Adrenomedullin Prevents Sex-Dependent Impairment of Autoregulation during Hypotension after Piglet Brain Injury through Inhibition of ERK MAPK Upregulation

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Abstract

Cerebrospinal fluid (CSF) adrenomedullin (ADM) levels are increased in female, but remain unchanged in male, piglets after fluid percussion injury (FPI) of the brain. Subthreshold vascular concentrations of ADM restore impaired hypotensive pial artery dilation after FPI more in males than females. Extracellular signal-related kinase (ERK) mitogen-activated protein kinase (MAPK) is upregulated and contributes to reductions in cerebral blood flow (CBF) after FPI. We hypothesized that ADM prevents sex-dependent impairment of autoregulation during hypotension after FPI through inhibition of ERK MAPK upregulation. FPI increased ERK MAPK more in males than in females. CBF was unchanged during hypotension in sham animals, was reduced more in males than in females after FPI during normotension, and was further reduced in males than in females during hypotension and after FPI. ADM and the ERK MAPK antagonist U 0126 prevented reductions in CBF during hypotension and FPI more in males than in females. Transcranial Doppler (TCD) blood flow velocity was unchanged during hypotension in sham animals, was decreased during hypotension and FPI in male but not in female pigs, and was ameliorated by ADM. Intracranial pressure (ICP) was increased after FPI more in male than in female animals. ADM blunted elevated ICP during FPI and hypotension in males, but not in females. ADM prevented reductions in cerebral perfusion pressure (CPP) during FPI and hypotension in males but not in females. The calculated autoregulatory index was unchanged during hypotension in sham animals, but was reduced more in males than females during hypotension and FPI. ADM prevented reductions in autoregulation during hypotension and FPI more in males than females. These data indicate that ADM prevented loss of cerebral autoregulation after FPI in a sex-dependent and ERK MAPK-dependent manner.

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

Introduction

PEDIATRIC TRAUMATIC BRAIN INJURY (pTBI) is a global public health concern (Langlois et al., 2005; Newacheck et al., 2004). Boys are disproportionately affected and young children sometimes have devastating outcomes (Langlois et al., 2005). Hypotension is common and worsens outcome after TBI (Coates et al., 2005). Hypotension can lead to cerebral ischemia when cerebral autoregulation is impaired. However, empirically increasing blood pressure after TBI may potentially cause harm if cerebral hyperemia is present and cerebral autoregulation is impaired. Consequently, improving cerebral autoregulation may be critical to preventing cerebral ischemia during hypotension. Preventing cerebral hyperemia increases

blood pressure and improves outcome (Tsuji et al., 1998). Since ethical constraints preclude mechanistic studies of cerebral autoregulation in children, we used an established porcine model of fluid percussion injury (FPI) that mimics many of the pathophysiological features of pTBI to corroborate clinical findings after pTBI (Armstead, 2000; Dickerson and Dobbing, 1967). Piglets offer the unique advantage of a gyrencephalic brain containing substantial white matter, which is more sensitive to ischemic/TBI damage, and is thus similar to human brain. Our data suggest that the newborn pig is more cerebrohemodynamically sensitive to FPI than juvenile pigs, and impaired autoregulation post-insult may be caused by an age-dependent decrease in the production of calcitonin gene-related peptide (CGRP) (Armstead, 2000).

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Adrenomedullin (ADM) is a 52-amino-acid peptide belonging to the CGRP family. In rats, ADM mRNA expression is upregulated after ischemia and may be cerebroprotective, particularly after stroke (Miyashita et al., 2006; Wang et al., 1995). ADM increases cerebral blood flow (CBF) and prevents ischemia after middle cerebral artery occlusion (Dogan et al., 1997). Marked increases in cerebrospinal fluid (CSF) ADM levels in children occur after severe TBI, and CBF was positively correlated with CSF ADM (Robertson et al., 2001), suggesting that ADM may be neuroprotective and participate in the regulation of CBF after TBI (Baskaya et al., 1995; Juhl et al., 2006). However, the correlation of CSF ADM with CBF in the setting of pTBI was not considered in terms of gender.

Adult data suggest a neuroprotective role of ovarian hormones in females after stroke and TBI (Alkayed et al., 1998; Davis et al., 2006; O'Connor et al., 2005), but little is known about them in pTBI. Of the three published studies (Donders and Woodward, 2003; Donders and Hoffman, 2002; Morrison et al., 2004), in the last two reports the authors wrote that male gender is a risk factor for reduced speed and efficiency of information processing after pTBI. Male neonatal cortical astrocytes are more sensitive to oxygen-glucose deprivation than female cells (Du et al., 2004), suggesting sex differences in sensitivity to ischemia (Liu et al., 2007). We reported lower CSF ADM levels and more blunted pial artery dilation during hypotension in male than in female pigs after FPI (Armstead and Vavilala, 2007). We also demonstrated the ability of subthreshold vascular concentrations of ADM to restore impaired hypotensive cerebrovasodilation after FPI in a sex-dependent manner. However, the mechanism of ADM-mediated cerebroprotection was not explored.

