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Connexin hemichannel blockade is neuroprotective after, but not during, global cerebral ischemia in near-term fetal sheep

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ABSTRACT

There is increasing evidence that connexin hemichannels, the half gap junctions that sit unopposed in the cell membrane, can open during ischemia and that blockade of connexin43 hemichannels after cerebral ischemia can improve neural outcomes. However, it is unclear whether connexin blockade during ischemia is protective. In the present study global cerebral ischemia was induced by 30 min of bilateral carotid artery occlusion in near-term (128 ± 1 day gestation age) fetal sheep. A specific mimetic peptide that blocks connexin43 hemichannels was infused into the lateral ventricle for either 1 h before and during ischemia (intra-ischemia group, n = 6) or for 25 h starting 90 min after the end of ischemia (post-ischemia group, n = 7). The vehicle was infused in the ischemia-vehicle group (n = 6) and sham-controls received sham occlusion plus vehicle (n = 10). The post-ischemia group showed enhanced recovery of EEG power from day five until the end of the experiment (−5 ± 1.6 dB) compared to ischemia-vehicle (−13 ± 1.9 dB, $p < 0.05$) and intra-ischemia infusion (−14.4 ± 3.6 dB, $p < 0.05$). Post-ischemic infusion was associated with higher neuronal counts compared to ischemia-vehicle and intra-ischemia in the cortex ($p < 0.05$) but not the CA1 and CA3 regions of the hippocampus. Oligodendrocyte cell counts in the intragyral and periventricular white matter were significantly higher in the post-ischemia group compared to ischemia-vehicle and intra-ischemia infusion ($p < 0.05$). These large animal data support the hypothesis that connexin hemichannel opening after, but not during, ischemia contributes to the spread of white and gray matter injury of the developing brain.

Introduction

Hypoxic–ischemic injury in the developing brain is characterized by a striking, progressive evolution of damage into previously uninjured areas over days to weeks after the insult (Thornton et al., 1998; Williams et al., 1992). Although the mechanisms of this spread remain unclear, recent evidence implicates connexin hemichannels in the propagation of injury. Hemichannels, or connexons, are half of a gap junction channel that sits in the unopposed membrane of a cell, before the formation of new channels. Opening of connexin hemichannels has been associated with ischemia, as well as oxygen glucose deprivation, metabolic inhibition or low extracellular calcium ion (Ca^{2+}) levels (Contreras et al., 2002; Decrock et al., 2009; Kondo et al., 2000; Li et al., 1996; Orellana et al., 2010). This may cause disruption of the resting membrane potential, release of cytotoxic levels of ATP (Kang et al., 2008) and glutamate (Ye et al., 2003), and uptake of water resulting in cell swelling and rupture (Quist et al., 2000; Rodriguez-Sinovas et al., 2007).

Despite a growing body of evidence that hemichannels contribute to ischemic brain injury, it remains highly controversial whether hemichannels open during or only after ischemia. In neuronal cultures hemichannels opened rapidly during oxygen glucose deprivation (Thompson et al., 2006). However, astrocytic cell cultures only showed evidence of hemichannel opening after 1 h of reoxygenation following oxygen glucose deprivation (Orellana et al., 2010). Consistent with this, in near-term fetal sheep prolonged blockade of connexin43 hemichannels with a specific mimetic peptide starting 90 min after reperfusion significantly improved neural outcomes, as shown by reduced seizures, improved recovery of electroencephalogram (EEG) power and sleep state cycling, with greater oligodendrocyte survival and intermediate neuronal survival between sham controls and vehicle treated animals (Davidson et al., 2012b).

In the present study, we therefore investigated whether connexin hemichannels contribute independently to the propagation of injury during, compared to after global cerebral ischemia, in chronically instrumented near-term fetal sheep at 0.85 gestation. Brain maturation at this age in the sheep is equivalent to the human infant at term (Dobbing and Sands, 1970; McIntosh et al., 1979). This is a well

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characterized paradigm that allows long-term, real-time monitoring of many physiological parameters including brain activity and temperature, carotid artery blood flow, heart rate, blood pressure and body movements (Gunn et al., 1997; Williams et al., 1992).

Materials and methods

Fetal surgery

All procedures were approved by the Animal Ethics Committee of The University of Auckland. In brief, 29 time-mated Romney/Suffolk fetal sheep were instrumented using sterile technique at 118–124 days gestation (term is 145). Food, but not water was withdrawn 18 h before surgery. Ewes were given 5 mL of Streptocin (procaine penicillin (250,000 IU/mL) and dihydrostreptomycin (250 mg/mL, Stockguard Laboratories Ltd., Hamilton, New Zealand)) intramuscularly for prophylaxis 30 min prior to the start of surgery. Anesthesia was induced by I.V. injection of Alfaxan (Alphaxalone, 3 mg/kg, Jurox Pty Ltd, Rutherford, New South Wales, Australia), and general anesthesia maintained using 2–3% isoflurane in O_2. The depth of anesthesia, maternal heart rate and respiration were constantly monitored by trained anesthetic staff. Ewes received a constant infusion isotonic saline drip (at an infusion rate of approximately 250 mL/h) to maintain fluid balance.

