Urokinase Plasminogen Activator Impairs SNP and PGE₂ Cerebrovasodilation after Brain Injury through Activation of LRP and ERK MAPK

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Abstract

Pial artery dilation in response to prostaglandin (PG)E₂ and the nitric oxide (NO) releaser sodium nitroprusside (SNP) are blunted after fluid percussion brain injury (FPI), whereas responses to papaverine are unchanged. Urokinase plasminogen activator (uPA) and ERK mitogen-activated protein kinase (MAPK) are upregulated and contribute to the impairment of cerebrohemodynamics seen after FPI. PA vascular activity is mediated through the low-density lipoprotein receptor (LRP). Therefore, we investigated the role of uPA, LRP, and ERK MAPK in the impaired cerebrovasodilation response to PGE₂ and SNP after FPI. Lateral FPI (2 atm) was induced in anesthetized piglets equipped with a closed cranial window. Cerebrospinal fluid (CSF) ERK MAPK was quantified by enzyme-linked immunosorbent assay (ELISA). Pretreatment with soluble uPA receptor (suPAR), which antagonizes the vascular action of uPA, blunted the impairment of SNP and PGE₂-mediated dilation seen after FPI. Pretreatment with the LRP antagonist RAP, a monoclonal antibody against LRP (Mab ag LRP) and the ERK MAPK antagonist, U 0126, all provided similar protection, whereas control immunoglobulin G (IgG) had no effect. Responses to papaverine were unchanged after FPI. Upregulation of ERK MAPK phosphorylation in CSF after FPI was blunted in animals pretreated with suPAR, RAP, Mab ag LRP, or U 0126, whereas control IgG had no effect. These data indicate that uPA contributes to the impairment of SNP and PGE₂-mediated cerebrovasodilation seen after brain injury through activation of LRP and ERK MAPK.

Key words: cerebral circulation; newborn; plasminogen activators; signal transduction

Introduction

RELAXATION OF BLOOD VESSELS can be mediated by several mechanisms, including cyclic AMP (cAMP), cyclic GMP (cGMP), and potassium (K⁺) channels (Faraci and Heistad, 1998). Prostaglandins (PG) are present in cortical periarachnoid cerebrospinal fluid (CSF) in concentrations that are in the vasoactive range under resting conditions and contribute to the regulation of cerebral hemodynamics in the newborn pig (Leffler et al., 1993). The predominant dilator prostaglandins in the piglet are PGE₂ and PGI₂, which elicit cerebrovasodilation via cAMP, cGMP, and K⁺ channel-dependent mechanisms (Armstead, 1998; Leffler et al., 1993). The prostaglandin system is the prominent vasodilator mechanism in the newborn pig, but the relative importance of the nitric oxide (NO) system increases with maturation

(Willis and Leffler, 1999; Zuckerman et al., 1996). The prototypic NO releaser sodium nitroprusside (SNP) elicits vasodilation via cGMP in a K⁺-dependent manner in the piglet (Armstead, 1996). We have previously observed that PGE₂ and SNP-induced pial artery dilation is impaired after fluid percussion brain injury (FPI) in the piglet (Armstead, 1997, 1998), a model of traumatic brain injury (TBI) (Gennarelli, 1994). Since prostaglandins and NO mediate dilation to numerous physiologic stimuli, it is important to understand the effects of TBI on these two key signaling mediators. However, the mechanisms that contribute to impairment of vasodilation in response to PGE₂ and SNP after FPI are incompletely understood.

Urokinase and tissue plasminogen activator (uPA and tPA) are serine proteases that convert plasminogen to the active protease plasmin (Collen and Lijnen, 1991). Our previ-

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ous studies show that exogenous uPA produces pial artery dilation in the pig (Armstead et al., 2005). The effect of plasminogen activators (PA) on vascular activity is mediated through the low-density lipoprotein receptor (LRP) (Bu et al., 1992). EEIIMD, a peptide derived from the endogenous PA inhibitor PAI-1, and soluble uPA receptor (suPAR), each inhibit PA-mediated vasodilation without compromising catalytic activity (Armstead et al., 2005; Nassar et al., 2004; Akkawi et al., 2006). The concentration of uPA in the CSF is elevated after TBI in the piglet (Armstead et al., 2006), while suPAR blunts pial artery vasoconstriction associated with FPI (Armstead et al., 2005), suggesting its contribution to impaired cerebral hemodynamics post-insult.