Mitogen-activated protein kinase (MAPK) is part of a family of at least three kinases (extracellular signal-related kinase [ERK], p38, and c-Jun N-terminal kinase [JNK]) (Laher and Zhang, 2001). After FPI, ERK MAPK is upregulated and contributes to reductions in CBF and impaired dilation by potassium channel agonists such as CGRP (Armstead et al., 2009; Ross and Armstead, 2003). Therefore, we explored the hypothesis that ADM prevents the sex-dependent impairment of autoregulation during hypotension after FPI through inhibition of ERK MAPK upregulation.

Methods

Closed cranial window and brain injury procedures

Newborn pigs (1–5 days old, weighing 1.0–1.4 kg) of both genders were studied. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. The animals were sedated with isoflurane (1–2 MAC). Anesthesia was maintained with α -chloralose (30–50 mg/kg supplemented with 5 mg/kg/h IV). A catheter was inserted into a femoral artery to monitor blood pressure and to sample blood gas tension 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 as monitored rectally.

A cranial window was placed in the parietal skull of the anesthetized animals. This window consisted of three parts: a stainless steel ring, a circular glass cover-slip, 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 CSF, of the following composition (all 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, Pco₂ 46 mm Hg, and Po₂ 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.

CBF was measured in the cerebral cortex using radioactively-labeled microspheres, as previously detailed (Armstead, 1999). Briefly, a known amount of radioactivity in 15- μ m 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 sec before microsphere injection and continued for 2 min after the injection. The reference withdrawal rate was 1.03 mL/min. After each experiment, the pig was sacrificed and the brain removed and weighed. CBF 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/min), C is counts per minute (cpm) in the tissue sample, R is the rate of withdrawal of the reference blood sample (in mL/min), and CR is the total counts in the reference blood sample. CBF so determined reflects flow to the cerebral cortex and hippocampus both ipsilateral and contralateral to the injury site.

The method used to induce brain FPI has 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 the 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, and 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.

Six experimental groups were studied: (1) sham control, (2) FPI, vehicle pre-treated, (3) FPI pre-treated with ADM (0.1 mg/kg IV), (4) FPI pre-treated with the putative ADM antagonist ADM 22-52 (10^{-7} M), (5) FPI pre-treated with U 0126 (1 mg/kg IV), and (6) FPI post-treated with ADM. Hypotension was induced by the rapid withdrawal of either 5–8 or 10–15 mL of blood/kg to induce moderate or severe hypotension (decreases in mean arterial blood pressure of 25% and 45%, respectively) (Armstead, 1999). These decreases in blood pressure were maintained constant for 10 min by titration of additional blood withdrawal or blood reinfusion (Armstead, 1999). The vehicle for all agents was 0.9% saline, except for the ADM antagonist, for which we used dimethyl sulfoxide (100 μ L) diluted with 9.9 mL 0.9% saline. In sham control animals, responses to hypotension (moderate and severe) and papaverine (10^{-8} and 10^{-6} M) were obtained initially and then again 1 h and 4 h later in the presence of the agent vehicle. In drug-treated FPI animals, ADM, ADM 22-52, or U 0126 was administered 30 min before FPI, and the responses to hypotension and papaverine were obtained at 1 h and 4 h after injury. In a subset of animals ADM was administered 30 min after FPI (post-treatment). The order of agonist administration was randomized within the drug treatment groups. Vasodilator responses to ADM (10^{-9} or 10^{-7} M) were also obtained in the absence and presence of ADM 22-52 (10^{-7} M).

Determination of autoregulatory index (ARI) via transcranial Doppler (TCD) ultrasonography

A Spencer Technologies PMD-100 TCD monitor (Spencer Technologies, Seattle, WA) was used to insonate the cerebral vessels prior to and after FPI in the absence and presence of induced hypotension, and to measure the middle cerebral artery blood velocity (V_{mca}) and calculate the autoregulatory index (ARI). By definition, $ARI = \% \Delta CVR / \% \Delta CPP$, where cerebrovascular resistance, $CVR \approx V_{\text{cerebral blood vessel}} / CPP$, or cerebral perfusion pressure, which is mean arterial pressure (MAP) – ICP (intracranial pressure). It is inferred that $ARI < 0.04$ indicates impaired cerebral autoregulation, $ARI > 0.4$ indicates intact cerebral autoregulation, $ARI = 0$ indicates absent autoregulation, and $ARI = 1.0$ reflects perfect autoregulation. An Integra Camino monitor (Integra Neurosciences, Plainsboro, NJ) was used to measure ICP, and ARI was calculated using CPP in all animals. We placed the TCD probe in the posterior portion of the cortex.

