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European Journal of Pharmacology 474 (2003) 249–254
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PTK, ERK and p38 MAPK contribute to impaired NMDA-induced vasodilation after brain injury

William M. Armstead*

Department of Anesthesia and Pharmacology, University of Pennsylvania, Philadelphia, PA 19104, USA

Received 19 June 2003; accepted 24 June 2003

Abstract

N-Methyl-D-aspartate (NMDA)-induced pial artery dilation is reversed to vasoconstriction following fluid percussion brain injury (FPI). This study investigated the contribution of activation of protein tyrosine kinase (PTK) and the extracellular signal-regulated kinase (ERK) and p38 isoforms of mitogen-activated protein kinase (MAPK) in impaired vasodilation to NMDA after fluid percussion brain injury in pigs equipped with a closed cranial window. NMDA (10^{-8}, 10^{-6} M)-induced vasodilation was reversed to vasoconstriction following fluid percussion brain injury, but such responses were partially restored by genistein (4′,5,7-trihydroxy isoflavone), U0126 [1,4-diamino-2,3-dicyano-1,4-bis (0-aminophenylmercapto)butadiene] and SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole], PTK, ERK and p38 MAPK inhibitors (9 ± 1% and 16 ± 1%, sham control; − 6 ± 2% and − 11 ± 3%, fluid percussion brain injury; and 3 ± 1% and 6 ± 1%, fluid percussion brain injury-genistein, respectively). However, the robustness of the protection to NMDA dilation was significantly greater for U0126 vs. SB203580 (4 ± 1% and 7 ± 1% vs. 1 ± 1% and 1 ± 2%). Similar results were observed for glutamate. These data show that PTK, ERK and p38 MAPK activation contribute to impaired NMDA cerebrovasodilation after fluid percussion brain injury. These data suggest that activation of the ERK isoform of MAPK contributes to such impairment more than the p38 MAPK isoform.

Keywords: Cerebral circulation; Excitatory amino acid; Signal transduction; Neurotrauma

1. Introduction

Traumatic brain injury is a leading cause of morbidity and mortality in infants and children (Duhaime et al., 1987; Rodriguez and Brown, 1990). Fluid percussion brain injury (FPI) is an experimental model for blunt head trauma (Gennarelli, 1994). Although the effects of brain injury have been well documented in adult animal models (McIntosh et al., 1989), less has been reported on the effects of brain injury in the newborn or the mechanisms underlying such changes.

Glutamate is an important excitatory amino acid transmitter in the brain. It can bind to any of three different ionotropic receptor subtypes named after specific synthetic analogues: *N*-methyl-D-aspartate (NMDA), kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA). Activation of NMDA receptors has been observed to elicit cerebrovascular dilation and may represent one of the mechanisms for the coupling of local cerebral metabolism to blood flow (Faraci and Breese, 1981). One contribution to neurological damage following traumatic brain injury is thought to be cerebrovascular dysfunction. Although activation of the NMDA receptor may contribute to altered cerebrovascular regulation following traumatic brain injury (McIntosh, 1993), the effects of such injury on the vascular action of NMDA have been less well appreciated. Interestingly, NMDA-induced pial artery dilation has been observed to be impaired following fluid percussion brain injury (Armstead, 2000c). However, the mechanism for such impairment post insult is incompletely understood.

Activation of protein kinase C (PKC) is thought to contribute to the cerebral vasospasm associated with pathologic conditions such as subarachnoid hemorrhage (Laher and Zhang, 2001). Activation of PKC, in turn, promotes interaction with other more distal signaling pathways, such as protein tyrosine kinase (PTK) and its substrate, mitogen-activated protein kinase (MAPK), also thought to contribute to cerebral vasospasm (Laher and Zhang, 2001). MAPK itself is actually a family of at least three kinases: extracel-

* Department of Anesthesia, University of Pennsylvania, 3400 Spruce Street, Philadelphia, PA 19104, USA. Tel.: +1-215-573-3674; fax: +1-215-349-5078.
E-mail address: armsteaw@uphs.upenn.edu (W.M. Armstead).

