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Endothelin and the neurovascular unit in pediatric traumatic brain injury

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Abstract

Objective—This study characterized the association between endothelin-1, cerebral hemodynamics, and histopathology after fluid percussion brain injury in the newborn pig.

Methods—Lateral fluid percussion injury was induced in newborn pigs equipped with a closed cranial window. Cerebral blood flow was determined with radiolabeled microspheres and cerebrospinal fluid endothelin-1 was measured by radioimmunoassay.

Results—Cerebrospinal fluid endothelin-1 was increased from 26 ± 4 to 296 ± 37 pg/ml ($\sim 10^{-10}$ M) at 8 hours following fluid percussion injury. Post-injury treatment (30 minutes) with the endothelin-1 antagonist BQ-123 (1 mg/kg, intravenous) blocked pial artery vasoconstriction to topical endothelin-1 ($\sim 10^{-10}$ M) and blunted fluid percussion injury-induced reductions in cerebral blood flow at 8 hours post-insult (56 ± 6 and 26 ± 4 ml/minute versus 57 ± 6 and 40 ± 4 ml/minute; 100 g for cerebral blood flow before injury and 8 hours post-fluid percussion injury in vehicle and BQ-123 post-treated animals, respectively). Fluid percussion injury resulted in neuronal cell loss and decreased microtubule associated protein 2 immunoreactivity in the parietal cortex, which were blunted by BQ-123.

Discussion—These data indicate that fluid percussion injury-induced changes in cerebral hemodynamics are associated with neuronal damage and that endothelin-1 contributes to fluid percussion injury-induced histopathologic changes.

Keywords

Cerebral hemodynamics; Pediatric traumatic brain injury; Endothelin; Histopathology

Introduction

Traumatic brain injury is the most common cause of brain injury and the leading cause of death and disability in children.¹ While the effects of traumatic brain injury have been extensively investigated in models involving adult animals,² less is known about traumatic brain injury in the newborn/infant. Traumatic brain injury can cause uncoupling of blood flow and metabolism, resulting in cerebral ischemia or hyperemia.³ Although cerebral hyperemia was historically considered the cause of diffuse brain swelling after traumatic brain injury in the pediatric setting,⁴ recent evidence suggests that cerebral hypoperfusion is

the dominant derangement.⁵ Decreases in cerebral blood flow and pial artery diameter, along with impaired vasodilator responsiveness are greater in newborn than in juvenile pigs after fluid percussion brain injury,⁶ a model of concussive head injury.⁷ These data support the potential importance of hypoperfusion in post-traumatic outcome and the concept that, in contrast to the juvenile pig, cerebral hemodynamics in the newborn pig is more sensitive to brain injury.⁶ Piglets offer the unique advantage in elucidating these pathways by virtue of having a gyrencephalic brain that contains substantial white matter, which is more sensitive to ischemic/traumatic brain injury damage than grey matter, similar to humans.

The neurovascular unit, which focuses attention on the dynamic interactions between cerebral blood vessels and the neurons they serve, has been recognized as being important in achieving an integrated response to central nervous system ischemic injury, such as traumatic brain injury.⁸ An important goal of this research area is an examination of the genes and proteins that are uniquely expressed by the neurovascular unit and how such profiles change under pathologic conditions. Identification of a pattern specific expression within the neurovascular unit following fluid percussion injury may lend insight into the contributing factors in the loss of cerebral hemodynamic control post-insult. Dysregulation of cerebral blood flow is thought to contribute to neurologic outcome and therefore often is an important therapeutic target.

Microtubule associated protein 2 is an abundant neuronal cytoskeletal protein that is localized almost exclusively in dendrites and perikarya.⁹ It is thought to be important for microtubule stabilization and cytoarchitecture of neurons.^{9,10} Numerous studies of ischemia and fluid percussion injury have used a loss of microtubule associated protein 2 immunoreactivity as a sensitive marker of neuronal damage.^{11–13}

Endothelin-1 is a 21 amino acid peptide with potent vasoconstrictor properties.¹⁴ Our previous studies have shown that endothelin-1 is released following fluid percussion injury and contributes to age dependent cerebral hemodynamic impairment in the pig.¹⁵ For example, cerebral blood flow is reduced following fluid percussion injury, while the endothelin-1 antagonist BQ-123 ameliorated reductions in cerebral blood flow in an age dependent manner.¹⁵ Because neuronal cell death underlies ischemic brain injury, this study was designed to characterize the association between endothelin-1, cerebral hemodynamics, and acute neuronal damage after fluid percussion injury in the newborn pig.