Enzyme-linked immunosorbent assay (ELISA)

Commercially available ELISA kits were used to quantify CSF ADM and ERK MAPK (Assay Designs, Ann Arbor, MI) concentrations. Phosphorylated ERK MAPK enzyme values were normalized to the total ERK MAPK form value, and expressed as percentage of the control condition.

Immunohistochemistry

Four hours after FPI, the animal was sacrificed and two thin slices of parietal cortex were cut parallel to the brain surface.

These slices were placed in 4% paraformaldehyde for 24 h at 4°C, and then were subjected to cryo-sectioning. Sections of the parietal cortex from piglet brains after FPI and from uninjured control pigs were fixed with methanol for 10 min, incubated in citrate buffer (pH 6.0) at 90°C for 15 min for antigen unmasking, endogenous peroxidase was blocked with 0.3% H_2O_2 , and the sections were stained with anti-phospho-ERK rabbit monoclonal antibody (2 μ g/mL, #4376; Cell Signaling, Belmont, CA), or with rabbit anti-ADM human serum (diluted 1:5000, #H010-01; Phoenix Pharmaceuticals, Billerica, MA), with mouse anti-neuronal nuclei monoclonal antibody (NeuN; 5 μ g/mL) as a neuronal marker (#MAB377; Chemicon International, CITY?, STATE?), or with mouse IgG₁ as a negative control. This was followed by secondary biotinylated anti-mouse or anti-rabbit IgG (1:200), and by incubation with HRP-conjugated streptavidin. Peroxidase was detected using the avidin-biotin complex ABC Kit (Vector Laboratories, Burlingame, CA) counterstained with hematoxylin. Positive staining was visualized by the brown-colored 3,3-diaminobenzidine (DAB) reaction product.

Statistical analysis

Pial artery diameter, CBF, and CSF ERK MAPK isoform values were analyzed using ANOVA for repeated measures. If the value was significant, the data were then analyzed using Fisher's 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 values.

Results

FPI upregulates cerebral cortex ADM and ERK MAPK in a sex-dependent manner in the pig

Figure 1 shows immunocytochemical data demonstrating the presence of ADM in newborn male pigs (A), and little to no upregulation after FPI (1.9 ± 0.1 atm) (B). NeuN staining identified neurons (C) after FPI in a newborn female, along with an upregulation of ADM. However, these data cannot distinguish between the possibility that neurons serve as the cellular site of origin for ADM, or whether it just binds to these cells. In the female pig cerebral cortex at baseline (sham control) (D), there was somewhat more staining for ADM compared to the male, but a robust elevation in the amount of this antigen was seen after FPI (1.9 ± 0.1 atm) (C and E). Panel F serves as a negative control, in that there was minimal IgG staining. ERK MAPK staining was detectable at baseline (G), and robustly increased after FPI in the male (H). In contrast, ADM (0.1 mg/kg IV) pre-treatment blunted ERK MAPK upregulation after FPI, essentially back to sham control levels, in male pigs (I). ERK MAPK staining was similarly detectable in sham females (J), but upregulation was more modest in females after FPI (K), compared to males after FPI (H). ADM (0.1 mg/kg IV) pre-treatment similarly blunted ERK MAPK upregulation after FPI in female pigs (L), compared to female FPI pigs treated with vehicle (K).

FPI produces phosphorylation of CSF ERK MAPK, which is blunted by ADM in a sex-dependent manner

Total and phosphorylated (activated) ERK MAPK in cortical periarachnoid CSF was determined prior to FPI (0 time),

