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

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Submitted 23 February 2010; accepted in final form 30 April 2010

Armstead WM, Riley J, Kiessling JW, Cines DB, Higazi AA. PAI-1-derived peptide EEIIMD prevents impairment of cerebrovasodilation by augmenting p38 MAPK upregulation after cerebral hypoxia/ischemia. Am J Physiol Heart Circ Physiol 299: H76–H80, 2010. First published April 30, 2010; doi:10.1152/ajpheart.00185.2010.—Babies are frequently exposed to cerebral hypoxia and ischemia (H/I) during the perinatal period as a result of stroke, problems with delivery, or postdelivery respiratory management. The sole approved treatment for acute stroke is tissue type plasminogen activator. H/I impairs pial artery dilation (PAD) induced by hypercapnia and hypotension, the impairment aggravated by type plasminogen activator and attenuated by the plasminogen activator inhibitor-1-derived peptide EEIIMD. Mitogen-activated protein kinase (MAPK), a family of at least three kinases, ERK, p38, and JNK, is upregulated after H/I and ERK contribute to impaired cerebrovasodilation. This study determined the roles of p38 and JNK MAPK in the impairment of dilatation post-H/I in pigs equipped with a closed cranial window and the relationship between alterations in MAPK isoforms and EEIIMD-mediated cerebrovascular protection. Cerebrospinal fluid-phosphorylated (activated) p38, and c-Jun-NH2-terminal kinase (JNK), is upregulated after H/I and JNK is decreased by EEIIMD administration 1 h postinjury. PAD in response to hypercapnia and hypotension was blunted by H/I, but dilatation was maintained by EEIIMD. Further impaired by the p38 antagonist SB-203580 but unchanged by the JNK antagonist SP-600125. Isoproterenol-induced PAD was unchanged by H/I, EEIIMD, SB-203580, and SP-600125. These data indicate that postinjury treatment with EEIIMD attenuated impaired cerebrovasodilation post-H/I by upregulating p38 but not JNK. These data suggest that plasminogen activator inhibitor-1-based peptides and 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.

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

Mitogen-activated protein kinase (MAPK), a family of at least three kinases [extracellular signal-related kinase (ERK), p38, and c-Jun-NH2-terminal kinase (JNK)], is upregulated after cerebral ischemia (2, 12, 18). Our recent studies show that uPA contributes to impaired stimulus-induced cerebrovascular dilation following cerebral H/I in the newborn pig through the upregulation of ERK MAPK, whereas the ERK MAPK antagonist U-0126 partially prevents vascular impairment (4). The upregulation of JNK MAPK and the protection against neuronal toxicity by antagonists of this MAPK isoform have been reported in rodent models of focal ischemia (7, 12). It has been speculated that JNK MAPK might contribute similarly to cerebrovasodilator outcome after cerebral H/I in the piglet. Using a combined biochemical and pharmacological approach, we investigated the roles of p38 and JNK MAPK in the H/I dilator impairment in piglets and the relationship between the changes in these MAPK isoforms and EEIIMD-mediated cerebrovascular protection postinsult.

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 anes-
thetized 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 MgCl\(_2\), 1.5 CaCl\(_2\), 132
NaCl, 6.6 urea, 3.7 dextrose, and 24.6 NaHCO\(_3\). This artificial CSF
was warmed to 37°C and had the following chemistry: pH 7.33, PCO\(_2\) at
no greater than 100 mmHg. As the cerebral ischemic response
was withdrawn as necessary to maintain mean arterial blood pressure
pressure from rising inordinately (Cushing response), venous blood
monitored via a sidearm of the cranial window. To prevent the arterial
diastolic arterial blood pressure (19, 20). Intracranial pressure was
pressure 15 mmHg greater than the numerical mean of systolic and
CSF into a hollow bolt in the cranium to maintain an intracranial
pressure. The pial arterial vessel diameter was measured with
a microscope, a camera, a video output screen, and a video mi-
croscale.