Materials and Methods

Newborn pigs (1–5 days old, 1.2–1.6 kg) of either sex were used in these experiments. All protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Animals were sedated with isoflurane (1–2 minimum alveolar concentration). Anesthesia was maintained with alpha-chloralose [30–50 mg/kg supplemented with 5 mg/kg/hour, intravenous (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 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 the anesthetized animals. This window consisted of three parts: (1) a stainless steel ring, (2) a circular glass coverslip, and (3) 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 cerebrospinal fluid, 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 cerebrospinal fluid was warmed to 37°C and had the following chemistry: pH 7.33, 46 mm Hg p_{CO_2} , and 43 mm Hg p_{O_2} which was similar to that of endogenous cerebrospinal fluid. Pial arterial vessel diameter was measured with a microscope, a camera, a video output screen and a video microscaler.

Cerebral blood flow was measured in the cerebral cortex using radioactively labeled microspheres.¹⁵ Briefly, a known amount of radioactivity in 15- μm microspheres (300 000–800 000 spheres) was injected into the left ventricle and the injection line flushed with 1 ml of saline. Withdrawal of reference blood samples was begun 15 seconds before microsphere injection and continued for 2 minutes after the injection. The reference withdrawal rate was 1.03 ml/minute. After each experiment, the pig was terminated and the brain was removed and weighed. Cerebral blood flow was determined by counting cerebral cortex brain tissue samples in a gamma counter. The energy from each nuclide was separated by differential spectroscopy. Aliquots of the actual microsphere solutions injected were used for overlap calculations. The count in each milliliter per minute of blood flow was determined by dividing the counts in the reference withdrawal by the rate of reference withdrawal. Thus blood flow can be calculated as $Q = C \times R \times CR^{-1}$, where Q is brain blood flow (in ml/minute), C is counts per minute (in cpm) in the tissue sample, R is the rate of withdrawal of reference blood sample (in ml/minute), and CR is the total counts in the reference blood sample. Cerebral blood flow so determined reflects flow to the cerebral cortex both ipsilateral and contralateral to the injury site.

Methods for brain fluid percussion injury have been described previously.^{2,6} A device designed by the Medical College of Virginia was used. A small opening was made in the parietal bone contralateral to the cranial window. A metal shaft was sealed into the opening on the 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 injury was induced by striking the piston with a 4.8 kg pendulum. The intensity of the injury (1.9–2.3 atm with a constant duration of 19–23 milliseconds) 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 μm) and arterioles (resting diameter: 50–70 μm) were examined to determine whether segmental differences in the effects of fluid percussion injury could be identified. Typically, 2–3 ml of artificial cerebrospinal fluid was flushed through the window over a 30-second period, and excess cerebrospinal fluid was allowed to run off through one of the needle ports. For sample collection, 300 μl of the total cranial window volume of 500 μl was collected by slowly infusing artificial cerebrospinal fluid into one side of the window and allowing the cerebrospinal fluid to drip freely into a collection tube on the opposite side.

Three types of experiments were performed (all $n=5$): (1) sham control, (2) fluid percussion injury treated with vehicle (saline), and (3) fluid percussion injury treated with BQ-123 (1 mg/kg, i.v.). Vehicle or BQ-123 was administered 30 minutes post-fluid percussion injury. Topical endothelin-1 (10^{-10}M) was administered before and at 8 hours post-fluid percussion

injury. The percentage changes in artery diameter value were calculated based on the diameter in the control period before endothelin-1 administration before and after fluid percussion injury to normalize for brain injury-induced vasoconstriction.

Tissue Fixation, Histology, and Immunohistochemistry

After termination, the brains were perfused via the carotid artery with 800 ml of heparinized saline followed by 800 ml of 4% paraformaldehyde in phosphate buffered saline. The brains were then removed and post-fixed in 4% paraformaldehyde for 24 hours. The brains were blocked into 0.5 cm coronal sections for gross examination. All blocks were cryoprotected in sucrose and frozen in isopentane at -30°C . Frozen sections of $40\text{ }\mu\text{m}$ were obtained from the front face of each block and divided as follows: one series of sections was mounted on microscope slides and stained with cresyl violet, while an adjacent series was evaluated for microtubule associated protein 2 immunoreactivity (clone AP-20; Sigma Chemical Co., St Louis, MO, USA) using previously published techniques.¹³ Briefly, tissue sections were washed in tris buffered saline pH 7.4 containing 0.1% Triton X-100, blocked with 5% normal horse serum, and incubated with anti-microtubule associated protein 2 antibody at 4°C overnight. Tissues were then washed with Triton X-100 and incubated with biotinylated donkey anti-mouse immunoglobulin G (1:1000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2 hours. The antigen antibody complex was visualized using the ABC technique (Vector Laboratories, Inc., Burlingame, CA, USA) with nickel-enhanced 3,3'-diaminobenzidine as the chromogen.