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doi:10.1016/S0014-2999(03)02012-0

lular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) (Laher and Zhang, 2001). Previous studies in the piglet have observed a role for superoxide (O_2^-) generated via PKC activation in the impairment of NMDA pial artery dilation following cerebral hypoxia/ischemia (Armstead, 2000b). Since fluid percussion injury in the piglet produces cerebrovascular hypoperfusion associated with a decrease in the saturation of hemoglobin for oxygen (Armstead and Kurth, 1994), ischemia may result from fluid percussion brain injury.

Therefore, the present study was designed to investigate the role of PTK, ERK and p38 MAPK activation in impaired NMDA and glutamate pial artery dilation after fluid percussion brain injury. In order to accomplish this aim, responses to NMDA and glutamate were obtained before and after fluid percussion brain injury in untreated animals and in those animals pretreated prior to FPI with pharmacologic inhibitors. These inhibitors were genistein (4′,5,7-trihydroxy isoflavone) for PTK, U0126 [1,4-diamino-2,3-dicyano-1,4-bis (0-aminophenylmercapto)butadiene] for EKR MAPK and SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole) for p38 MAPK, respectively.

2. Materials and methods

Newborn (1–5 days old, 1.3–2.1 kg) pigs of either sex were used in these experiments. All protocols were approved by the Institutional Animal Care and Use Committee. Animals were sedated with isoflurane (1–2 minimum alveolar concentration). 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 for 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 mechanically 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 $MgC1_2$, 1.5 $CaCI_2$, 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, P_{CO_2} 46 mm Hg and m P_{O_2} 43 mm Hg, which was similar to that of endogenous CSF. Pial arterial vessels were observed with a dissecting microscope, a television camera mounted on the microscope and a video output screen. Vascular diameter was measured with a video microscaler.

Methods for brain fluid percussion brain injury 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. Fluid percussion brain injury 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 ms) 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.

2.1. Protocol

Two types of pial arterial vessels, small arteries (resting diameter, 120–160 μm) and arterioles (resting diameter, 50–70 μm) were examined to determine whether segmental differences in the effects of FPI could be identified. Typically, 2–3 ml of 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.

Eight types of experiments using a total of 90 animals were performed (all $n=6$): (1) sham control, (2) sham control with coadministered genistein, (3) sham control with coadministered U0126, (4) sham control with coadministered SB203580, (5) fluid percussion brain injury, (6) fluid percussion brain injury pretreated with genistein, (7) fluid percussion brain injury pretreated with U0126 and (8) fluid percussion brain injury pretreated with SB203580. In experiments designed to investigate the influence of fluid percussion brain injury on vascular responses to excitatory amino acids, NMDA and glutamate (10^{-8}, 10^{-6} M) were topically applied 60 min before and 60 min after fluid percussion brain injury. The PTK inhibitor genistein (10^{-6}, 10^{-5} M), the ERK MAPK inhibitor U0126 (10^{-6}, 10^{-5} M) or the p38 MAPK inhibitor SB203580 (10^{-5}, 10^{-4} M) were applied 30 min prior to fluid percussion brain injury, such agents remained on the brain surface during fluid percussion brain injury and responses after fluid percussion brain injury obtained during the continued ad-

ministration of each agent. Sham control experiments were designed to obtain responses to agonists initially and then again 60 min later. All agents were obtained from Sigma Aldrich.

2.2. Statistical analysis

Pial artery diameter and systemic artery pressure values were analyzed using analysis of variance 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 ± S.E. of the absolute values or as percentage changes from control values.

3. Results

3.1. Role of PTK activation in impaired NMDA vasodilation after fluid percussion brain injury

Topical NMDA and glutamate (10^{-8}, 10^{-6} M) elicited reproducible pial small artery (120–160 μm) and arteriole (50–70 μm) vasodilation in sham control animals (data not shown). However, NMDA- and glutamate-induced vasodilation was reversed to vasoconstriction within 1 h after fluid percussion brain injury (Figs. 1 and 2). This post injury vasoconstriction induced by these two agents was reversed to dilation by pretreatment with the PTK inhibitor, genistein (10^{-6}, 10^{-5} M). On a percentage basis, pial small artery dilation to NMDA was inhibited by 171 ± 20% and 164 ± 8%, while glutamate was inhibited by 143 ± 10% and 151 ± 10%, respectively. Pretreatment with genistein (10^{-6} M), however, resulted in significantly less inhibition

