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Neutralizing anti-interleukin-1β antibodies modulate fetal blood–brain barrier function after ischemia

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ABSTRACT

We have previously shown that increases in blood–brain barrier permeability represent an important component of ischemia–reperfusion related brain injury in the fetus. Pro-inflammatory cytokines could contribute to these abnormalities in blood–brain barrier function. We have generated pharmacological quantities of mouse anti-ovine interleukin-1β monoclonal antibody and shown that this antibody has very high sensitivity and specificity for interleukin-1β protein. This antibody also neutralizes the effects of interleukin-1β protein in vitro. In the current study, we hypothesized that the neutralizing anti-interleukin-1β monoclonal antibody attenuates ischemia–reperfusion related fetal blood–brain barrier dysfunction. Instrumented ovine fetuses at 127 days of gestation were studied after 30 min of carotid occlusion and 24 h of reperfusion. Groups were sham operated placebo-control- (n = 5), ischemia-placebo- (n = 6), ischemia-anti-IL-1β antibody- (n = 7), and sham-control antibody- (n = 2) treated animals. Systemic infusions of placebo (0.154 M NaCl) or anti-interleukin-1β monoclonal antibody (5.1 ± 0.6 mg/kg) were given intravenously to the same sham or ischemic group of fetuses at 15 min and 4 h after ischemia. Concentrations of interleukin-1β protein and anti-interleukin-1β monoclonal antibody were measured by ELISA in fetal plasma, cerebrospinal fluid, and parietal cerebral cortex. Blood–brain barrier permeability was quantified using the blood-to-brain transfer constant (K_i) with α-aminoisobutyric acid in multiple brain regions. Interleukin-1β protein was also measured in parietal cerebral cortices and tight junction proteins in multiple brain regions by Western immunoblot. Cerebral cortical interleukin-1β protein increased ($P < 0.001$) after ischemia–reperfusion. After anti-interleukin-1β monoclonal antibody infusions, plasma anti-interleukin-1β monoclonal antibody was elevated ($P < 0.001$), brain anti-interleukin-1β monoclonal antibody levels were higher ($P < 0.03$), and interleukin-1β protein concentrations ($P < 0.03$) and protein expressions ($P < 0.001$) were lower in the monoclonal antibody-treated group than in placebo-treated-ischemia–reperfusion group. Monoclonal antibody infusions attenuated ischemia–reperfusion-related increases in K_i across the brain regions ($P < 0.04$), and K_i showed an inverse linear correlation ($r = -0.65$, $P < 0.02$) with anti-interleukin-1β monoclonal antibody concentrations in the parietal cortex, but had little effect on tight junction protein expression. We conclude that systemic anti-interleukin-1β monoclonal antibody infusions after ischemia result in brain anti-interleukin-1β antibody uptake, and attenuate ischemia–reperfusion-related interleukin-1β protein up-regulation and increases in blood–brain barrier permeability across brain regions in the fetus. The pro-inflammatory cytokine, interleukin-1β, contributes to impaired blood–brain barrier function after ischemia in the fetus.

Abbreviations: BBB, blood–brain barrier; ECoG, electrocorticogram; IC, internal control; IL-1β, interleukin-1β; I/R, ischemia/reperfusion; K_i, blood-to-brain transfer constant; mAb, monoclonal antibody; RBCs, red blood cells; TJ, tight junction protein.

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Introduction

Hypoxic–ischemic brain injury is a major contributor to neurologic abnormalities originating in the perinatal period (Vannucci, 2000; Volpe, 2012) and there is evidence to suggest that the onset of brain injury in neonates can commence before birth (Vannucci, 2000; Volpe, 2012). We have recently demonstrated that impaired blood–brain barrier (BBB) function represents a formerly unappreciated major component of hypoxic–ischemic brain injury in the fetus and that decreases in the expression of tight junction (TJ) proteins are associated with increases in BBB permeability (Chen et al., 2012). Therefore, we postulated that these changes could represent a therapeutic target to attenuate ischemia-related abnormalities in fetal BBB function.

Pro-inflammatory cytokines are important mediators of brain injury that cause or exacerbate brain injury after perinatal insults (Leviton et al., 2010, 2011). Interleukin-1β (IL-1β) is a key mediator of inflammatory responses and one of the best-characterized early response pro-inflammatory cytokines. We have previously shown increases in the expression of IL-1β in the cerebral cortex of fetal sheep after brain ischemia (Sadowska et al., 2012). In addition, elevated levels of IL-1β in the brain can accentuate injury and result in adverse neurological outcomes after hypoxic–ischemic injury (Cai et al., 2000; Pantoni et al., 1998). Therefore, IL-1β could represent an attractive target to attenuate injury to the fetal BBB.