Endothelin-1 Analysis

Commercially available kits (Peninsula Laboratories, Inc., Belmont, CA, USA) were used to determine the concentration of endothelin-1 in cerebrospinal fluid.¹⁵ All samples and standards were assayed in duplicate. All experimental values fell within the linear range of the assay.

Statistical Analysis

Pial artery diameter, cerebral blood flow, and endothelin-1 levels were analyzed using analysis of variance for repeated measures. If the F value was significant, the data were then analyzed by Fisher's protected least significant difference test. An alpha 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

Cortical periarachnoid cerebrospinal fluid endothelin-1 concentration was increased within 8 hours of fluid percussion injury (Fig. 1A). Topical administration of endothelin-1 (10^{-10}M), which is approximately the concentration observed in cerebrospinal fluid after fluid percussion injury, elicited reproducible pial small artery and arteriole vasoconstriction in sham control piglets. Endothelin-1 induced vasoconstriction was potentiated after fluid percussion injury, but blocked by BQ-123 (1 mg/kg, i.v.; Fig. 1B). Fluid percussion injury produced a decrease in cerebral blood flow which was blunted by BQ-123 post-treatment (Fig. 1C). BQ-123 had no effect on baseline cerebral blood flow before fluid percussion injury.

Figure 2 illustrates neuronal staining of the parietal cortex with either cresyl violet (top left panel) or microtubule associated protein 2 (top right panel) in a representative animal from the uninjured vehicle treated group (group 1). At 8 hours following fluid percussion injury (group 2 animals), a loss of cresyl violet staining, suggestive of neuronal loss, was observed (middle left panel). Loss of neuronal staining was equivalent bilaterally and was

accompanied by the appearance of glial and/or inflammatory cells characterized by small intensely stained cell soma. A similar loss of dendritic microtubule associated protein 2 staining was visible bilaterally (middle right panel). In addition, a few darkly stained neurons and punctate labeling of microtubule associated protein 2 were visible, indicative of neuronal degeneration. In animals post-treated with BQ-123 (group 3), a neuronal cell loss was markedly attenuated bilaterally (bottom left panel). Similarly, the cortex was remarkably unchanged in microtubule associated protein 2 immunoreactivity (bottom right panel) compared to uninjured piglets (top right panel).

Values for physiological variables were obtained at the beginning, the end, and at 8 hours post-fluid percussion injury. These data show that blood chemistry (pH, $p\text{CO}_2$, and $p\text{O}_2$) was not different at the end compared to the beginning of the experiment. There were no group differences in blood chemistry or mean level of fluid percussion injury (2.1 ± 0.1 atm).

Discussion

Results of the present study show that there is a marked loss of cortical neurons following fluid percussion injury in the newborn pig using cresyl violet and microtubule associated protein 2 as indices of histopathology. The loss of microtubule associated protein 2 appears to be concurrent with neuronal degeneration. Because administration of the endothelin-1 antagonist BQ-123 blunted the injury induced histopathologic changes, these data indicate that endothelin-1 contributes to cerebral cortical neuropathologic alterations. Cresyl violet staining has previously been used to reveal pyknosis and neuronal cell loss after traumatic brain injury in the adult pig,¹⁶ while microtubule associated protein 2 has served as an index of neuronal damage after fluid percussion injury in the adult rat.¹³ Results of the present study are the first to show that endothelin-1 contributes to neuronal cell degeneration and loss of microtubule associated protein 2 immunoreactivity following fluid percussion injury in the newborn pig. Nonetheless, a limitation of the present study is that histopathologic data are qualitative and not quantitative in nature.