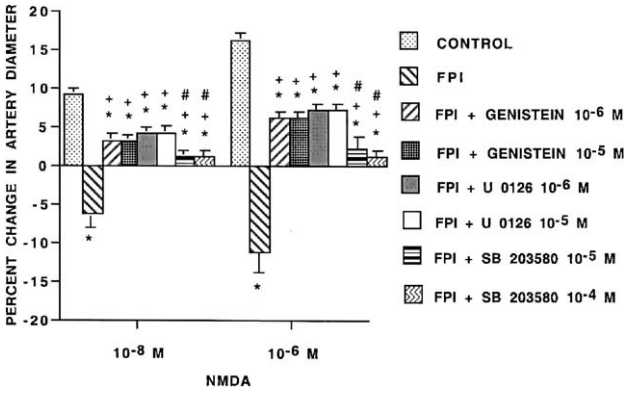

Fig. 1. Influence of NMDA (10^{-8}, 10^{-6} M) on pial small artery diameter before (control), after FPI, after FPI in genistein (10^{-6} M) pretreated animals, after FPI in genistein (10^{-5} M) pretreated animals, after FPI in U0126 (10^{-6} M) pretreated animals, after FPI in U0126 (10^{-5} M) pretreated animals, after FPI in SB203580 (10^{-5} M) pretreated animals and after FPI in SB203580 (10^{-4} M) pretreated animals. $n = 6$. $^{*}p < 0.05$ vs. control, $^{+}p < 0.05$ vs. absence of pretreatment, $^{\#}p < 0.05$ vs. U1026 pretreated value.

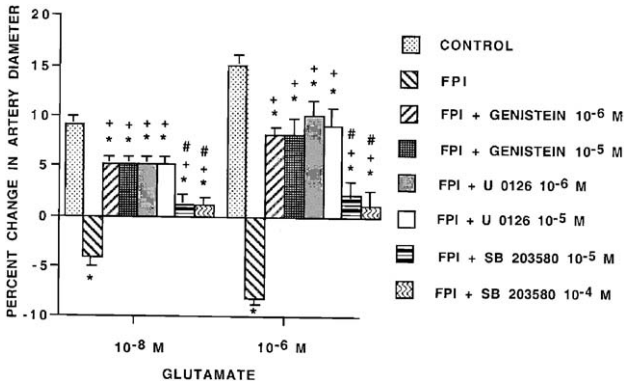

Fig. 2. Influence of glutamate (10^{-8}, 10^{-6} M) on pial small artery diameter before (control), after FPI, after FPI in genistein (10^{-6} M) pretreated animals, after FPI in genistein (10^{-5} M) pretreated animals, after FPI in U0126 (10^{-6} M) pretreated animals, after FPI in U0126 (10^{-5} M) pretreated animals, after FPI in SB203580 (10^{-5} M) pretreated animals and after SB203508 (10^{-4} M) pretreated animals. $n = 6$. $^{*}p < 0.05$ vs. control, $^{+}p < 0.05$ vs. absence of pretreatment, $^{\#}p < 0.05$ vs. U0126 pretreated value.

for NMDA and glutamate dilation post insult (65 ± 6% and 61 ± 4% for NMDA and 48 ± 6% and 49 ± 5% for glutamate, respectively). No significant differences were observed for data obtained in animals pretreated with 10^{-6} M genistein vs. those pretreated with genistein (10^{-5} M). Similar results were observed in pial arterioles.

3.2. Role of ERK and p38 MAPK activation in impaired NMDA vasodilation after fluid percussion brain injury

Post injury vasoconstriction to NMDA and glutamate was also reversed to vasodilation by pretreatment with either the ERK or p38 MAPK inhibitors U0126 (10^{-6}, 10^{-5} M) or SB203580 (10^{-5}, 10^{-4} M) (Figs. 1 and 2). However, U0126 restored dilation to NMDA and glutamate to a significantly greater extent than SB203580 (Figs. 1 and

