PAI-1-derived peptide EEIIMD prevents impairment of cerebrovasodilation by augmenting p38 MAPK upregulation after cerebral hypoxia/ischemia

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PERINATAL CEREBRAL hypoxia/ischemia (H/I) has many causes, unclear pathophysiology, no specific mechanism-related treatment, and poor outcome. Neonatal stroke may occur in as many as 1 in 4,000 births (22). In newborns with stroke, complications such as hypoxic/ischemic events are common (9). Maternal and perinatal coagulopathy predispose to perinatal stroke (10, 16) with 30% of neonatal strokes being due to thrombosis (8). A better understanding of the pathophysiological responses that occur in children after cerebral H/I is needed to develop mechanism-based approaches to therapy.

One contributor to neurological damage after H/I is thought to be cerebrovascular dysfunction. For example, hypotenion leads to the loss of cerebrovascular regulation promoting tissue ischemia, whereas cerebrovasoconstriction associated with hypopacina contributes to periventricular leukomalacia in the perinate (25). Using a piglet model, we have shown that pial artery dilation in response to hypotension and hypercapnia is blunted after cerebral H/I (4, 13, 19, 20).

Recombinant tissue type plasminogen activator (tPA) is the only Food and Drug Administration-approved treatment for stroke (15). However, tPA exhibits deleterious as well as beneficial effects that profoundly constrain its clinical utility. In addition to its salutary role in reperfusion, tPA contributes to excitotoxic neuronal cell death (23) and increases stroke infarct volume in mice (26). We have also observed that a topical administration of tPA or urokinase plasminogen activator (uPA) to the piglet cerebral cortex potentiates an impairment of pial artery dilation caused by H/I (5). The plasminogen activator inhibitor-1 (PAI-1)-derived peptide, EEIIMD, blocks tPA- and uPA-mediated effects on vascular contractility mediated by their interaction with the low-density lipoprotein receptor without inhibiting fibrinolytic activity (6, 21). uPA is upregulated after cerebral H/I in the piglet (4). Pretreatment with EEIIMD prevents the impairment of hypercapnic and hypotensive dilation after H/I (5), suggesting that endogenous plasminogen activators contribute to cerebral hemodynamic outcome postsult.

MATERIALS AND METHODS

Closed cranial window technique and cerebral H/I. Newborn pigs (1–5 days, and 1.2–1.6 kg) of either sex were studied. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Animals were anesthetized with

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isoflurane (1 to 2 mean alveolar concentration), maintained with α-chloralose (30–50 mg/kg, supplemented with 5 mg·kg⁻¹·h⁻¹ iv). A catheter was inserted into a femoral artery to monitor blood pressure. The trachea was cannulated, and the animals were ventilated with room air. A heating pad was used to maintain the animals at 37–39°C, monitored rectally.

A cranial window was placed in the parietal skull of these anesthetized animals. This window consisted of three parts: a stainless steel ring, a circular glass coverslip, and three ports consisting of 17-gauge hypodermic needles attached to three precut holes in the stainless steel ring. For placement, the dura was cut and retracted over the cut bone edge. The cranial window was placed in the opening and cemented in place with dental acrylic. The volume under the window was filled with a solution, similar to cerebrospinal fluid (CSF), of the following composition: (in mM) 3.0 KCl, 1.5 MgCl₂, 1.5 CaCl₂, 132 NaCl, 6.6 urea, 3.7 dextrose, and 24.6 NaHCO₃. This artificial CSF was warmed to 37°C and had the following chemistry: pH 7.33, PaCO₂ 35 mmHg, and P O₂ of 150 mmHg. Hypoxia (PO₂ of 20–30 mmHg) was maintained for 20 min. Hypoxia (PO₂ of 20–30 mmHg) was subsided, the shed blood was returned to the animal. Cerebral ischemia was monitored via a sidearm of the cranial window. To prevent the arterial diastolic arterial blood pressure (19, 20), intracranial pressure was monitored by expressing the data as a percentage of control (total). H/I induced a marked phosphorylation of p38 MAPK within 1 h postinjury (Fig. 1). EEIIMD (1 mg/kg iv) administered 30 min before or 1 h after H/I potentiated the phosphorylation of p38 MAPK (Fig. 1). In contrast, CSF p38 MAPK concentration was unchanged by the administration of the inactive analog EEIIMR (1 mg/kg iv) and the JNK MAPK antagonist SP-600125 (1 mg/kg iv) pre- or postinjury (Fig. 1). The purported p38 MAPK antagonist SB-230580 (1 mg/kg iv) and combined SB-203580 + EEIIMD blocked p38 MAPK phosphorylation (Fig. 1). CSF JNK MAPK was unchanged by H/I, EEIIMD, EEIIMR, SP-600125, and SB-203580 (data not shown). CSF ERK MAPK was upregulated by H/I and blunted by EEIIMD (102 ± 5, 289 ± 39, and 126 ± 10% for control, H/I, and H/I + EEIIMD, respectively).