The present study further shows that fluid percussion injury induced histopathology was associated with elevated cortical periarachnoid cerebrospinal fluid endothelin-1 concentration.¹⁵ The present experimental design does not allow for conclusion to be drawn regarding the cellular site of origin for endothelin-1 detected in cerebrospinal fluid. Potential sites include neurons, glia, vascular smooth muscle, and endothelial cells. Using a weight drop traumatic brain injury model in the rat, endothelin-1 mRNA expression was enhanced post-insult in endothelial, smooth muscle cells, and pericytes/macrophages of the hippocampus and cerebral cortex.¹⁷ In our study, systemically administered BQ-123 blocked vasoconstriction to topical exogenous endothelin-1, indicating that this antagonist was able to cross the blood brain barrier in a sufficient quantity to block responses to the respective agonist. These data strengthen conclusions regarding the contribution of endogenously released endothelin-1 to fluid percussion injury associated histopathology. For example, in animals given BQ-123, there was less histopathology. The observation that greater reductions in cerebral blood flow in vehicle treated animals were associated with greater histopathology than the corresponding changes in histopathology in BQ-123 treated animals where there was a less reduction in cerebral blood flow is supportive of the dynamic interaction between cerebral hemodynamics and neuronal cell integrity in the context of the neurovascular unit, and the role of endothelin-1 as a mediator in that dynamic relationship. Since calpain has been implicated in the development of vasospasm and ischemic cell death,^{18,19} mechanisms which activate calpain could link altered cerebral hemodynamics to neuronal cell loss. Endothelin-1 elevates intracellular calcium concentration, which may activate calpain²⁰. Therefore, endothelin-1 induced calpain activation may link cerebral hemodynamic changes after fluid percussion injury to histopathology.

Though endothelin-1 appears to be an important candidate linking reductions in cerebral blood flow to histopathology after fluid percussion injury in the newborn pig, it should be noted that some of our other recent studies have implicated additional potential mediators in this role in the context of the neurovascular unit. For example, endogenous plasminogen activators such as tissue plasminogen activator and urokinase plasminogen activator are upregulated in brain parenchyma and cerebrospinal fluid and contribute to hypoperfusion and neuronal cell loss after fluid percussion injury in the piglet.^{21,22} Alternatively, diminished upregulation of endogenous cerebrovasodilator peptides such as adrenomedullin has been shown to contribute to fluid percussion injury associated hypoperfusion and histopathology in a cause–effect relationship.²³ The latter studies support the generalization of the neurovascular unit concept that cerebral hemodynamics influences histopathologic outcome after traumatic brain injury.

In conclusion, results of the present study indicate that fluid percussion injury induced changes in cerebral hemodynamics are associated with neuronal damage and that endothelin-1 contributes to fluid percussion injury induced histopathologic changes.

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References

- Rodriguez JG. Childhood injuries in the United States. A priority issue. *Am J Dis Child*. 1990; 144:625–6. [PubMed: 2346145]
- Wei EP, Dietrich WD, Povlishock JT, Navari RM, Kontos HA. Functional, morphological, and metabolic abnormalities of the cerebral microcirculation after concussive brain injury in cats. *Circ Res*. 1980; 46:37–47. [PubMed: 7349916]
- Richards HK, Simac S, Piechnik S, Pickard JD. Uncoupling of cerebral blood flow and metabolism after cerebral contusion in the rat. *J Cereb Blood Flow Metab*. 2001; 21:779–81. [PubMed: 11435789]
- Bruce DA, Alavi A, Bilaniuk L, Dolinskas C, Obrist W, Uzzell B. Diffuse cerebral swelling following head injuries in children: the syndrome of ‘malignant brain edema’. *J Neurosurg*. 1981; 54:170–8. [PubMed: 7452330]
- Adelson PD, Clyde B, Kochanek PM, Wisniewski SR, Marion DW, Yonas H. Cerebrovascular response in infants and young children following severe traumatic brain injury: a preliminary report. *Pediatr Neurosurg*. 1997; 26:200–7. [PubMed: 9436831]
- Armstead WM, Kurth CD. Different cerebral hemodynamic responses following fluid percussion brain injury in the newborn and juvenile pig. *J Neurotrauma*. 1994; 11:487–97. [PubMed: 7861442]
- Gennarelli TA. Animate models of human head injury. *J Neurotrauma*. 1994; 11:357–68. [PubMed: 7837277]
- Lo EH, Broderick JP, Moskowitz MA. tPA and proteolysis in the neurovascular unit. *Stroke*. 2004; 35:354–6. [PubMed: 14757877]
- De Camilli P, Miller PE, Navone F, Theurkauf WE, Vallee RB. Distribution of microtubule associated protein 2 (MAP2) in the nervous system of the cat studied by immunofluorescence. *Neuroscience*. 1984; 11:819–46.
- Friedrich P, Aszodi A. MAP2: a sensitive crosslinker and spacer in dendritic architecture. *FEBS Lett*. 1991; 295:5–9. [PubMed: 1765166]
- Matesic DF, Lin CS. Microtubule associated protein 2 as an early indication of ischemia induced neurodegeneration in the gerbil forebrain. *J Neurochem*. 1994; 63:1012–20. [PubMed: 8051544]
- Miyazawa T, Bonnekoh P, Hossman KA. Temperature effect on immunostaining of micro-tubule associated protein 2 and synaptophysin after 30 min of forebrain ischemia in rats. *Acta Neuropathol*. 1993; 85:526–32. [PubMed: 8493860]