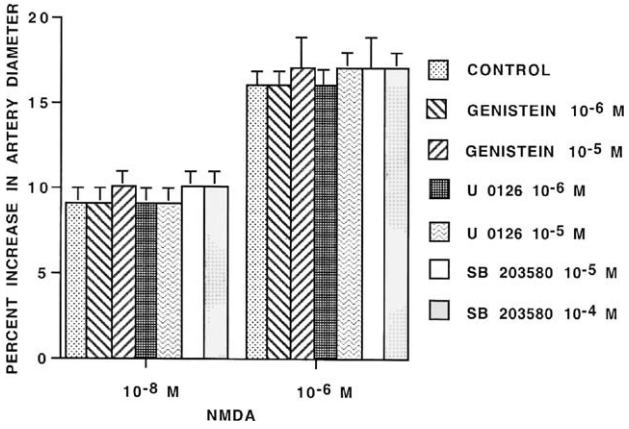

Fig. 3. Influence of NMDA (10^{-8}, 10^{-6} M) on pial small artery diameter before (control), after genistein (10^{-6} M), after genistein (10^{-5} M), after U0126 (10^{-6} M), after U01126 (10^{-5} M), after SB203580 (10^{-5} M) and after SB203580 (10^{-4} M). $n = 6$.

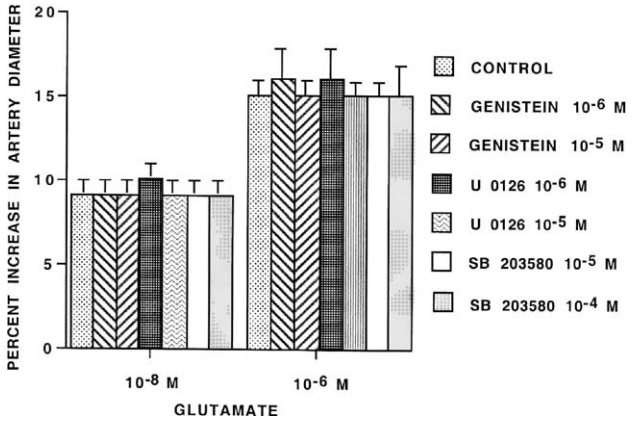

Fig. 4. Influence of glutamate (10^{-8}, 10^{-6} M) on pial small artery diameter before (control), after genistein (10^{-6} M), after genistein (10^{-5} M), after U0126 (10^{-6} M), after U0126 (10^{-5} M), after SB203580 (10^{-5} M) and after SB203580 (10^{-4} M). $n=6$.

2). On a percentage basis, NMDA pial small artery dilation was inhibited by $54 \pm 7\%$ and $55 \pm 6\%$ while that of glutamate inhibited by $43 \pm 11\%$ and $39 \pm 11\%$ in animals pretreated with U0126 (10^{-6} M) . Such values were significantly different from that obtained in animals pretreated with SB203580 (10^{-5} M) ($86 \pm 10\%$ and $96 \pm 15\%$ for NMDA and $87 \pm 12\%$ and $87 \pm 12\%$ for glutamate, respectively). No significant differences in protection of responses were observed between animals pretreated with U0126 10^{-6} M vs. U0126 10^{-5} M or SB203580 10^{-5} M vs. SB203580 10^{-4} M. However, values for both U0126 and SB203580 pretreated animals were significantly different from those obtained in untreated fluid percussion brain injury animals described above. Similar values and statistical differences between pretreated and untreated animals were observed for responses in pial arterioles.

3.3. Influence of PTK, ERK and p38 MAPK inhibitors on pial artery diameter and NMDA vasodilation in sham control animals

Neither genistein, U0126 nor SB203580 had any significant effect on pial artery diameter. Coadministration of either genistein (10^{-6}, 10^{-5} M), U0126 (10^{-6}, 10^{-5} M) or SB203580 (10^{-5}, 10^{-4} M) with NMDA (10^{-8}, 10^{-6} M) or glutamate (10^{-8}, 10^{-6} M) had no effect on pial artery dilation to these excitatory amino acids (Figs. 3 and 4). Similar data were obtained in pial arterioles.