Mitogen-activated protein kinase (MAPK), a key intracellular signaling system, is a family of at least three kinases, extracellular signal-related kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) (Laher and Zhang, 2001). TBI induces the expression of neurotrophin-related mRNA and receptors (Hicks et al., 1999), which subsequently triggers downstream MAPK cascades (Otani et al., 2002) through interactions with high-affinity tyrosine kinase receptors (Bonni et al., 1999). Upregulation of PAs contributes to impairment of cerebrovasodilation in response to activation of the *N*-methyl-D-aspartate (NMDA) receptor after FPI (Armstead et al., 2005). Independent studies show that ERK MAPK activation contributes to blunted NMDA dilation after FPI (Armstead 2003), suggesting that PAs impair NMDA dilation after FPI via an ERK MAPK-dependent mechanism. We hypothesize that uPA contributes to impairment of PGE₂ and SNP pial artery dilation after FPI through activation of LRP and ERK MAPK.

Methods

Materials

Recombinant single chain urokinase (uPA) was expressed in S2 cells, purified by antibody affinity chromatography, and characterized for affinity to the cellular and soluble uPA receptor (suPAR), for PA activity and for fibrinolytic activity in the presence and absence of suPAR, as described previously (Higazi et al., 1996a,b, 1998). uPA migrates as a homogeneous ~50-kDa protein on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions. The LRP antagonist, recombinant receptor-associated protein (RAP) (Bu et al., 1992) was generated in *Escherichia coli* (DH5 α /pGEX-2T plasmid) expressing GST-RAP (kind gift of Dr. T. Willnow, University of Potsdam, Germany) after induction with IPTG. RAP was purified on glutathione-sepharose (Amersham) and was eluted using 10mM glutathione in phosphate-buffered saline (PBS) and buffer exchange to PBS using an Amicon Unit centrifugal device (10,000 MWCO). The preparation of RAP was passed twice through Acticlean Etox (Sterogene) column to remove the endotoxin. RAP migrated as a single band on SDS-PAGE and inhibited the internalization of ¹²⁵I uPA in murine embryonic fibroblasts (MEF-1) cells, which is known to be LRP dependent.

Closed cranial window and brain injury procedures

Newborn pigs (1–5 days old, 1.2–1.6 kg) of either sex were studied. All protocols were approved by the Institutional An-

imal Care and Use Committee of the University of Pennsylvania. Animals were sedated with isoflurane (1–2 MAC). Anesthesia was maintained with α -chloralose (30–50 mg/kg supplemented with 5 mg/kg/h i.v.). A catheter was inserted into a femoral artery to monitor blood pressure, and to sample blood gas tensions and pH. Drugs to maintain anesthesia were administered through a second catheter placed in a femoral vein. 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 pre-cut 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 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, p_{CO2} 46 mm Hg, and p_{O2} 43 mm Hg, which was similar to that of endogenous CSF. Pial arterial vessel diameter was measured with a microscope, a camera, a video output screen, and a video microscaler.

The methods used to induce brain FPI have been described previously (Wei et al., 1980). A device designed by the Medical College of Virginia was used. A small opening was made in the parietal skull contralateral to the cranial window. A metal shaft was sealed into the opening on top of intact dura. This shaft was connected to the transducer housing, which was in turn connected to the fluid percussion device. The device itself consisted of an acrylic plastic cylindrical reservoir 60 cm long, 4.5 cm in diameter, and 0.5 cm thick. One end of the device was connected to the transducer housing, whereas the other end had an acrylic plastic piston mounted on O-rings. The exposed end of the piston was covered with a rubber pad. The entire system was filled with 0.9% saline. The percussion device was supported by two brackets mounted on a platform. FPI was induced by striking the piston with a 4.8-kg pendulum. The intensity of the injury (usually 1.9–2.3 atm with a constant duration of 19–23 msec) was controlled by varying the height from which the pendulum was allowed to fall. The pressure pulse of the injury was recorded on a storage oscilloscope triggered photoelectrically by the fall of the pendulum. The amplitude of the pressure pulse was used to determine the intensity of the injury.