Recent evidence suggests that anti-IL1β neutralizing antibodies can reduce the biological effects of pro-inflammatory cytokines (Yasothan and Kar, 2008). However, despite been recent advances in the potential therapeutic use of monoclonal antibodies (mAbs) (Breda et al., 2011), effective mAbs have not been developed or approved to treat perinatal brain injury. We have recently generated and purified pharmacological quantities of a specific mouse anti-ovine IL-1β mAb (anti-IL-1β mAb) using previously reported protocols and reagents (Rothel et al., 1997) for use i our in vivo fetal sheep studies. We also confirmed that this neutralizing anti-IL-1β mAb is sensitive and specific for the ovine IL-1β protein and that it effectively neutralizes the biological effects of the ovine IL-1β in vitro (Chen et al., 2013).

The BBB, blood–cerebral spinal fluid (CSF), and CSF–brain barriers are important barriers during brain development (Ek et al., 2012; Stonestreet et al., 1996). The cerebrovascular endothelial cells of the BBB are connected by tight junction proteins, and maintain homeostasis of the central nervous system (CNS) by limiting the entry of substances that could alter neuronal function (Ek et al., 2012). Evidence suggests that BBB damage is an important component of hypoxic–ischemic brain injury in the fetus and neonate (Chen et al., 2012; Inder and Volpe, 2000). The integrity of the TJ seal between adjacent endothelial cells is also critical to CNS homeostasis after hypoxic–ischemic injury (Abbott et al., 2010; Chen et al., 2012). However, limited information is available regarding the effects of hypoxia–ischemia on BBB function and the ability of therapeutic agents to attenuate ischemia-related increases in BBB permeability during the perinatal period (Chen et al., 2012) because most studies have been performed in rodents in which vascular access is not feasible (Fernandez-Lopez et al., 2012; Muramatsu et al., 1997).

The neurodevelopment of the fetal sheep brain has many similarities to that of the human fetal and neonatal brains (Back et al., 2006; Barlow, 1969). The development of the sheep fetus at 127 days of gestation is approximately similar to that of the near term human infant (Back et al., 2006; Gunn et al., 1997). In addition, procedures in the current study such as systemic intravenous infusions of anti-IL-1β mAb and physiological kinetic measures of BBB permeability are not readily achievable in fetuses of smaller animals such as rodents.

Given these considerations, we tested the hypotheses that intravenous infusions of anti-IL-1β mAb result in mAb penetration into the brain parenchyma and CSF, attenuate ischemia-related increases in IL-1β protein and BBB permeability, and modify TJ protein expression after ischemic injury in the brain of the ovine fetus.

Materials and methods

The present study was performed after approval by the Institutional Animal Care and Use Committees of The Alpert Medical School of Brown University and Women & Infants Hospital of Rhode Island and in accordance to the National Institutes of Health Guidelines for the use of experimental animals.

Animal preparation, study groups, and experimental design

Surgery was performed under 1–2% isoflurane anesthesia on 20 mixed breed pregnant ewes at 120–122 days of gestation as previously described (Chen et al., 2012). In brief, polyvinyl catheters were placed in fetal brachial veins and arteries and in the femoral artery of the ewe for blood pressure and heart rate monitoring and to obtain blood samples. An amniotic fluid catheter was also placed for pressure monitoring as a referent to correct for the fetal arterial blood pressure values.

After exposure of the fetal carotid arteries, the lingual arteries and vertebral–occipital anastomosis were ligated to restrict blood flow from non-cerebral and vertebral sources, respectively. Two inflatable 4-mm vascular occluders (In Vivo Metric, Healdsburg, CA, USA) were placed around each carotid artery along with ultrasonic flow probes (Transonic Systems Inc., Ithaca, NY, USA). In order to determine the fetal electrocorticogram (ECoG), two pairs of screws (Small Parts, Inc., Miami Lakes, FL, USA) were placed on the dura and a reference electrode was sewn to the scalp (Gunn et al., 1997) and connected to a recorder (ADInstruments, Colorado Springs, CO, USA).

The fetal sheep were studied at 85% of gestation (125–128 days of gestation, 127 ± 1.3, mean ± standard deviation). Full term gestation in sheep is 145–147 days. Five–seven days after surgery, the ewes were assigned to four groups as follows: instrumented sham control fetuses treated with placebo (Sham-PL, n = 5) or with anti-IL-1β mAb (Sham-mAb, n = 2) and experimental ischemic fetuses exposed to 30 min of carotid occlusion and 24 h of reperfusion, then treated with placebo (Isch-PL, n = 6) or anti-IL-1β mAb (Isch-mAb, n = 7). The placebo-treated sham fetuses were sham operated control sheep from our previous studies (Chen et al., 2012) to avoid unnecessary usage of this large animal resource. This was justified because the studies employed identical study design and methodology as in our former work (Chen et al., 2012).

