameters that were not affected by Diaz or subsequent 5-HD administration.

**Fig. 3.** Effect of I/R on iloprost (Ilo)-induced pial arteriolar vasodilation. Arteriolar responses to Ilo (1–10 μg/ml) were recorded 15 min before and 1 h after 10 min of global cerebral ischemia followed by reperfusion. Ilo elicited concentration-dependent pial arteriolar vasodilation, which was severely attenuated after I/R in vehicle- and Diaz-treated piglets. Thus Diaz did not preserve Ilo-induced vasodilation. *P < 0.05 vs. before I/R.
5-HD + Diaz + I/R was therefore not caused by a nonspecific effect of the drug. These results suggest the importance of mitochondria in the mechanism of cerebrovascular endothelial damage inflicted by I/R. The link between mitochondria and the prevention of the endothelial dysfunction induced by I/R is unknown. The most obvious mechanism, coupling mitoKATP channel opening by Diaz to endothelium protection, would be a decrease in mitochondrial ROS production during I/R. Diaz reduced ROS production in the heart after I/R (16) and in neuronal cell cultures after oxygen and glucose deprivation (22). However, ROS seem to play only a minor role in the attenuation of hypercapnia-induced vasodilation after I/R, because superoxide anion scavengers were unable to preserve/restore posts ischemic vascular reactivity (30). Nevertheless, it remains unproven whether the applied ROS scavengers could prevent the deleterious effects of ROS produced in the endothelial mitochondria, especially effects of ROS that are unrelated to superoxide. Indeed, nociceptin/orphanin FQ, an opioid peptide released during I/R, was demonstrated to play a role in the attenuation of hypercapnia-induced vasodilation after I/R (20), and ROS were suggested to be mediators of nociceptin/orphanin FQ-elicited vascular damage (1).

In conclusion, Diaz protects postischemic vascular reactivity to CO2, an ischemia-sensitive indicator of endothelial function in the newborn pig. This vascular protection probably aids the reestablishment of adequate perfusion of the brain tissue after I/R; therefore, it may augment the direct neuroprotective effect of Diaz observed in vivo.

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