3.4. Blood chemistry and injury intensity level

The arterial blood gas and the pH for the piglets at the beginning and end of the experiment was no different in all experimental groups (e.g., 7.45 ± 0.02, 34 ± 3, 91 ± 7 vs. 7.44 ± 0.02, 32 ± 4, 93 ± 6 mm Hg for pH, P_{CO_2} and P_{O_2}). The injury intensity level was 1.9 ± 0.1 atm.

4. Discussion

Results of the present study show that pial artery dilation in response to NMDA receptor activation and glutamate was reversed to vasoconstriction following fluid percussion brain injury, consistent with previous observations (Armstead, 2000c, 2001a,b; Kulkarni and Armstead, 2000, 2002). New data in this study show that dilation to NMDA and glutamate was partially restored in animals pretreated with genistein, U0126 and SB203580, indicating the role of PTK, ERK and p38 MAPK activation in impaired dilation to these excitatory amino acids post insult. Because the protection with U0126 was significantly greater than that observed with SB203580, these data interestingly suggest that activation of the ERK isoform of MAPK is more important than that of the p38 isoform in such insult associated impairment of excitatory amino acid pial artery dilation. Such differences in the relative role of the kinases probably was not due to insufficient blockade of the respective kinase pathways in that the two concentrations of kinase inhibitors used provided data that was not significantly different from one another regarding protection of responses to the excitatory amino acids post fluid percussion brain injury. Further, vasodilation to NMDA and glutamate was unchanged in the presence of these kinase inhibitors in sham control animals. These data therefore suggest that protection observed post brain injury is not due simply to a nonspecific enhancement of dilation post insult. However, because neither genistein, U0126 nor SB203580 fully restored dilation to NMDA and glutamate after fluid percussion brain injury, these data also suggest that mechanisms in addition to those (JNK MAPK and others) contribute as well. Further, since the protection afforded was approximately equivalent in both pial small arteries and arterioles, these data indicate that there are minimal regional vascular differences in the contributions of these signal transduction pathways in excitatory amino acid dilator impairment. Finally, because these antagonists did not significantly affect pial artery diameter by themselves, these data suggest that PTK, ERK and p38 MAPK activation minimally contribute to basal resting pial artery tone in the piglet.

Previous studies have observed that NMDA induced pial artery dilation was impaired after brain injury in an age dependent manner (Armstead, 2000c, 2001a,b). For example, NMDA was reversed from a vasodilator to a vasoconstrictor within 1 h of fluid percussion brain injury and responses remained impaired for at least 72 h in the newborn pig (Armstead, 2000c). In the juvenile, however, NMDA dilation was only attenuated within 1 h of fluid percussion brain injury and such impairment only lasted for 8 h (Armstead, 2000c). Interestingly, NMDA receptor activation has been observed to contribute to impaired cerebrovascular autoregulation during hypotension after fluid percussion brain injury in age dependent manner (Armstead, 2002). One explanation for this observation could relate to the physiologic antagonism of the modified NMDA vascular activity (conversion to vasoconstrictor) on

hypotension associated pial dilation. The more modest role of NMDA in impaired hypotensive cerebral autoregulation in the juvenile, then, could relate to the similar more modest alteration in NMDA vascular activity by fluid percussion brain injury (less physiologic antagonism) in this age group.

Although the mechanism by which NMDA impairs hypotensive cerebral autoregulation after fluid percussion brain injury is largely unknown, considerably more information is available regarding mechanisms contributory to impaired NMDA dilation after fluid percussion brain injury. For example, FPI releases the opioid nociceptin/orphanin FQ (Armstead, 2000a), which, in turn contributes to the age dependent impairment of NMDA dilation after brain injury (Armstead, 2000c). Generation of superoxide anion (O_2^-) in a cyclooxygenase dependent manner by nociceptin/orphanin FQ has been observed to contribute to impairment of NMDA dilation after fluid percussion brain injury (Kulkarni and Armstead, 2000, 2002). Further, both endothelin and vasopressin contribute to the release of nociceptin/orphanin FQ following fluid percussion brain injury to indirectly impair NMDA dilation after FPI (Armstead, 2001a,b). Alternatively, endothelin and vasopressin may more directly impair such vasodilation post insult via cyclooxygenase and protein kinase C dependent O_2^- generation (Armstead, 1999, 2001a,b). Interestingly, endothelin has also been observed to impair hypotensive cerebral autoregulation after fluid percussion brain injury in an age dependent manner (Armstead, 1999). It is presently uncertain if NMDA might reciprocally affect endothelin dependent mechanisms to contribute to its observed role in impaired hypotensive cerebral autoregulation post injury. Although the above could suggest that PTK and MAPK inhibitors might afford protection by scavenging of O_2^- post fluid percussion brain injury, such does not appear to be the case. For example, using nitroblue tetrazolium reduction as an index of O_2^- generation, it was recently observed that the amount of O_2^- generation was equivalent in untreated and PTK/MAPK inhibitor pretreated animals (Ross and Armstead, in press). These data suggest that the PTK and MAPK inhibitors used in that study did not scavenge oxygen free radicals or inhibit their production.