Ischemia was induced after baseline determinations on the morning of study by inflation of the occluders with sterile water for 30 min. Reperfusion then continued for 24 h after the deflation of the occluders. We have previously shown that the permeability of the BBB is increased for at least 48 h after ischemia in fetal sheep (Chen et al., 2012). Systemic intravenous infusions of neutralizing monoclonal anti-IL-1β mAb [5.1 ± 0.6 mg/kg, mean ± standard deviation (SD) or placebo (0.154 M NaCl, 15 ml)] were administered over a 6-h interval after the onset of reperfusion. The initial phase of the anti-IL-1β or placebo mAb infusion was given over 2 h beginning 15 min after ischemia. Additional anti-IL-1β mAb or placebo infusions were also given over 2 h beginning 4 h after ischemia. The infusion paradigm was designed to achieve early-sustained increases in systemic mAb levels in order to expose the cerebral microvasculature to mAb for a prolonged time after ischemia and before onset of BBB permeability studies. This infusion paradigm was designed so that the fetal sheep would receive a relatively slow exposure to the mAb because we were not able to predict physiological responses of the fetal sheep to systemic inhibition of IL-1β.

BBB permeability was measured for 1 h starting 24 h after the termination of ischemia. At the end of the studies, a hysterotomy was performed under intravenous pentobarbital anesthesia, the fetus withdrawn from the uterus, and the brain quickly removed. The ewe was euthanized with pentobarbital (100–200 mg/kg). Immediately before removal of the brain, 0.5–1 ml of CSF was obtained from the cisterna magnum of the fetal sheep via a direct puncture of the allantoic

membrane with a 23-gauge needle. CSF samples with visible blood contamination were not saved for analysis. One-half of the fetal brain was dissected to measure regional BBB permeability as we previously described in detail (Stonestreet et al., 1996) and the remainder of the brain dissected and frozen to determine the cerebral cortical uptake of anti-IL-1β mAb, IL-1β concentrations, IL-1β, TNF-α, and IL-6 protein expressions, and TJ protein expression.

Physiological determinations

Fetal arterial blood gases, pH, heart rate, mean arterial blood pressure (MABP), hematocrit, and lactate values were measured twice at baseline and sequentially after ischemia. ECoG and carotid arterial blood flow were obtained at baseline, during, and after ischemia. Blood gases and pH were determined on a blood gas analyzer (model 248, Siemens, Washington, DC, USA) corrected to 39.5 °C for the fetuses and to 38.5 °C for ewes. Carotid arterial blood flow was measured with the flow probes (Small Animal Blood Flow Meter: T206, Transonic Systems Inc., Ithaca, NY, USA). Although carotid blood flow is not a direct measurement of cerebral blood flow, carotid arterial and cerebral blood flow are closely related (van Bel et al., 1994). Carotid arterial blood flow was recorded and averaged over 5 min at each measurement interval and presented as percent change from the baseline values in each group.

The ECoG files were visually inspected by a board certified epileptologist (J.G.), who was unaware of the group identity. The recorded ECoG signals were digitized (16 bits, 1000 Hz) and stored. Signal processing was performed off line with MATLAB® (Mathworks, Natick, MA) on data segments from the study periods which were extracted from the ECoG recordings of each fetus. The duration of each ECoG segment averaged 15 min in each group. The length of the analyzed ECoG segments was not different among groups. We analyzed the ECoG data to determine the frequency range that suggested the largest differences among the study periods. Next, grand average power spectral densities (PSD) were used to estimate the frequency ranges. Preliminary examination of fetal sheep exposed to ischemia and reperfusion suggested that a discriminatory frequency band of 10–100 Hz was optimal for analysis (Chen et al., 2012). Segments in this band were then subdivided into ten-second non-overlapping windows to calculate the power of the extracted data. A 10-s window was selected based upon requirements for the window to be long enough for accurate power analysis estimates and short enough to eliminate potential artifacts. A fifth order Butterworth band pass filter of 10–100 Hz and a 60 Hz notch filter were applied to each window. The window mean was subtracted and the variance of the de-meaned windows used as the window power because the power of a sample with a zero mean is equal to the sample variance (Shiavi, 2007). The overall power of each segment was calculated as an average power for the windows within the segment that did not contain discontinuities caused by artifacts. The power of the segments was normalized to a zero mean and unit standard deviation for each fetus to account for offset power differences among the fetuses. Finally, the baseline power for each group was subtracted from the power for the rest of the segments before analysis. Seizures were determined by rhythmic discharges or spikes and wave patterns with definite evolution in frequency, location, or morphology lasting at least 10 s by an epileptologist (J.G.). Evolution of amplitude alone was not qualified.