EEIIMD prevents, whereas the p38 MAPK antagonist SB-203580 aggravates, impairment of cerebrovasodilation after H/I. Two levels of hypercapnia, hypotension, and isoproterenol elicited reproducible graded pial small artery (120 to 160 μm) and arteriole (50 to 70 μm) dilation in sham-operated control animals (data not shown). Pial small artery dilation in response to hypercapnia and hypotension was blunted 1 and 4 h after H/I, whereas responses to isoproterenol were unchanged (Figs. 2, 3, and 4). Similar reductions in responses were seen in arterioles (data not shown). Pre- and posttreatment with EEIIMD, but not EEIIMR, prevented the impairment of pial artery dilation in response to hypercapnia and hypotension, while having no effect on vasodilation in response to isoproterenol (Figs. 2–4).

However, vascular responses to hypercapnia and hypotension post-H/I were reversed to vasoconstriction by pre- and postinjury treatment with the p38 MAPK antagonist SB-203580 (Figs. 2 and 3). The coadministration of SB-203580 with EEIIMD blocked the protection of vascular responses to hypercapnia and hypotension post-H/I observed with EEIIMD alone, resulting in a vasoconstriction to these two stimuli (Figs. 2 and 3). Isoproterenol-induced pial artery vasodilation was unchanged by SB-203580 (Fig. 4). The JNK MAPK antagonist SP-600125 had no influence on vascular responses to hypercapnia, hypotension, or isoproterenol.
enol after H/I (Figs. 2–4). Similar observations were made in pial arterioles (data not shown).

Blood chemistry and mean arterial blood pressure. Blood chemistry values were collected before and after all experiments. There were no statistical differences between sham-operated control, H/I, and H/I drug-treated animals. Hypoxia decreased PO2 to 34 ± 110062 mmHg. Low levels of hypercapnia raised PCO2 to 56 ± 6 mmHg, and high levels of hypercapnia raised the PCO2 to 75 ± 8 mmHg. Carbon dioxide levels were kept constant during periods of hypoxia, and oxygen levels were kept constant during periods of hypercapnia. Mean arterial blood pressure was modestly decreased at 1 h post-H/I (64 ± 9 and 54 ± 9 mmHg for control and 1 h post H/I, respectively).

DISCUSSION

There are two principal new findings to be derived from this study. First, these data show that intravenous postinjury treatment with the PAI-1-derived peptide EEIIMD prevents the impairment of vasodilator responses to hypercapnia and hypotension after cerebral H/I. These data support the involvement of the upregulation of plasminogen activators in the impairment in vascular reactivity seen postinsult. In prior studies, we had shown that EEIIMD applied locally as a proof of principle preinjury provided protection (5). Here we show the comparable effectiveness when EEIIMD is given through the more clinically relevant intravenous route. The protection afforded by EEIIMD appears selective since the inactive analog EEIIMR failed to restore vasodilation in response to hypercapnia and hypotension postinsult. Because the vasodilation in response to isoproterenol was unchanged by cerebral H/I (3–5, 13, 14), these data also suggest that whereas the intrinsic reactivity of pial arteries was preserved, the activation of this reactivity was somehow inhibited by H/I. Mechanistically, we have previously observed that the ERK MAPK inhibitor U-0126 partially

Fig. 1. Phosphorylation of p38 MAPK in cerebrospinal fluid before cerebral hypoxia/ischemia (H/I) (0 min) and as a function of time (in h) after H/I in vehicle or treated with EEIIMD, EEIIMR, SP-600125, SB-203580, or combined SB-203580 + EEIIMD (all, 1 mg/kg iv); n = 5 pigs. Data are expressed as percentages of control by ELISA determination of phospho-MAPK and total MAPK isoform and subsequent normalization to total form. A: pretreatment 30 min before H/I. B: posttreatment 1 h after H/I. *P < 0.05 vs. corresponding 0 time value; +P < 0.05 vs. corresponding H/I nontreated value; #P < 0.05 vs. corresponding EEIIMD alone value.