FIG. 1. Typical sections of the parietal cortex from sham control, FPI, and FPI + ADM-treated newborn male and female piglets. (A) Uninjured sham control newborn male treated with vehicle and stained with rabbit anti-ADM human serum. (B) FPI newborn male treated with vehicle and stained with rabbit anti-ADM. (C) FPI newborn female treated with vehicle and stained with mouse anti-neuronal nuclei monoclonal antibody ($5 \mu\text{g/mL}$) as a neuronal marker along with rabbit anti-ADM. (D) Uninjured sham control newborn female treated with vehicle and stained with rabbit anti-ADM. (E) FPI newborn female treated with vehicle and stained with rabbit anti-ADM. (F) FPI newborn female vehicle stained with mouse IgG as a negative control. (G) Sham control newborn male treated with vehicle and stained with anti-phospho-ERK rabbit monoclonal antibody ($2 \mu\text{g/mL}$). (H) FPI newborn male vehicle-treated stained with anti-phospho-ERK rabbit monoclonal antibody. (I) FPI newborn male ADM (0.1 mg/kg IV) treated and stained with anti-phospho-ERK rabbit monoclonal antibody. (J) Sham control newborn female treated with vehicle and stained with anti-phospho-ERK rabbit monoclonal antibody. (K) FPI newborn female treated with vehicle and stained with anti-phospho-ERK rabbit monoclonal antibody. (L) FPI newborn female treated with ADM (0.1 mg/kg IV) and stained with anti-phospho-ERK rabbit monoclonal antibody. Original magnification is $100\times$ for all panels, except for inserts for which it is $400\times$ ($n = 2$ animals per group; FPI, fluid percussion injury; ADM, adrenomedullin; ERK, extracellular signal-related kinase).

and at 1 h and 4 h post-FPI. Phospho-ERK MAPK was increased from 749 ± 25 to $1266 \pm 82 \text{ pg/mL}$ ($p < 0.05$), while the total ERK MAPK isoform was increased from 750 ± 21 to $787 \pm 25 \text{ pg/mL}$ ($p = 0.14$) in males at 1 h after FPI. Because the quantity of both phospho and total ERK MAPK isoforms changed after FPI as determined by ELISA of CSF, expression of the data as a percentage of control (total) normalizes values and allows for easier interpretation of data. Vehicle, agonist, or antagonist was administered 30 min prior to FPI. FPI produced a marked phosphorylation (activation) of CSF ERK MAPK within 1 h (Fig. 2A and B). ERK MAPK phosphorylation induced by FPI was significantly greater in male than in female pigs (Fig. 2). Pre-treatment with ADM (0.1 mg/kg IV) blunted the phosphorylation of ERK MAPK post-injury in both sexes (Fig. 2A and B). However, ERK MAPK phosphorylation was

blunted by exogenous ADM to a significantly greater extent in the male than in the female. Conversely, topical administration of the truncated peptide ADM 22-52 (10^{-7} M) potentiated ERK MAPK phosphorylation post-FPI in both sexes. ERK MAPK phosphorylation in the presence of ADM 22-52 was greater in the male than in the female. However, on a percentage basis, the potentiation of ERK MAPK phosphorylation by ADM 22-52 was greater in the female than in the male. U 0126 (1 mg/kg IV), a putative ERK MAPK antagonist, blocked ERK MAPK phosphorylation after FPI (Fig. 2). ADM, ADM 22-52, and U 0126 had no effect on ERK MAPK phosphorylation before FPI. ADM 22-52 (10^{-7} M) blocked ADM-induced (10^{-9} and 10^{-7} M) pial artery vasodilation (7 ± 1 and 12 ± 1 versus 0 ± 1 and $1 \pm 1\%$ for ADM 10^{-9} and 10^{-7} M in the absence and presence of ADM 22-52, respectively; $n = 6$).

FIG. 2. Phosphorylation of ERK MAPK in CSF prior to FPI (0 time), and as a function of time after FPI, in pigs treated with vehicle (FPI), ADM (0.1 mg/kg IV) + FPI, ADM 22-52 (10^{-7} M) + FPI, or U 0126 (1 mg/kg IV) + FPI ($n = 6$). Time 0 is the time point immediately before injury. Data were analyzed by repeated measures ANOVA and expressed as percentage of control by ELISA determination of phospho ERK MAPK and total ERK MAPK isoforms, and subsequent normalization to the total form. (A) Male animals. (B) Female animals (* $p < 0.05$ compared with the corresponding value at time point 0; + $p < 0.05$ compared with the corresponding vehicle-treated value; # $p < 0.05$ compared with the corresponding female value; FPI, fluid percussion injury; ADM, adrenomedullin; ERK, extracellular signal-related kinase; MAPK, mitogen-activated protein kinase; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay).

ADM and U 0126 prevent sex-dependent reductions in pial artery diameter and CBF after FPI