IL-1β protein and anti-IL-1β mAb production and purification

Recombinant ovine IL-1β protein encoded by pGEX-2T vector was generated and purified and anti-IL-1β mAb generated using mouse hybridoma cells with previously described methods (Rothel et al., 1997). Purification of anti-IL-1β mAb was performed as we previously described (Chen et al., 2013) with additional modifications as follows. The immunoglobulin G (IgG) from the anti-IL-1β mAb was purified from cell culture supernatants by affinity chromatography on Protein G Sepharose (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Bound antibodies were eluted using 0.1 M glycine–HCl (pH 2.3) and neutralized to pH 7.4 after elution by adding 1 M Tris buffer. The eluted antibodies were passed through an anion-exchange column (CIMmultus QA, BIASeparations, Wilmington, DE, USA) to remove potential endotoxin contamination. Bound antibodies were eluted from the column by a buffer containing 200 mM NaCl, whereas the endotoxin was retained on the column. The antibody solution was concentrated on Millipore ultrafiltration devices with 30-kDa cut off membranes and passed to 0.2-micron syringe sterile filter (Millipore Corp., Chicago, IL, USA). The pGEX-2T vectors and mouse hybridoma cells were generously provided by the Commonwealth Scientific and Industrial Research Organization (CSIRO, Livestock Industries, Victoria, Australia).

Blood–brain barrier permeability measurements

BBB function was measured with α-[^{14}C]-aminoisobutyric acid (AIB, 103 Da, American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) as previously described in detail (Stonestreet et al., 1996). Twenty-four hours after the termination of carotid artery occlusion, [^{14}C]-AIB was injected intravenously. Arterial plasma concentrations of [^{14}C]-AIB were obtained at fixed intervals before and after the injection. Brain vascular volume was determined by administering technetium-99^{m} (^{99m}Tc, Cardinal Health, RI, USA) radiolabeled red blood cells (RBCs) to the fetus 2 min before the end of the studies (Chen et al., 2012). Four ml of whole blood obtained from the ewe was drawn into a syringe containing 0.8 ml of acid citrated-dextrose and was incubated with Tin-pyrophosphate for 15 min and ^{99m}Tc for an additional 15 min. The ^{99m}Tc-labeled RBCs were washed, resuspended in 0.154 M NaCl to a volume of 4 ml, and administered intravenously to the fetus. The radiolabeling efficiency of the RBCs exceeded 99%.

Fetal brains were dissected into the following regions: frontal, parietal, and occipital cerebral cortices, white matter, caudate nucleus, hippocampus, cerebellum, thalamus, superior and inferior colliculi, pons, medulla, and spinal cord. Plasma and tissue samples were prepared and radioactivity quantified for α-[^{14}C]-AIB. Knowledge of the plasma concentration profile and tracer concentration in the parenchyma allows calculation of the blood-to-brain transfer constant, K_i (μl·g brain^{-1}·min^{-1}) given by:

$$K_i = A_{br} / \int_o^t c_p(T)\ dT$$

where A_{br} is the amount of tracer that crossed the BBB from blood to brain during the tracer study (dpm·g^{-1}), and c_p is the tracer concentration in plasma (dpm·μl^{-1}) at time t (min). A_{br} is obtained by correcting the total amount of isotope measured in the tissue A_m (dpm·g^{-1}) for the residual part remaining in the brain vasculature space, which is measured by ^{99m}Tc-labeled RBCs. Thus, $A_{br} = A_m - V_p c_p$, where V_p is the blood volume of brain tissue (μl·g^{-1}) and c_p is the concentration of tracer in the terminal plasma sample (dpm·g^{-1}). $V_p = A^{\dagger}_m / c^{\dagger}_p$, where $A^{\dagger}_m$ and $c^{\dagger}_p$ have the same definitions as A_m and c_p above except that they apply to the ^{99m}Tc-labeled RBCs (Chen et al., 2012).

Protein extraction

Tissue was prepared to measure parenchymal anti-IL-1β mAb brain uptake as follows. Frozen brain tissue (0.53–0.64 g) was placed in 1 ml of lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1 mM benzethonium chloride (Sigma-Aldrich, Inc., St. Louis, MD, USA), 0.1% sodium-dodecyl-sulfate (SDS, Bio-Rad Laboratories, Hercules, CA, USA), 1% Igepal (Sigma-Aldrich), and 0.5% sodium deoxycholate (Sigma-Aldrich). The tissues were homogenized on ice for 30 s, and sonicated for 10 s at 10 mV. After 30 min incubation on ice, the

homogenates were centrifuged at 16,000 *g* for 30 min. The supernatant was aliquoted and frozen at −80 °C until assays.

The protein extraction procedures for cytokines (IL-1β, IL-6, and TNF-α) and TJ proteins from brain tissue have been previously described in detail (Chen et al., 2012; Sadowska et al., 2012). Briefly, brain tissue was extracted for the pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in buffer F (10 mM Tris–HCl pH 7.05, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 μM $ZnCl_2$, 0.1 mM $NaVO_4$, 1% Triton-X100), for TJ transmembrane proteins (occludin, claudin-1 and claudin-5) in Triton/Deoxycholate/SDS buffer (100 mM NaCl, 1% Triton-X100, 0.5% sodium deoxycholate, 0.2% SDS, 2 mM EDTA, and 1 mM benzamidine hydrochloride (Sigma-Aldrich)), and for cytoplasmic plaque proteins (ZO-1 and -2) in urea buffer (6 M urea, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 10 mM Tris–HCl pH 8.0, and 1% Triton-X100). The buffers were supplemented with 1% complete protease inhibitor cocktail (Roche, Nutley, NJ, USA). Total protein concentrations of the homogenates were determined with a bicinchoninic acid protein assay (BCA, Pierce, Rockford, IL, USA).

