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

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Diazoxide preserves hypercapnia-induced arteriolar vasodilation after global cerebral ischemia in piglets. Am J Physiol Heart Circ Physiol 289: H368–H373, 2005. First published February 25, 2005; doi:10.1152/ajpheart.00887.2004.—Diazoxide (Diaz), an activator of mitochondrial ATP-sensitive K⁺ (mitoKATP) channels, is neuroprotective, but the mechanism of action is unclear. We tested whether Diaz preserves endothelium-dependent (hypercapnia) or -independent arteriolar vasodilation responses. Anesthetized, ventilated piglets (n = 48) were equipped with closed cranial windows. Changes in diameter of pial arterioles were determined with intravital microscopy in response to graded hypercapnia (5–10% CO₂-21% O₂-balance N₂, n = 25) or Ilo (0.1–1 µg/ml, n = 18) before and 1 h after 10 min of global I/R. Experimental groups were pretreated with vehicle, NS-398 (a selective cyclooxygenase-2 inhibitor, 1 mg/kg), Diaz (3 mg/kg), or 5-HD (20 mg/kg) + Diaz. Potential direct effects of Diaz and 5-HD on hypercapnic vasodilation were also tested in the absence of I/R (n = 5). To confirm the direct effect of Diaz on mitochondria, mitochondrial membrane potential in cultured piglet cerebrovascular endothelial cells was monitored using Mito Tracker Red. Hypercapnia resulted in dose-dependent pial arteriolar vasodilation, which was attenuated by ~70% after I/R in vehicle- and NS-398-treated animals. Diaz and 5-HD did not affect the CO₂ response. Diaz significantly preserved the posts ischemic vasodilation response to hypercapnia, but not to Ilo. Diaz depolarized mitochondria in cultured piglet cerebrovascular endothelial cells, and 5-HD completely abolished the protective effect of Diaz, both findings indicate a role for mitoKATP channels in preserving vasodilation.

Inadequate respiration and/or cerebral hypoperfusion may lead to asphyxia or cerebral ischemia-reperfusion (I/R) in neonates and are significant causes of perinatal morbidity/mortality (6). Compared with stroke management in adults, perinatal stroke management has a major advantage, because in developed countries nearly all babies can receive instant medical care. Effective and low-risk neuroprotective treatment (even pretreatment in some cases) can, in theory, reduce irreversible neurological damage.

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 Ca²⁺ 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⁺ (mitoKATP) channels (17). We recently showed that Diaz pretreatment limits Ca²⁺ influx into brain mitochondria and prevents mitochondrial swelling and that these beneficial effects are reversed by coapplication of the mitoKATP 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 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 CO₂/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 mitoKATP 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; Biochrome, Vienna, Austria) followed by an injection of α-chloralose (40 mg/kg iv; Sigma, St. Louis, MO). Supplemental doses of α-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 gas values 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 P02 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 microscope. 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 cranium 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 reinfused.

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 cranial window was flushed with aCSF, and arteriolar diameters were allowed to return to baseline values.

Drug treatment. In group 1 (n = 7), arteriolar responses to graded hypercapnia were recorded before and 1 h after I/R. The animals were given vehicle or Diaz intravenously 15 min before I/R. Treatment of group 2 (n = 5) was that same as that described for group 1, except group 2 was given NS-398 (1 mg/kg iv) 15 min before I/R. Treatment of group 3 (n = 8) was the same as that described for group 1, except group 3 was given Diaz (3 mg/kg iv) 15 min before I/R. Treatment for group 4 (n = 5) was the same as that described for group 3, except group 4 was also given 5-HD (20 mg/kg iv) 15 min before Diaz. In group 5 (n = 5), the arteriolar responses to graded hypercapnia were repeatedly determined three times: 1) with no treatment; 2) 15 min after treatment with Diaz (3 mg/kg iv), and 3) 15 min after treatment with 5-HD (20 mg/kg iv). In group 6 (n = 9), the arteriolar responses to Ilo (0.1–1 μg/ml) were recorded before and 1 h after I/R. The animals received the vehicle or Diaz 15 min before I/R. Treatment for group 7 (n = 9) was the same as that described for group 6, except group 7 was given Diaz (3 mg/kg iv) 15 min before I/R.

Piglet cerebral endothelial cell culture. We isolated and cultured primary piglet cerebral endothelial cells according to the protocol what we used previously to culture rat cerebral endothelial cells (21).

Drugs. Ilo (Sigma) was dissolved in aCSF. NS-398 (Sigma) was dissolved in dimethyl sulfoxide (10 μg/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 μm (group 1), 99 ± 8 vs. 98 ± 7 μm (group 2), 105 ± 4 vs. 96 ± 8 μm (group 3), 97 ± 5 vs. 105 ± 4 μm (group 4), 115 ± 12 vs. 116 ± 9 μm (group 6), and 124 ± 14 vs. 121 ± 15 μm (group 7). Neither Diaz nor 5-HD affected the baseline diameters; in group 5, the values were 107 ± 7, 98 ± 6, and 105 ± 9 μm before Diaz, after Diaz, and after 5-HD, respectively.

Graded hypercapnia significantly elevated the arterial PCO2 levels, with simultaneous reductions in arterial pH, during repeated challenges in all experimental groups. For instance, in group 3, 5% and 10% CO2 elevated PCO2 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 mitoKATP 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

![Fig. 1. Effect of ischemia-reperfusion (I/R) on hypercapnia-induced pial arteriolar vasodilation. Arteriolar responses to 5–10% CO2 ventilation were recorded 15 min before and 1 h after 10 min of global cerebral ischemia followed by reperfusion. Hypercapnia elicited concentration-dependent pial arteriolar vasodilation, which was markedly attenuated after I/R in vehicle- and NS-398-treated piglets. Diazoxide (Diaz) preserved vascular responsiveness to CO2. Vasodilation in response to 5% CO2 remained unchanged after I/R and was only slightly, but statistically significantly, decreased in response to 10% CO2. 5-Hydroxydecanoate (5-HD) abolished the beneficial effect of Diaz; vasodilation in response to 5–10% CO2 was virtually absent after I/R in these animals. *P < 0.05 vs. before I/R.

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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 cerebral 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/signal transduction pathway in the VSM. The present study confirmed this attenuation of Ilo-induced dilation. 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 arteriolar 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...
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 postischemic 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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