ADM (0.1 mg/kg IV) produced equivalent decreases in mean arterial blood pressure ($15 \pm 6\%$ and $17 \pm 6\%$), and increases in pial artery diameter ($11 \pm 3\%$ and $12 \pm 4\%$) in male and female pigs (Fig. 3). Baseline CBF was greater in female than in male piglets. FPI produced greater pial small artery vasoconstriction and reductions in CBF in male than in female newborn pigs (Figs. 3 and 4). Exogenous ADM (0.1 mg/kg IV) pre-treatment 30 min prior to injury blunted pial artery vasoconstriction and reductions in CBF post-FPI (Figs. 3 and 4). On a percentage basis, the protection afforded by ADM from reductions in pial artery diameter and CBF were greater in the males than in the females. The ADM antagonist ADM 22-52 (0.1 mg/kg IV) had no effect on reductions in pial artery diameter and CBF post-FPI in the males, but potentiated reductions in pial artery diameter and CBF in the females post-FPI. The ERK MAPK antagonist U 0126 (1 mg/kg IV) blunted reductions in pial artery diameter and CBF post-FPI. On a percentage basis, the protection afforded by U 0126 was greater in the males than in the females. In a subset of animals, ADM (0.1 mg/kg IV) post-treatment at 30 min after FPI produced similar prevention of pial small artery vasoconstriction and reductions in CBF in male and female piglets as those seen with ADM pre-treatment ($-22 \pm 2\%$, $-6 \pm 1\%$, and $-7 \pm 1\%$

reductions in pial small artery diameter at 1 h post-FPI in male vehicle, ADM pre-treated, and ADM post-treated male piglets, respectively; $n = 3$). Similar observations were made in pial arterioles.

ADM and U 0126 prevent sex-dependent loss of pial artery dilation during hypotension after FPI

Moderate and severe hypotension decreased mean arterial blood pressure by $24 \pm 1\%$ and $44 \pm 1\%$, respectively. Prior to FPI, hypotensive pial small artery dilation was significantly less in male than in female pigs (Fig. 5), a finding similar to that recently published elsewhere (Armstead and Vavilala, 2007). Within 1 h of FPI, hypotensive pial artery dilation was impaired in both sexes, but the degree of impairment was greater in the males than in the females (Fig. 5). The intensity of the brain injury insult was equal in both sexes (1.9 ± 0.1 atm). Pre-treatment with ADM (0.1 mg/kg IV) 30 min prior to injury partially protected hypotensive pial artery dilatory responses in both sexes (Fig. 5). However, the amount of protection seen was greater in the males than in the females (Fig. 5). Conversely, pre-treatment with ADM 22-52 (10^{-7} M), the putative ADM antagonist, resulted in greater impairment of hypotensive pial artery dilation than did vehicle post-injury in the females (Fig. 5). Pre-treatment with U 0126 prevented impairment of hypotensive pial artery dilation in both sexes

FIG. 3. Influence of FPI on pial artery diameter in vehicle (FPI), ADM (10^{-10} M), ADM 22-52 (10^{-7} M), and U 0126 (10^{-6} M) pre-treated newborn male (A) and female (B) pigs ($n=6$). Data were analyzed by repeated measures ANOVA (* $p < 0.05$ compared with the corresponding FPI vehicle value; + $p < 0.05$ compared with the corresponding female value; FPI, fluid percussion injury; ADM, adrenomedullin; ANOVA, analysis of variance).

FIG. 4. Influence of FPI on total CBF in vehicle (FPI), ADM (0.1 mg/kg IV), ADM 22-52 (0.1 mg/kg IV), or U 0126 (1 mg/kg IV) pre-treated newborn male (A) and female (B) pigs ($n=3$). Time point 0 is the time immediately before injury. Data were analyzed by repeated measures ANOVA (* $p < 0.05$ compared with the corresponding time point 0 value; + $p < 0.05$ compared with the corresponding vehicle pre-treated value; # $p < 0.05$ compared with the corresponding female value; CBF, cerebral blood flow; FPI, fluid percussion injury; ADM, adrenomedullin; ANOVA, analysis of variance).

FIG. 5. Influence of moderate and severe hypotension on pial artery diameter before (time point 0) and after FPI in newborn male (A) and female (B) pigs pretreated with vehicle (FPI), ADM (0.1 mg/kg IV), ADM 22-52 (10^{-7} M), or U 0126 (1 mg/kg IV; $n=6$). Data were analyzed by repeated measures ANOVA (* $p < 0.05$ compared with the corresponding time point 0 value; + $p < 0.05$ compared with the corresponding vehicle-treated value; # $p < 0.05$ compared with the corresponding female value; FPI, fluid percussion injury; ADM, adrenomedullin; ANOVA, analysis of variance).

(Fig. 5). However, the protection was greater in the males than in the females. In a subset of animals, ADM (0.1 mg/kg IV) post-treatment given 30 min after FPI produced similar prevention of loss of pial small artery vasodilation during hypotension in male and female piglets to that observed with ADM pre-treatment. The responses were $11 \pm 1\%$ and $20 \pm 1\%$ during moderate and severe hypotension for sham control animals, $1 \pm 1\%$ and $2 \pm 1\%$ for FPI-vehicle animals, $8 \pm 1\%$ and $14 \pm 1\%$ for FPI-ADM pre-treated animals, and $8 \pm 1\%$ and $15 \pm 1\%$ for FPI-ADM post-treated male piglets, $n=3$. Papaverine-induced pial small artery vasodilation was unchanged by FPI, ADM, ADM 22-52, or U 0126 (data not shown). Similar observations were made in pial arterioles.