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-1β protein and anti-IL-1β mAb in plasma, CSF, and brain parenchyma were measured with ELISA assays. A sandwich ELISA was used to detect IL-1β protein in plasma, CSF, and brain parenchyma. The mouse anti-IL-1β mAb (2 μg/ml), which was also used for the infusions, was coated in a 96-well ELISA plate (Corning Inc., Corning, NY, USA) with a coating buffer (0.1 M carbonate/bicarbonate in ddH_2O, pH 9.6). After three washes, the plates were blocked with 200 μl per well of blocking buffer (1% sodium casein in washing buffer) at room temperature for 2 h. The wells were washed three times. Thereafter, 100 μl of 7.8–1000 pg/ml pure IL-1β protein as standards was added to the wells along with several blanks and the experimental samples in duplicate prepared as follows. Plasma samples were diluted 1:10, CSF samples were not diluted, and brain samples were diluted 1:5 or 1:10. The plates were then washed 5 times with washing buffer before adding 100 μl per well of rabbit anti-IL-1β polyclonal antibody (1:1000 dilution, LifeSpan BioSciences, Inc., Seattle, WA, USA) and then were incubated for 1 h at room temperature. After 5 washes, 100 μl per well of goat anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (1:2000 dilution, Alpha Diagnostic, San Antonio, Texas, USA) was added and incubated for 1 h at 25 °C. Subsequently, the plates were washed 5 times and developed with 100 μl of 3, 3′5, 5′-tetramethylbenzidine substrate solution (Invitrogen, Frederick, MD, USA) in the dark for 15–20 min. Once the maximum color intensity was attained, the reaction was stopped with 50 μl per well of 0.5 M H_2SO_4 (10%, w/w).

The sandwich ELISA was also used to validate the use of the mouse anti-IL-1β mAb in the ELISA assay in vitro. A constant amount of 100 μg/ml of anti-IL-1β mAb or a mouse mAb of the same isotype, raised against sheep (AbD Serotec, Raleigh, NC, USA) was pre-incubated with serial dilutions of duplicate purified ovine IL-1β protein for 1 h at 37 °C before assay. The concentration of free ovine IL-1β protein was determined from a standard curve of IL-1β protein with a range from 7.8 pg/ml to 1 ng/ml. The lower limit of detection was 1 pg/ml. The procedures for this sandwich ELISA were as described above.

An indirect ELISA with a lower limit of detection of 50 pg/ml was used to detect the anti-IL-1β mAb in plasma, CSF, and brain parenchyma. Purified ovine IL-1β protein was diluted to 0.5 μg/ml in coating buffer and 100 μl was added per well to a 96-well ELISA plate. After overnight incubation at 4 °C and five washes with PBS, the non-specific binding sites in the coated wells were blocked with 200 μl of blocking solution (1% sodium casein in PBS) for 2 h at room temperature. A standard curve of anti-IL-1β mAb from 50 pg/ml to 100 ng/ml was created by duplicate serial dilutions across the plate. One hundred μl of diluted plasma samples (1:100 or 1:400) or CSF samples (1:5) or brain extraction samples (1:5 or 1:10) in dilution buffer (0.05% Tween 20 plus blocking solution) was added to the remaining wells in duplicate. The plate was incubated for 2 h at room temperature and washed 5 times with PBS. One hundred μl of HRP-conjugated goat anti-mouse secondary antibody (Zymed) was diluted 1:1000 in diluting buffer and 100 μl added to each well. The plate was incubated for 1 h at room temperature, washed 5 times in PBS, and developed as described above. The lower limit of detection was 50 pg/ml.

The plates were read on a micro-plate reader (Model 680, Bio-Rad Laboratories) at 450 nm. A standard curve was plotted using three-parameter logistic regression analysis (SigmaPlot, Systat Software, Inc., San Jose, CA, USA). Non-specific mouse anti-sheep IgG1 mAb (AbD Serotec) was used as a negative control for mAb coating in the sandwich ELISA and for incubating with pre-coated IL-1β protein in the indirect ELISA. The optical densities of samples were read against the standard curve to determine sample IL-1β concentration.

In vitro IL-1β protein measured in the presence of mouse anti-ovine-non-specific IgG1 mAb and mouse anti-ovine-IL-1β mAb

To validate further that intravenous administration of the anti-ovine-IL-1β mAb could adequately bind to the ovine IL-1β protein and that mouse anti-ovine non-specific IgG1 mAb did not interfere with our detection of the IL-1β protein measured by ELISA, we performed an in vitro mAb validation assay as follows. IL-1β protein was measured on samples containing varying concentrations of ovine IL-1β protein (7.81 to 1000 pg/ml) in the presence of 0.154 M NaCl, 100 μg/ml of anti-ovine non-specific IgG1 mAb and 100 μg/ml of anti-ovine-IL-1β mAb.