Total cerebral ischemia was accomplished by infusing artificial
CSF into a hollow bolt in the cranium to maintain an intracranial
pressure 15 mmHg greater than the numerical mean of systolic and
diastolic arterial blood pressure (19, 20). Intracranial pressure was
monitored via a sidearm of the cranial window. To prevent the arterial
pressure from rising inordinately (Cushing response), venous blood
was withdrawn as necessary to maintain mean arterial blood pressure
at no greater than 100 mmHg. As the cerebral ischemic response
subsided, the shed blood was returned to the animal. Cerebral isch-
emia was maintained for 20 min. Hypoxia (P\(_{O_2}\) of ~35 mmHg) was
produced for 10 min before ischemia by decreasing the inspired O\(_2\)
via inhalation of N\(_2\), which was followed immediately by the total
cerebral ischemia. Hypotension was induced by the rapid withdrawal
of either 5–8 or 10–15 ml blood/kg to induce moderate or severe
hypotension (decreases in mean arterial blood pressure of 25 and 45%,
respectively) (3). Such drops in blood pressure were maintained
constant for 10 min by a titration of additional blood withdrawal or
blood reinfusion (2). Two levels of hypercapnia (low and high) were
induced via the inhalation of graded levels of a 10% CO\(_2\)-21%
O\(_2\)-balance N\(_2\) gas mixture for 10 min to produce levels of P\(_{CO_2}\)
of 50–60 mmHg for the low exposure and 70–80 mmHg for the high
exposure (13).

Protocol. Two types of pial vessels, small arteries (resting diam-
ter, 120–160 µm) and arterioles (resting diameter, 50–70 µm) were
examined to determine whether segmental differences in the effects of
H/I could be identified. Typically, 2 to 3 ml of artificial CSF were
flushed through the window over a 30-s period, and excess CSF was
allowed to run off through one of the needle ports.

Thirteen experimental groups of animals were studied (all, n = 5):
1) sham-operated control, vehicle treated; 2) H/I, vehicle pretreated;
3) H/I, pretreated with EEIIMD (1 mg/kg iv); 4) H/I, pretreated with
EEIIMR (1 mg/kg iv); the inactive analog of EEIIMD; 5) H/I,
pretreated with the JNK MAPK antagonist, SP-600125 (1 mg/kg iv);
6) H/I, pretreated with the p38 MAPK antagonist SB-203580 (1 mg/kg iv); 7) H/I, pretreated with combined EEIIMD and SB-203580;
8) H/I, posttreated with vehicle; 9) H/I, posttreated with EEIIMD; 10)
H/I, posttreated with EEIIMR; 11) H/I, posttreated with SP-600125;
and 12) H/I posttreated with SB-203580 and H/I posttreated with
combined EEIIMD and SB-203580. The vehicle for all agents was
0.9% saline, except for the MAPK antagonists, which was DMSO
(stock) diluted with saline, with a maximal ratio of 1:1,000. These two
types of vehicle-CSF controls had no significant effect on pial artery
diameter. In sham-operated control animals, responses to hypercapnia,
hypotension, and isoproterenol (10\(^{-8}\) and 10\(^{-6}\) M) were obtained
initially and then again 1 and 4 h later in the presence of agent vehicle.
In H/I vehicle animals, responses to vasoactive stimuli were obtained
initially and then again 1 and 4 h postsimult in the presence of vehicle.

RESULTS

H/I elevates CSF p38 MAPK, which is potentiated by EEIIMD, but
has no effect on CSF JNK MAPK. The activation (phosphoryla-
tion) state of the p38 and JNK MAPK isoforms was determined 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 hyper-
capnia 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 vasodi-
lation 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
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 vasocon-
striction 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 ± 2 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 EEIMR, 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.

![Graph](http://ajpheart.physiology.org.org)
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 vasoactivity 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 αvβ3 (17, 24) and that plasminogen activators can promote the formation of a signal-transducing complex between low-density lipoprotein receptor and αvβ3 (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 αvβ3-dependent process (14). However, other as yet undefined mechanisms, which may include the opioid nociceptin/orphanin FQ (14). Kiessling JW, Cines DB, Higazi AA, Armstead WM. Inhibition of c-Jun N-terminal kinase (JNK) and JNK interacting protein response in rat brain after transient middle cerebral artery occlusion. Neurosci Lett 284: 195–199, 2000.