ADM prevents sex-dependent loss of regional CBF autoregulation during hypotension after FPI

CBF in the parietal cortex and hippocampus was unchanged during hypotension prior to FPI (2.0 ± 0.1 atm), a finding supportive of intact cerebral autoregulation pre-insult (Fig. 6). After FPI, CBF in the cortex and hippocampus was reduced more in males than in females in animals with the same level of insult (Fig. 6). Regional CBF was further reduced during moderate and severe hypotension, but these reductions were greater in males than in females (Fig. 6). ADM (0.1 mg/kg IV) pre-treatment, however, helped to prevent loss of cerebral autoregulation, since reductions in regional CBF during hypotension were less than those occurring in animals not receiving ADM (Fig. 6). In a subset of animals, ADM

(0.1 mg/kg IV) post-treatment 30 min after FPI produced a similar level of prevention of loss of regional CBF autoregulation during hypotension to animals receiving ADM pre-treatment. In males, these values in the cerebral cortex were: 65 ± 9 for sham control, 26 ± 4 for FPI animals, 11 ± 2 for FPI-severe hypotension animals, all in the absence of ADM, and 63 ± 9 for sham control animals, 40 ± 5 for FPI animals, and 22 ± 4 for FPI-severe-hypotension animals in the presence of ADM post-treatment, respectively, $n=6$. In females, these values in the cerebral cortex were: 75 ± 10 , 39 ± 4 , 23 ± 4 versus 74 ± 10 , 49 ± 5 , and 33 ± 5 mL/min/100 g for sham control, FPI, and FPI + severe hypotension conditions in the absence and presence of ADM post-treatment, respectively ($n=6$).

ADM prevents sex-dependent reductions in cerebral perfusion pressure (CPP) during hypotension after FPI

ICP was increased after FPI more in male than in female animals subjected to equivalent insult levels (1.9 ± 0.1 atm), and was roughly equivalent to the mean arterial blood pressure (Fig. 7A). The ICP values seen during moderate and severe hypotension after FPI were also greater in males than in females (Fig. 7A). ADM pre-treatment decreased the peak ICP recorded at 3 min after FPI in both sexes (4 ± 1 to 20 ± 1 mm Hg versus 4 ± 1 to 13 ± 1 mm Hg, and 4 ± 1 to 13 ± 1 mm Hg versus 5 ± 1 to 10 ± 1 mm Hg, in males and females in the absence and presence of ADM, respectively). ADM pre-treatment blunted the elevation of ICP seen after FPI during normotension and subsequent hypotension in males, but not

FIG. 6. CBF in (A) parietal cortex and (B) hippocampus during control (C), moderate and severe hypotension (MH and SH) in sham, FPI, and FPI + ADM-treated (0.1 mg/kg IV) male and female pigs ($n = 6$). Data were analyzed by repeated measures ANOVA (* $p < 0.05$ compared to the corresponding sham value; + $p < 0.05$ compared to the corresponding control value; # $p < 0.05$ compared to the corresponding FPI value; & $p < 0.05$ compared to the corresponding male value; CBF, cerebral blood flow; FPI, fluid percussion injury; ADM, adrenomedullin; ANOVA, analysis of variance).

in females (Fig. 7A). CPP was decreased at 1 h post-FPI in the males, but not the females (Fig. 7B). CPP during hypotension after FPI was decreased more in males than in females (Fig. 7B). ADM had no effect on CPP in females, either at 1 h post-FPI or during subsequent hypotension (Fig. 7B). However, ADM pre-treatment did help to prevent the reduction in CPP seen in males at both 1 h post-FPI and during subsequent hypotension (Fig. 7B). ADM post-treatment had similar effects on ICP and CPP at 1 h post-FPI and during subsequent hypotension.

ADM prevents sex-dependent reductions in the autoregulatory index (ARI) during hypotension after FPI

Figure 8 shows typical tracings seen during normotension and severe hypotension, before and after FPI, as a function of

FIG. 7. (A) Intracranial pressure (ICP) and (B) cerebral perfusion pressure (CPP) during control (C), and moderate and severe hypotension (MH and SH) in sham, FPI, and FPI + ADM-treated (0.1 mg/kg IV) male and female pigs ($n = 6$). Data were analyzed by repeated measures analysis of variance (ANOVA) (* $p < 0.05$ compared to the corresponding sham value in A, and compared to the corresponding C value in B; + $p < 0.05$ compared to the corresponding FPI value in A, and compared to the corresponding sham value in B; # $p < 0.05$ compared to the corresponding male value in A, and compared to the corresponding FPI value in B; & $p < 0.05$ compared to the corresponding male value in B; FPI, fluid percussion injury; ADM, adrenomedullin).