Calculation of IL-1β protein and anti-IL-1β mAb concentrations in fetal brain

The amounts of IL-1β protein and anti-IL-1β mAb in brain parenchyma were determined by correcting for the IL-1β protein and anti-IL-1β mAb contained within the vascular space using previously published methods (Banks et al., 2002, 2005). Arterial plasma was collected immediately before the fetal cerebral cortex was dissected at 25 h after ischemia. A portion of fetal parietal cortex was weighed. The concentrations of IL-1β protein and anti-IL-1β mAb in plasma, CSF, and the amount of IL-1β protein and anti-IL-1β mAb in the parietal cortical parenchyma were determined. The brain vascular space measured by ^{99m}Tc-labeled RBCs and hematocrits obtained as described above and in our earlier report (Chen et al., 2012) facilitated the corrections for the IL-1β protein (ng/g brain) and anti-IL-1β mAb remaining within the brain vascular space. The equations to calculate the amounts of IL-1β protein and anti-IL-1β mAb that are contained within the brain parenchyma (ng/g brain) are shown in Table 1:

Western immunoblot detection and protein quantification

Twenty μg of total protein per well was fractionated by SDS-PAGE electrophoresis and transferred onto PVDF membranes (0.2 μm, Bio-Rad) using a semi-dry technique. Membranes were incubated with primary antibodies: mouse mAbs anti-occludin (Zymed, San Francisco, CA, USA), -claudin-1 (Zymed), -claudin-5 (Zymed), -ZO-1 (Zymed), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Imgenex, San Diego, CA, USA) at dilutions of 1:1000, 1:5000, 1:1000, 1:500, and 1:5000, respectively; and rabbit polyclonal antibodies anti-ZO-2 (Zymed), -IL-1β (Lifespan Biosciences), -IL-6 (Millipore Corp.), and -TNF-α (AbD Serotec) at dilutions of 1:500, 1:5000, 1:1000, and 1:5000, respectively. Peroxidase-labeled secondary antibodies were used; goat anti-mouse secondary antibodies (Zymed) at dilutions of 1:5000 for occludin, 1:10,000 for claudin-1, claudin-5, ZO-1 and GAPDH, and goat anti-rabbit secondary antibodies (Alpha Diagnostic) at a dilution of 1:10,000 for ZO-2, IL-1β, IL-6, and TNF-α.

Table 1
Equations to calculate amount of IL-1β protein and anti-IL-1β mAb in brain parenchyma.

1	Total brain to plasma ratio (μl/g) = $\frac{\text{Amount of protein or mAb in brain tissue (ng)}}{\text{Concentration of protein or mAb in plasma (ng/μl)} \times \text{Weight of brain tissue (g)}}$
2	Corrected brain to plasma ratio (μl/g) = Total brain to plasma ratio (μl/g) − Plasma volume (μl/g)
3	Plasma volume (μl/g) = Blood volume (μl/g) × (1 − Hct / 100)
4	Amount of IL-1β protein or anti-IL-1β mAb in brain parenchyma (ng/g brain) = Corrected brain to plasma ratio (μl/g) × Protein or mAb concentration in plasma (ng/μl)

As we have previously described in detail, all experimental samples were normalized to a reference internal control protein standard that was obtained from the cerebral cortex of one adult sheep (Chen et al., 2012). The use of the internal control standard, which is unique to our laboratory, allows for comparisons among large groups of study subjects examined on multiple immunoblots and improves the accuracy of the Western immunoblot quantification (Chen et al., 2012). GAPDH expression was also used as a loading control to ensure that equal amounts of protein were applied to each lane. Each immunoblot included samples from the study groups and internal control samples. The internal control samples were placed in three lanes, as the first, middle, and last samples on each immunoblot. A coefficient of variation for the internal control samples on each immunoblot was calculated. The values for the experimental samples were accepted as valid only when the percent coefficient of variation for the internal control samples was less than 20% on each immunoblot. Detection of IL-1β, IL-6, TNF-α, occludin, claudin-1, claudin-5, ZO-1, and ZO-2 bands at 19, 24, 51, 65, 17, 18, 225, and 160 kDa, respectively, was dependent on incubation with primary antibody, omission of the antibody resulted in the loss of the signal.

To verify that the intravenous anti-IL-1β mAb infusions given to the fetal sheep did not alter the accuracy of the Western immunoblot results for the measurement of IL-1β protein expression, a portion of the cerebral cortex from a placebo-treated ischemic fetus was divided into three sections. Each section was separately treated with placebo (0.154 M NaCl), 5 mg/kg of anti-IL-1β mAb, and 5 mg/kg of non-specific mouse anti-sheep IgG1 mAb (AbD Serotec) for 10 min and for an overnight incubation. Protein was then extracted from each cerebral cortical section, and IL-1β protein expression was measured as described above by Western immunoblot and compared.