Protocol

Two types of pial vessels, small arteries (resting diameter, 120–160 μ m) and arterioles (resting diameter, 50–70 μ m) were examined to determine whether segmental differences in the effects of FPI could be identified. Typically, 2–3 mL of artificial CSF were flushed through the window over a 30-sec period, and excess CSF was allowed to run off through one of the needle ports. For sample collection, 300 μ L of the total cranial window volume of 500 μ L was collected by slowly infusing artificial CSF into one side of the window and allowing the CSF to drip freely into a collection tube on the opposite side.

Seven experimental groups were studied (all $n = 6$): (1) sham control, (2) FPI, vehicle treated, (3) FPI treated with mouse immunoglobulin G (IgG; 10 $\mu\text{g}/\text{mL}$), (4) FPI treated with the soluble uPA inhibitor suPAR (10^{-7} M), (5) FPI treated with an antibody directed at the alpha chain of LRP (10 $\mu\text{g}/\text{mL}$ topical; American Diagnostica no. 3402), (6) FPI treated with the LRP antagonist RAP (10^{-5} M), and (7) FPI treated with the ERK MAPK inhibitor U 0126 (10^{-6} M, Sigma). The vehicle for all agents was 0.9% saline, except for the MAPK inhibitor, which used dimethyl sulfoxide (100 μL) diluted with 9.9 mL of 0.9% saline. In sham control animals, responses to PGE₂ (1, 10 ng/mL), SNP, and papaverine (10^{-8} , 10^{-6} M) were obtained initially, and then again 1 and 4 h later in the presence of the agent vehicle. In drug-treated FPI animals, IgG, suPAR, anti-LRP antibody (Ab), RAP, or U 0126 were administered 30 min before FPI, and the responses to PGE₂, SNP, and papaverine obtained at 1 and 4 h after injury. The order of agonist administration was randomized within animal drug treatment groups.

ELISA

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to quantify CSF uPA (Dipharma) and ERK MAPK (Assay Designs) concentration. Phosphorylated ERK MAPK enzyme values were normalized to total form and then expressed as percent of the control condition.

Statistical analysis

Pial artery diameter and CSF MAPK isoform values were analyzed using analysis of variance (ANOVA) for repeated measures. If the value was significant, the data were then analyzed by Fishers protected least significant difference test. An α level of $p < 0.05$ was considered significant in all statistical tests. Values are represented as mean $\pm$ SEM of the absolute value or as percentage changes from control value.

Results

uPA contributes to impairment of pial artery dilation to SNP and PGE₂ in an LRP and ERK MAPK-dependent manner

Topical sodium nitroprusside (SNP) (10^{-8} , 10^{-6} M), which releases NO, the prostaglandin PGE₂ (1, 10 ng/mL), and papaverine (10^{-8} , 10^{-6} M) elicit reproducible pial small artery (120–160 μm) and arteriole (50–70 μm) dilation in the newborn pig (data not shown). Within 1 h of FPI, pial small artery vasodilator responses to SNP and PGE₂ were blunted, but responses to papaverine were unchanged (Figs. 1–3). Pretreatment with the antagonist of the vascular action of uPA, suPAR (10^{-7} M), administered 30 min prior to FPI followed by continuous exposure of the cerebral cortical surface to this agent, blunted impairment of SNP and PGE₂ dilation post-injury while dilation in response to papaverine was unchanged (Figs. 1–3). Similarly, pretreatment with the LRP antagonist RAP (10^{-5} M), the monoclonal antibody against LRP (Mab ag LRP, 10 $\mu\text{g}/\text{mL}$), and the ERK MAPK antagonist, U 0126 (10^{-6} M) all blunted impairment of SNP and PGE₂ dilation post-injury, while papaverine-induced dilation was unchanged (Figs. 1–3). In contrast, mouse IgG had no effect on FPI-induced impairment of SNP and PGE₂ dilation (Figs. 1 and 2). Similar observations were made in pial small arteries at 4 h post-FPI and in pial arterioles at 1 and 4 h post-insult.