sex and ADM pre-treatment. Blood velocity in the middle cerebral artery decreased only modestly during severe hypotension prior to FPI in both sexes (Fig. 8). However, blood velocity decreased markedly during severe hypotension in the males after FPI, and was ameliorated by ADM pre-treatment (Fig. 8). Velocity decreased during hypotension in the females after FPI, but the magnitude of the decrease was not as large as that seen in the males. ADM similarly helped to prevent the reductions in velocity seen during hypotension after FPI in the females (Fig. 8). The ARI was unchanged during hypotension prior to FPI, but was reduced during hypotension after FPI in both male and female piglets (Fig. 9). However, the magnitude of the decrease in ARI was greater in male than in female piglets (Fig. 9). ADM pre-treatment helped to prevent reductions in ARI during hypotension after FPI in both male and female piglets, and eliminated the sex-dependent difference (Fig. 9). ADM post-treatment yielded

FIG. 8. Typical transcranial Doppler (TCD) tracings from paired groups of animals during normotension and severe hypotension (Sham-M, male sham controls; FPI-vehicle-M, FPI + vehicle males; FPI-ADM-M, FPI + ADM males; Sham-F, female sham controls; FPI-vehicle-F, FPI + vehicle females; FPI-ADM-F, FPI + ADM females; FPI, fluid percussion injury; ADM, adrenomedullin; P.I., peak interval, Dias., diastolic).

ARI values similar to those seen with pre-treatment (0.77 ± 0.05 , 0.23 ± 0.02 , 0.58 ± 0.05 , and 0.57 ± 0.08 for severe hypotension in the sham control, FPI, FPI + ADM pre-treatment, and FPI + ADM post-treatment male groups, respectively; $n = 3-6$).

Blood chemistry

There were no statistically significant differences in blood chemistry between sham control, FPI, and FPI + drug-treated animals before or after any of the experiments. The amplitude of the pressure pulse, which is used as an index of injury intensity, was equivalent in male and female FPI-vehicle and FPI + drug-treated animals (1.9 ± 0.1 atm).

Discussion

The results of this study show that ADM is upregulated in brain parenchyma after FPI in a sex-dependent manner, corroborating and strengthening prior observations showing the same sex-dependent pattern for ADM concentration changes in CSF induced by FPI (Armstead and Vavilala, 2007). We recently observed that FPI upregulates the ERK isoform of MAPK in CSF and brain parenchyma in an age-dependent manner, with higher levels seen in the newborn compared to the juvenile pig (Armstead et al., 2009). Results of the present study extend the latter observations, in that TBI-associated elevation of CSF ERK MAPK concentration and tissue immunoreactivity was greater in the male than in the

FIG. 9. Autoregulatory index (ARI) values for moderate and severe hypotension (MH and SH) in sham male, FPI male, FPI + ADM male, sham female, FPI female, and FPI + ADM female animals ($n=4-5$). Data were analyzed by repeated measures analysis of variance (ANOVA). (* $p < 0.05$ compared to the corresponding sham value; + $p < 0.05$ compared to the corresponding FPI vehicle value; # $p < 0.05$ compared to the corresponding female value; FPI, fluid percussion injury; ADM, adrenomedullin).

female newborn pig. A limitation of the closed cranial window technique for quantification of substances in the CSF is that neither the cellular site of origin nor the site of action can be determined. Double-labeling immunohistochemistry, such as that used in this study, may offer some insight. For example, NeuN staining suggests the involvement of neurons, though ADM and ERK MAPK staining were often observed in association with pial arteries. Nonetheless, the present study design precludes any conclusion as to whether ADM and ERK MAPK originate or simply bind to these cells after FPI. Additionally, there are no probes currently available that can selectively block action at vascular smooth muscle or endothelial cells, in order to discern the cell type mediating ADM cerebrovasodilation.

Exogenous ADM pre-treatment inhibits upregulation of tissue and CSF phosphorylated ERK MAPK after FPI in a sex-dependent manner. Specificity for the modulation of ERK MAPK upregulation by ADM is shown by the potentiation of FPI-induced elevated CSF ERK MAPK concentrations after giving the truncated peptide ADM 22-52. This analogue blocked ADM-induced pial artery dilation, suggesting that it is an ADM antagonist. U 0126, the putative ERK MAPK antagonist, blocked FPI-associated increases in CSF ERK MAPK, indicating its efficacy. Taken together, these data suggest a dynamic sex-dependent modulation of ERK MAPK upregulation by ADM in the setting of TBI.