In addition to being altered following fluid percussion brain injury, it should be noted that other models of cerebral insult also modify the vascular response to NMDA receptor activation. For example, global cerebral ischemia, hypoxia or combined hypoxia/ischemia blunt NMDA induced pial artery dilation (Armstead, 2000b; Busija et al., 1996; Bari et al., 1998). While there may be some similarities regarding mechanisms for NMDA dilator impairment in these various models of cerebral insult (e.g., O_2^-), the role of PTK, ERK and p38 MAPK activation is uncertain.

Previous studies have indicated that traumatic brain injury induces the expression of neurotrophin related mRNA and receptors (Hicks et al., 1999; McKeon et al., 1997; Oyesiku et al., 1999), which subsequently triggers downstream MAPK cascades through interactions with specific high-affinity tyrosine kinase receptors (Bonni et al., 1999). Interestingly, a recent study observed that lateral fluid percussion brain injury in the rat resulted in the bilateral expression of ERK and JNK but not p38 MAPK, perhaps due to insult associated release of glutamate (Otani et al., 2002a,b). Such results are consistent with the more robust protection of NMDA dilation after fluid percussion brain injury with the ERK inhibitor U0126 vs. that observed with the p38 inhibitor SB203580 observed in the present study. Further, results of the present study are the first to correlate such previously observed expression after fluid percussion brain injury with impaired vascular activity to a stimulus such as NMDA receptor activation.

Several studies have suggested the activation of JNK and p38 cascades to induce neuronal injury following middle cerebral artery occlusion (Hayashi et al., 2000) and spinal cord injury (Nakahara et al., 1999). In global cerebral ischemia, ERK has been associated with neuroprotection (Hu et al., 2000). Alternatively, sustained activation of ERK could be deleterious as inhibition of ERK reduced injury in a model of focal ischemic injury (Alessandrini et al., 1999). The mechanism(s) that mediate these deleterious effects of ERK remain to be fully characterized but could relate to amplification of excitotoxic glutamate release after brain injury (Alessandrini et al., 1999).

A caveat of the pharmacologic approach utilized in the present study relates to efficacy and specificity of the antagonists for PTK and MAPK. Since data from the present study are similar to that of a previous one in which a higher concentration of genistein or U0126 (e.g., 10^{-5} M) did not have any further protective effect in another cerebral injury model (hypoxia/ischemia) (Jagolino and Armstead, 2003) these data suggest that the lower concentration (10^{-6} M) used in this study was near maximally efficacious in the inhibition of PTK and ERK MAPK respectively. Similarly, in vitro dose response data indicate that SB203580 (10^{-5} M) was maximally efficacious (Bolla et al., 2002). Regarding selectivity, other studies point towards the concentration used in the present study for genistein and U0126 as being quite selective for PTK and ERK MAPK, respectively (Kim et al., 1998; Namura et al., 2001), although SB203580 may have some interaction with the JNK pathway, depending on dose used (Barone et al., 2001).

In conclusion, results of the present study show that PTK, ERK and p38 MAPK activation contribute to impaired NMDA cerebrovasodilation after fluid percussion brain injury. These data suggest that the activation of the ERK isoform of MAPK contributes to such impairment more than the p38 MAPK isoform.

Acknowledgements

I would like to thank John Ross for excellent technical assistance in the performance of the experiments. This research was supported by grants from the National Institute

of Health, the AHA-PA, DE Affiliate and the University of Pennsylvania Research Foundation.

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