13. Hicks RR, Smith DH, McIntosh TK. Temporal response and effects of excitatory amino acid antagonism on microtubule associated protein 2 immunoreactivity following experimental brain injury in rats. *Brain Res.* 1995; 678:151–60. [PubMed: 7620884]
14. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature.* 1988; 332:411–5. [PubMed: 2451132]
15. Armstead WM. Role of endothelin-1 in age dependent cerebrovascular hypotensive responses after brain injury. *Am J Physiol.* 1999; 274:H1884–94. [PubMed: 10564144]
16. Smith DH, Chen XH, Xu BN, McIntosh TK, Gennarelli TA, Meaney DF. Characterization of diffuse axonal pathology and selective hippocampal damage following inertial brain trauma in the pig. *J Neuropathol Exp Neurol.* 1997; 56:822–34. [PubMed: 9210879]
17. Rafols D, Steiner J, Rafols JA, Petrov T. Intracellular coexpression of endothelin-1 and inducible nitric oxide synthase underlies hypoperfusion after traumatic brain injury in the rat. *Neurosci Lett.* 2004; 362:154–7. [PubMed: 15193775]
18. Yamaura I, Tami E, Saido TC, Suzuki K, Minami N, Maeda Y. Calpain-calpastatin system of canine basilar artery in vasospasm. *J Neurosurg.* 1993; 79:537–43. [PubMed: 8410223]
19. Markgraf CG, Velayo NL, Johnson MP, McCarty DR, Medhi S, Koehl JR, et al. Six hour window of opportunity for calpain inhibition in local ischemia in rats. *Stroke.* 1998; 29:152–8. [PubMed: 9445345]
20. Simonson MS, Dunn ML. Cellular signaling by peptides of the endothelin gene family. *FASEB J.* 1990; 4:2989–3000. [PubMed: 2168326]
21. Armstead WM, Nassar T, Akkawi S, Smith DH, Chen XH, Cines DB, et al. Neutralizing the neurotoxic effects of exogenous and endogenous tPA. *Nat Neurosci.* 2006; 9:1150–5. [PubMed: 16936723]
22. Armstead WM, Cines DB, Bdeir KH, Bdeir Y, Stein SC, Higazi AA. uPA modulates the age dependent effect of brain injury on cerebral hemodynamics through LRP and ERK MAPK. *J Cereb Blood Flow Metab.* 2009; 29:524–33. [PubMed: 19050721]
23. Armstead WM, Kiessling JW, Bdeir K, Kofke WA, Vavilala MS. Adrenomedullin prevents sex dependent impairment of autoregulation during hypotension after piglet brain injury through inhibition of ERK MAPK upregulation. *J Neurotrauma.* 2010; 27:391–402. [PubMed: 20170313]

Figure 1.

(A) Influence of fluid percussion injury (fluid percussion injury) on cerebral spinal fluid endothelin-1 ($n=5$). (B) Influence of endothelin-1 (10^{-10} M) on pial artery diameter before (control) and after fluid percussion injury, and after fluid percussion injury in BQ-123 (1 mg/kg, i.v.) post-treated animals ($n=5$). (C) Influence of fluid percussion injury on cerebral blood flow (ml/minute, 100 g) in the absence and presence of BQ-123 ($n=5$). * $P<0.05$ compared to control value; + $P<0.05$ compared to vehicle value.

Figure 2. Cerebral cortex stained with either cresyl violet (left panels) or microtubule associated protein 2 (right panels). Conditions are: sham (top panels), fluid percussion injury (middle panels), and fluid percussion injury and BQ-123 (1 mg/kg, i.v.) post-treated (bottom panels).