Densitometrical analysis

Band intensities of Western immunoblots were analyzed with a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA). The Western immunoblots of IL-1β protein exhibited two bands at approximately 19 kDa. The lower band was used for quantitative analysis because not all animals exhibited distinct upper bands. IL-6 exhibited two closely associated bands at 24 kDa. The densitometry values for the two bands were not sufficiently distinct on all immunoblots to allow for separate band analyses and, consequently, both bands were used for quantitative analysis of IL-6. The three ZO-2 bands were also were combined in the final densitometry analysis. Experimental samples were normalized to the average of the three respective internal control samples on each immunoblot. The final values for each protein in each brain region represented an average of the densitometry values from at least two different immunoblots (range 2–6) and are presented as a ratio to the internal control sample.

Statistical analysis

The sham-placebo, ischemic-placebo, and ischemic-mAb groups were compared statistically. The two animals in the sham-mAb group were not included in the analysis. The two animals in the sham-mAb group were examined to ascertain that the neutralizing anti-IL1-β mAb by itself did not have major adverse side effects compared with the non-ischemic placebo-treated sham group. All physiological variables were similar to the sham-placebo group that we previously reported (Chen et al., 2012). As a result, additional animals were not enrolled in this group to limit unnecessary use of this large animal resource. Results are expressed as mean ± SD, with the exception of the ECoG and sham-mAb animals, which are expressed as median ± SD and mean ± range, respectively. The range for sham-mAb group was used because only two animals were examined. The median was used for the ECoG because the ECoG values in the placebo-treated ischemic and anti-IL-1β mAb-treated ischemic groups were not normally distributed. The Mann–Whitney *U* test was used to compare the ECoG recordings from the ischemia study periods to the normalized baseline ECoG values. Chi-square analysis was used to compare the presence or absence of the seizures among the study groups. Serial measurements of physiological variables and regional BBB permeability within and among the three study groups were compared by two-factor ANOVA for repeated measures. One-way ANOVA was used to detect differences among the three study groups with respect to changes in BBB permeability for each brain region, and in CSF and brain parenchymal IL-1β protein and anti-IL-1β mAb, and in protein expression of cytokines (IL-1β, IL-6, and TNF-α) and TJs. If a significant difference was found by ANOVA, the Fischer's least significant difference (LSD) post hoc test was used to detect differences among the groups and brain regions. The least square linear regression analysis was used to compare the changes in BBB permeability with the concentrations of anti-IL-1β mAb within the brain parenchyma. $P < 0.05$ was considered statistically significant.

Results

Gestational age, brain, and body weights

Gestational age (127 ± 2, 126 ± 2, and 126 ± 1 days), brain (42.7 ± 9.6, 36.5 ± 9.5, and 45.1 ± 7.5 g), and body weights (3.24 ± 0.8, 2.89 ± 0.4, 2.97 ± 0.6 kg) were not different among the placebo-treated sham (n = 5), placebo-treated ischemic (n = 6) and anti-IL-1β mAb-treated ischemic (n = 7) groups, respectively.

Physiological variables in the fetal sheep

The arterial pH, PO_2 and PCO_2, heart rate, MABP, lactate, and hematocrit values were within the normal physiologic range for our laboratory (Stonestreet et al., 1993) and did not change during the study periods within or among the groups (data not shown).

ECoG and carotid blood flow

The ECoG analysis and carotid blood flow values were determined to confirm that the ischemic exposure was adequate and comparable between the placebo-treated ischemic and anti-IL-1β mAb-treated ischemic groups. The fetal ECoG was attenuated during ischemia (Fig. 1A). On visual inspection of the ECoG signals (J.G.), both the placebo- and anti-IL-1β mAb-treated ischemic groups demonstrated attenuation of greater than 50% from baseline amplitude values during ischemia. In contrast, the non-ischemic fetuses did not exhibit changes in amplitude during sham treatment. Furthermore, a significant difference was not observed in the presence or absence of seizures ($P = 0.17$) among the study groups (zero of 5 in the placebo-treated sham, 3 of 6 in the placebo-treated ischemic and 3 of 7 in the anti-IL-1β mAb-treated ischemic groups). Baseline carotid arterial blood flow values did not differ ($P = 0.65$) among the groups