Total and phosphorylated ERK MAPK in cortical periarachnoid CSF was measured by ELISA under baseline (prior to FPI, 0 time) and injury (1 or 4 h) conditions. The activation (phosphorylation) state of this MAPK isoform was determined by expressing the data as a percent of control (total) to normalize these values. Vehicle alone (dimethyl sulfoxide [DMSO]; 100 μL) diluted in 9.9 mL of 0.9% saline or antagonist was administered 30 min prior to FPI. Phosphorylation of CSF ERK MAPK was upregulated within 1 h of FPI, but such upregulation was blunted in animals pre-

FIG. 1. Influence of SNP (10^{-8} , 10^{-6} M) on pial artery diameter before (control), 1 h after FPI, 1 h after FPI in animals pretreated with mouse IgG, 1 h after FPI in animals pretreated with suPAR (10^{-7} M), 1 h after FPI in animals pretreated with monoclonal antibody to LRP (Mab ag LRP, 10 $\mu\text{g}/\text{mL}$), 1 h after FPI in animals pretreated with RAP (10^{-5} M), and 1 h after FPI in animals pretreated with U 0126 (10^{-6} M), $n = 5$ –6. * $p < 0.05$ compared to corresponding control value; † $p < 0.05$ compared to corresponding non-pretreated value.

FIG. 2. Influence of PGE₂ (1, 10 ng/mL) on pial artery diameter before (control), 1 h after FPI, 1 h after FPI in animals pretreated with mouse IgG, 1 h after FPI in animals pretreated with suPAR (10^{-7} M), 1 h after FPI in animals pretreated with monoclonal antibody to LRP (MAb ag LRP, 10 μ g/mL), 1 h after FPI in animals pretreated with RAP (10^{-5} M), and 1 h after FPI in animals pretreated with U 0126 (10^{-6} M), $n = 5-6$. * $p < 0.05$ compared to corresponding control value; † $p < 0.05$ compared to corresponding non-pretreated value.

treated with suPAR, RAP, or anti-LRP (Fig. 4). In contrast, IgG had no effect on FPI-induced upregulation of ERK MAPK (Fig. 4). The ERK MAPK antagonist U 0126 blocked the upregulation of CSF ERK MAPK, as expected (Fig. 4).

Effect of suPAR, anti-LRP, RAP, IgG, and U 0126 effects on pial artery diameter in sham control piglets

Administration of suPAR, anti-LRP antibody, RAP, IgG, and U 0126 to the cerebral cortical surface had no effect on pial artery diameter under sham control conditions.

Blood chemistry

There were no statistical differences in blood chemistry between sham control, FPI, and FPI antagonist-treated animals before or after all experiments. The amplitude of the

pressure pulse, used as an index of injury intensity, was equivalent in FPI-vehicle and FPI-antagonist animals (1.9 ± 0.1 atm).

Discussion

There are several important new findings in this study. First, since suPAR partially prevented impairment of SNP and PGE₂ vasodilation after FPI, these data indicate that endogenous uPA released after brain injury (Armstead et al., 2006) contributes to derangement of two fundamental controllers of cerebrovascular dilator capacity. Impairment of pial artery reactivity by uPA is not an epiphenomenon in that responses to papaverine, which produces vasodilation independent of PGs and NO, were unchanged by FPI (Armstead 1997) or by suPAR. The concentrations of PGE₂ were chosen to mimic physiologic and pathologic conditions of

FIG. 3. Influence of papaverine (10^{-8} , 10^{-6} M) on pial artery diameter before (control), 1 h after FPI, 1 h after FPI in animals pretreated with mouse IgG, 1 h after FPI in animals pretreated with suPAR (10^{-7} M), 1 h after FPI in animals pretreated with monoclonal antibody to LRP (MAb ag LRP, 10 μ g/mL), 1 h after FPI in animals pretreated with RAP (10^{-5} M), and 1 h after FPI in animals pretreated with U 0126 (10^{-6} M), $n = 5-6$.

FIG. 4. Phosphorylation of ERK MAPK in cortical periarachnoid CSF prior to FPI (0 min), as a function of time (h) after FPI in vehicle (FPI), mouse IgG + FPI, suPAR (10^{-7} M) + FPI, monoclonal LRP antibody (Mab ag LRP, $10 \mu\text{g/mL}$) + FPI, RAP (10^{-5} M) + FPI, and U 0126 (10^{-6} M) + FPI animals, $n = 5-6$. Data expressed as percent of control by ELISA determination of phospho MAPK and total MAPK isoforms and subsequent normalization to total form. * $p < 0.05$ compared with corresponding 0 time value; † $p < 0.05$ compared with corresponding FPI non-pretreated value.

the cerebral circulation (Leffler et al., 1993; Willis and Leffler, 1999; Armstead, 1998).