Fig. 2. Influence of hypotension (moderate and severe) on pial artery diameter in newborn pigs before (control) and after cerebral H/I or treated with EEIIMD, EEIIMR, SP-600125, SB-203580, or combined SB-203580 + EEIIMD (all, 1 mg/kg iv); n = 5 pigs. A: pretreatment 30 min before H/I. B: posttreatment 1 h after H/I. *P < 0.05 vs. corresponding control value; +P < 0.05 vs. corresponding nontreated H/I value; #P < 0.05 vs. corresponding EEIIMD alone value.
prevented the impaired reactivity to hypercapnia and hypotension (4, 13), indicating that the upregulation of this MAPK isoform (4) probably contributed to the H/I-induced impairment of the activation of reactivity.

In contrast, the results of the present study show that the p38 MAPK inhibitor SB-203580 aggravated the H/I-induced impairment of cerebrovasodilation, whereas the concentration of p38 MAPK in the CSF was elevated after injury. The coadministration of SB-203580 with EEIIMD blocked the protection of vascular responses to hypercapnia and hypotension after H/I. These data support the second key finding of this study, which indicate that p38 MAPK is protective in the setting of cerebral H/I. Because EEIIMD, but not EEIIMR, potentiated the elevation of CSF p38 MAPK concentration, these data suggest that this PAI-1-derived peptide produces protection, in part, in a p38 MAPK-dependent mechanism. The coadministration of SB-203580 with EEIIMD blocked the protection afforded by the former, strengthening the key role of p38 MAPK in responsiveness to vasodilator stimuli post H/I. The administration of SB-203580 also blocked p38 MAPK upregulation after H/I, indicating that the dose of this drug was efficacious and had crossed the blood brain barrier in sufficient concentration.

However, CSF JNK MAPK concentration was unchanged, and the respective antagonist, SP-600125, had no effect on cerebrovasodilator impairment post H/I. These data indicate that JNK MAPK appears to play a lesser, if any, role in the vascular impairment induced by global cerebral H/I in the piglet. These data are in contrast to the mechanism of injury caused by focal cerebral ischemia in the rat where JNK MAPK was upregulated and mediated neuronal cell toxicity (7, 12). It is presently uncertain whether these differences relate to model of cerebral ischemia (focal vs. global), age, and/or species but suggest the need for caution when extrapolating results across these parameters. A limitation of the closed cranial window technique to quantify CSF MAPK concentration is that the cellular site of origin cannot be determined. Potential sources include neurons, glia, vascular smooth muscle, and endothelial sources.
Our prior studies indicated that uPA was upregulated after H/I and contributed to an impaired cerebrovasodilation in an ERK MAPK-dependent mechanism (4). In the context of the present results, the dynamic interactive changes of ERK and p38 MAPK, the one opposing and the other promoting vasodilation, appear to yield the ultimate cerebrohemodynamic outcome after this central nervous system injury. Shifting the MAPK isoform profile with the administration of EEIIMD (blunting ERK and promoting p38 upregulation) yields a protection of cerebrovasodilation after H/I. A quantification of ERK MAPK in CSF appears to parallel changes in brain parenchyma under H/I conditions (4).

The PAI-1-derived peptide EEIIMD inhibits the vasooactivity of tPA and uPA without inhibiting their fibrinolytic activity (6, 21). Since EEIIMD binds to the docking site of tPA and uPA but does not inhibit their plasminogen activator activity, these data suggest that the effect of these plasminogen activators is mediated through their signal-transducing activities. We have previously shown that uPA binds directly to αβ3 (17, 24) and that plasminogen activators can promote the formation of a signal-transducing complex between low-density lipoprotein receptor and αβ3 (1). The results of a more recent study extend these initial observations and indicate that uPA released after cerebral H/I impairs pial artery dilation induced by hypercapnia and hypotension through an integrin αβ3-dependent process (14). However, other as yet undefined mechanisms, which may include the opioid nociceptin/orphanin FQ and/or the activation of the N-methyl-D-aspartate receptor (2), may also contribute to the cerebrovascular derangement following cerebral H/I.

In conclusion, the data in the present study indicate that an intravenous administration of EEIIMD postinjury limits cerebrovasodilator impairment induced by H/I by upregulating p38 but not JNK. These data suggest that EEIIMD or other approaches to upregulate p38 may offer a novel approach to increase the benefit-to-risk ratio of thrombolytic therapy for diverse central nervous system disorders associated with H/I.

**REFERENCES**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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