During normotension, FPI produces greater reductions in parietal cortical and hippocampal blood flow in male than female newborn piglets, which expands prior observations that pial arteries vasoconstrict more in males than in females after equivalent levels of FPI (Armstead and Vavilala, 2007), data corroborated by the present study. Similarly, ADM-induced sex-dependent prevention of FPI-associated reductions in regional CBF during normotension extend prior findings showing the same action of ADM on pial artery vasoconstriction post-injury (Armstead and Vavilala, 2007), data also corroborated by the present study. New data from this study strengthen this interaction, in that the ADM truncated pep-

tide, ADM 22-52, reversed the actions of ADM, suggesting that this analogue functions as an ADM-like antagonist. U 0126 blunted pial artery vasoconstriction and reductions in regional CBF after FPI, findings similar to recent observations of others (Armstead et al., 2009). Taken together, these data indicate that the sex-dependent ADM modulation of ERK MAPK upregulation may be a newly-recognized explanation for the clinical observation that boys have worse outcomes than girls after head injury. Translationally, the observation that post-treatment with ADM prevents brain injury-induced pial artery vasoconstriction and reductions in regional CBF suggests the identification of an endogenous system that may serve as a novel target for intervention in the critical care treatment of brain-injured children.

A key new finding of this study is the evidence that sex-dependent modulation of ERK MAPK upregulation by ADM in the setting of TBI may explain why newborn male piglets exhibit greater impairment of pial artery dilation during hypotension than female newborn piglets after FPI (Armstead and Vavilala, 2007). New data show that exogenous ADM given pre- or post-treatment protects against reductions in parietal cortical and hippocampal blood flow during combined hypotension and brain injury in a sex-dependent manner. These data extend prior observations that ADM pre-treatment preserved pial artery dilation during hypotension after FPI in a sex-dependent manner (Armstead and Vavilala, 2007), a finding also reproduced in the present study. Potentiation of inhibition of pial artery dilation during hypotension after FPI in a sex-dependent manner by the ADM analogue ADM 22-52, together with the protection afforded by the ERK MAPK antagonist U 0126, further supports the hypothesis that sex-dependent ADM modulation of ERK MAPK upregulation associated with FPI contributes to impairment of cerebral autoregulation during hypotension post-injury.

Determination of the ARI under conditions of combined FPI and hypotension in the piglet in this study was used as a frame of reference to qualitatively compare the gold standard

basic science methodology for characterization of cerebral hemodynamic impairment (radiolabeled microsphere CBF determination) with that used clinically (transcranial Doppler) (Freeman et al., 2008). Several key pieces of information emerged. First, ICP was increased after FPI more in the male than in the female at an equivalent insult level. The ICP values seen during moderate and severe hypotension after FPI were also higher in the male than in the female. Second, while ADM decreased the peak ICP in both sexes, it blunted the elevation of ICP seen after FPI during both normotension and hypotension more in the male than in the female. Third, CBF was decreased more in the male than in the female after FPI. ADM prevented the reduction in CBF seen after FPI and combined FPI and hypotension in the male, but did not do so in the female. An ARI of 1 by definition designates perfect autoregulation, while a value of 0 designates absent autoregulation. The ARI was unchanged during hypotension prior to FPI, indicating intact cerebral autoregulation. ARI was reduced during hypotension after FPI in both male and female piglets, though the magnitude of the decrease was greater in the male than in the female. ADM helped to prevent reductions in ARI in both male and female piglets, though the magnitude of the protection was greater in the male than in the female. These observations point to salient sex-dependent differences in the hemodynamic effects seen in animals subjected to equivalent insults, and indicate that upregulation of an endogenous protective system may have markedly different effects on post-injury cerebral hemodynamics in males and in females. These data suggest that the reductions in ICP seen after ADM administration indicate that it may be useful in improving CPP post-injury. Importantly, the changes in ARI and its manipulation by ADM were similar to those observed via determination of CBF by radiolabeled microspheres. These data suggest that ARI may be a useful indicator of cerebral hemodynamic status in brain-injured patients. The potential significance of this cannot be overstated, given the correlation between ARI and Glasgow Coma Scale scores seen in pediatric brain-injured patients (Freeman et al., 2008; Vavilala et al., 2006).

In conclusion, the results of this study suggest that different critical care pathways should be implemented for the treatment of male and female pediatric TBI patients. Administration of the peptide ADM and inhibitors of ERK MAPK may offer a novel approach to the treatment of pediatric TBI, particularly in boys. Future experiments should be directed toward identification of the time frame post-TBI when administration of ADM-like drugs will exert their maximal benefit.

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Author Disclosure Statement

No competing financial interests exist.

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