Importantly, suPAR inhibits uPA-mediated vasodilation without compromising its catalytic activity (Armstead et al., 2005; Nassar et al., 2004; Akkawi et al., 2006). Nonetheless, prior observations showing that uPA promotes vasodilation makes the observed protection by suPAR, somewhat counter-intuitive. It might be anticipated that application of a second dilator in the setting of upregulation of uPA expression by FPI should have resulted in an additive or synergistic effect. Preservation of dilation to PGE₂ and SNP after FPI with suPAR pretreatment, therefore, suggests an interaction of uPA with signaling systems unique to these vasodilators. The mechanism which promotes interaction with these alternative signaling systems (e.g., ERK MAPK) under TBI conditions is uncertain, but may involve a different binding site for the uPA-suPAR complex, a competition with uPA for binding to LRP, or a dose-dependent parabolic effect that is shifted by suPAR. However, it is clear that the protective effect of suPAR did not result from a general nonspecific potentiation of vascular responsiveness, as there was no effect on dilation induced by papaverine.

The second major finding of the present study relates to mechanisms involved in uPA-mediated inhibition pial artery dilation in response to SNP and PGE₂ after FPI. Responses to SNP and PGE₂ were protected with the monoclonal antibody to LRP and not by IgG. Finding similar protection of vasodilator responses by the LRP antagonist, RAP, strengthen the proposed role of this receptor in uPA-mediated vasodilator impairment after brain injury. U 0126, an ERK MAPK antagonist, also blunted impairment of SNP and PGE₂-mediated vasodilation after FPI. FPI is followed by an

increase in the CSF concentration of phosphorylated (activated) ERK MAPK. The monoclonal antibody to LRP and RAP blunted this elevation in ERK MAPK post-insult. Together, the biochemical data support and corroborate the pharmacological data to indicate that uPA inhibits dilation to these two vasodilator stimuli through an ERK MAPK-dependent process. The ability of U 0126 to block elevation of CSF ERK MAPK concentration after FPI indicates that the dose of this ERK MAPK antagonist was appropriate. Taken together, these data indicate that uPA impairs dilation in response to SNP and PGE₂ after brain injury in an LRP and ERK MAPK-dependent manner.

TBI is the leading cause of brain injury, death, and disability in children (Rodriguez, 1990). While the effects of TBI have been extensively investigated in adult animal models (Wei et al., 1980), less is known about its effect in the newborn/infant. TBI can uncouple flow and metabolism, resulting in cerebral ischemia or hyperemia (Richards et al., 2001). Although cerebral hyperemia was historically considered the cause of diffuse brain swelling after TBI in the pediatric setting (Bruce et al., 1981), recent evidence suggests that cerebral hypoperfusion is the dominant derangement (Adelson et al., 1997). Decreases in cerebral blood flow and pial artery diameter, along with impaired vasodilator responsiveness are greater in newborn than in juvenile pigs following FPI (Armstead and Kurth, 1994; Armstead et al., 2005). These data support the potential importance of hypoperfusion in outcome post-TBI and the idea that the newborn's cerebral hemodynamics is more sensitive to brain injury (Armstead and Kurth, 1994). Piglets offer the unique advantage of a gyrencephalic brain containing substantial white matter, which is more sensitive to ischemic/TBI damage than rodent brain and more similar to humans.

Clinically, it has been suggested that coagulopathy contributes to outcome in pediatric inflicted brain injury (Hymel et al., 1997). Our prior studies have observed the upregulation of uPA after TBI in the piglet (Armstead et al., 2006). The present study using biochemical and pharmacologic approaches investigated the role of endogenous uPA in the derangement of two fundamental controllers of cerebrovascular dilator capacity after TBI. Results of this study are the first to document that endogenous uPA contributes to loss of cerebrohemodynamic dilator reserve through LRP and ERK MAPK-dependent mechanisms. These data suggest that modulation of uPA expression and/or action may preserve cerebrohemodynamic control after TBI and thereby improve outcome. Translationally, these data further suggest that administration of agents like suPAR which preserve the catalytic activity of uPA but block the deleterious signal transducing actions of this PA may result in improved outcome for TBI patients. Future experiments should be directed towards identification of the timeframe post-TBI when administration of suPAR-like drugs may exert maximal benefit.

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Author Disclosure Statement

The authors have no conflict of interest to declare.

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