Following a maternal midline abdominal incision and exteriorization of the fetus, both fetal brachial arteries were catheterized with polyvinyl catheters to measure mean arterial blood pressure. An amniotic catheter was secured to the fetal shoulder. ECG electrodes (Cooner Wire Co., Chatsworth, California, USA) were sewn across the fetal chest to record fetal heart rate. The vertebral–occipital anastomoses were ligated and inflatable carotid occluder cuffs were placed around both carotid arteries (Gunn et al., 1997; Williams et al., 1992). A 3S Transonic ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY) was placed around the right carotid artery. Using a 7 stranded stainless steel wire (AS633–5SSF; Cooner Wire Co.), two pairs of EEG electrodes (AS633–5SSF; Cooner Wire Co.) were placed on the dura over the parasagittal parietal cortex (10 mm and 20 mm anterior to bregma and 10 mm lateral) and secured with cyanoacrylate glue. A reference electrode was sewn over the occiput. A further two electrodes were sewn in the nuchal muscle to record electromyographic activity as a measure of fetal movement. A thermistor was placed over the parasagittal dura 30 mm anterior to bregma. An intracerebroventricular catheter was placed into the left lateral ventricle (6 mm anterior and 4 mm lateral to bregma). The uterus was then closed and antibiotics (80 mg Gentamicin, Pharmacia and Upjohn, Rydalmere, New South Wales, Australia) were administered into the amniotic sac. The maternal laparotomy skin incision was infiltrated with a local analgesic, 10 mL 0.5% bupivacaine plus adrenaline (AstraZeneca Ltd., Auckland, New Zealand). All fetal catheters and leads were exteriorized through the maternal flank. The maternal long saphenous vein was catheterized to provide access for post-operative maternal care and euthanasia.

Post-operative care

Sheep were housed together in separate metabolic cages with access to food and water ad libitum. They were kept in a temperature-controlled room (16 ± 1 °C, humidity 50 ± 10%), in a 12 h light/dark cycle. Antibiotics were administered daily for four days I.V. to the ewe (600 mg benzylpencillin sodium, Novartis Ltd, Auckland, New Zealand, and 80 mg Gentamicin, Pharmacia and Upjohn). Fetal catheters were maintained patent by continuous infusion of heparinized saline (20 U/mL at 0.15 mL/h) and the maternal catheter was maintained by daily flushing.

Data recording

Data recordings began 24 h before the start of the experiment and continued for the remainder of the experiment. Data were recorded and saved continuously to disk for off-line analysis using custom data acquisition programs (LabView for Windows, National Instruments, Austin, Texas, USA). Arterial blood samples were taken for pre-ductal pH, blood gas, base excess (Ciba-Corning Diagnostics 845 blood gas analyzer and co-oximeter, Massachusetts, USA), glucose and lactate measurements (YSI model 2300, Yellow Springs, Ohio, USA). All fetuses had normal biochemical variables for their gestational ages (Wibbens et al., 2005).

Experimental protocols

Experiments were performed at 128 ± 1 d gestation. Ischemia was induced by reversible inflation of the carotid occluder cuffs with saline for 30 min. For connexin43 hemichannel blocking, a peptide (H-Val-Asp-Cys-Phe-Leu-Ser-Arg-Pro-Thr-Glu-Lys-Thr-OH (Auspep, Vic, AU)) that mimics the second extracellular loop of connexin43 ('Peptide 5' reported in O'Carroll et al., 2008) was infused into the left lateral ventricle via the intracerebroventricular catheter attached to an external pump. Control fetuses received cerebral ischemia followed by infusion of the vehicle via the intracerebroventricular catheter (ischemia-vehicle, n = 7). The post-ischemia infusion dose group (n = 6) received 50 μmol/kg/h for 1 h followed by 50 μmol/kg/24 h for 24 h, dissolved in artificial cerebrospinal fluid (aCSF), at a rate of 1 mL/h for 25 h starting 90 min after the end of the occlusion. The intra-ischemia infusion group (n = 6) received 50 μmol/kg/h starting 1 h prior to the beginning of the occlusion and continued until the end of the occlusion. aCSF was infused in the ischemia-vehicle group (n = 7). The sham control group received a sham carotid artery occlusion (n = 10). All animals were killed at seven days post ischemia with an overdose of sodium pentobarbitone (9 g I.V. to the ewe; Pentobarb 300, Chemstock International, Christchurch, N.Z.).

Fluorescent peptide infusion

A single animal received intracerebroventricular infusion of fluorescein tagged mimetic peptide as per the post-ischemia infusion protocol. The fetus was killed at the end of the infusion period and the brain was infused with 4% paraformaldehyde. 180 μM slices were cut on a vibratome and mounted on glass slides. Images were taken on a confocal microscope (Olympus Fluoview FV1000, Center Valley, PA).

Immunocytochemistry

The fetal brains were perfusion fixed with 10% phosphate-buffered formalin. Slices (10 μm thick) were cut using a microtome (Leica Jung RM2035). Slides were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol. Slides were washed in 0.1 mol/L phosphate buffered saline (PBS). Antigen retrieval was performed using the citrate buffer boil method followed by incubation in 1% H_20_2 in methanol for anti-neuronal nuclei (NeuN) and in PBS for Olig2. Blocking was performed in 3.5% normal horse serum (NHS) for NeuN and normal goat serum (NGS) for Olig2 for 1 h at room temperature. Sections were labeled with 1:400 mouse NeuN monoclonal antibody (Chemicon International, Temecula, CA, USA) or 1:400 Olig2 (Chemicon International) overnight at 4 °C. Sections were incubated in biotin-conjugated secondary 1:200 anti-mouse (NeuN) or 1:200 anti-rabbit IgG (Olig2, a marker for oligodendrocytes at all stages of the lineage (Jakovcevski et al., 2009), Vector Laboratories, Inc., Burlingame, USA) in 3.5% NHS. Slides were then incubated in ExtrAvidin® (1:200, Sigma-Aldrich Pty. Ltd.) in PBS for 2 h at room temperature and then reacted in diaminobenzidine