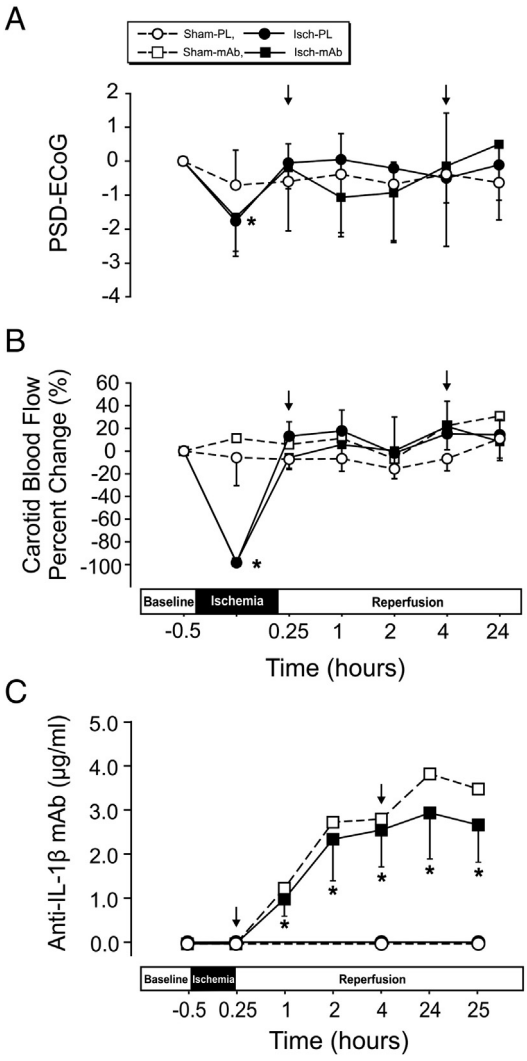

Fig. 1. Changes in ECoG, carotid blood flow and anti-IL-1β mAb concentration during the study. Arrows indicate the timing of the placebo or anti-IL-1β mAb infusions. Non-ischemic placebo-treated sham control (Sham-PL), non-ischemic anti-IL-1β mAb-treated sham control (Sham-mAb), placebo-treated ischemic (Isch-PL), and anti-IL-1β mAb-treated ischemic (Isch-mAb) groups are indicated as open circles connected by dashed lines, open square connected by dashed lines, closed circle connected by solid lines and closed square connected by solid lines, respectively. (A) ECoG decreased during ischemia. ECoG for Sham-PL (n = 5), Isch-PL (n = 4) and Isch-mAb (n = 7) groups plotted against study time in hours on X-axis, before ischemia at baseline, and during ischemia and reperfusion. Isch-PL (n = 4) because of inadequate ECoG recordings in two of the fetal sheep in this group. Y-axis is the power spectral densities, PSD-ECoG plotted as the difference from the individually averaged baseline ECoG values. Values are shown as median ± SD. *$P < 0.05$ compared to baseline within Isch-PL and Isch-mAb groups, but not the sham control group. (B) Carotid blood flow plotted as percent change from baseline for the Sham-PL (n = 5), Isch-PL (n = 6), Isch-mAb (n = 7), and Sham-mAb (n = 2) groups plotted against study time in hours. Values are shown as mean ± SD. *$P < 0.001$ versus baseline within the ischemic groups (Isch-PL and Isch-mAb). (C) Anti-IL-1β mAb concentrations increase in fetal plasma after the infusions. Concentration (μg/ml) of anti-IL-1β mAb in fetal plasma plotted on the Y-axis against study time in hours on X-axis. The Sham-PL (n = 5), Isch-PL (n = 6), Isch-mAb (n = 7), and the sham-mAb (n = 2) groups. The increase in plasma anti-IL-1β mAb concentration was detected within 45 min after the onset infusions. *$P < 0.001$ versus baseline values.

(308 ± 42, 298 ± 82, 298 ± 20, $ml \cdot min^{-1} \cdot 100\ g^{-1}$ in the placebo-treated sham, placebo-treated ischemic, and anti-IL-1β mAb-treated ischemic groups, respectively). Carotid arterial blood flow approached zero within 1 min of onset of ischemia, remained in this range for the 30-min duration of ischemia (Fig. 1B), and did not differ among the groups after ischemia.

Anti-IL-1β mAb and IL-1β protein concentrations in fetal plasma

The plasma levels of anti-IL-1β mAb increased within 45 min after the onset of the intravenous anti-IL-1β mAb infusions in the anti-IL-1β mAb-treated ischemic group (Fig. 1C, ANOVA: main effects for mAb concentration over the study times, $F = 38.0$, $P < 0.001$). Plasma antibody concentrations remained relatively stable in the anti-IL-1β mAb-treated ischemic group from four until 25 h after onset of the mAb infusions (Fig. 1C, ANOVA: main effects for mAb concentrations at 4, 24 and 25 h after the onset of the intravenous infusions, $F = 1.52$, $P = 0.26$).

Systemic fetal plasma concentrations of IL-1β protein (baseline 421 ± 109 pg/ml, mean ± SD, n = 20) did not change during or after exposure to 30 min of brain ischemia (ANOVA: main effects for plasma protein IL-1β concentrations, $F = 0.10$, $P = 0.90$, data not shown), suggesting that transient cerebral ischemia did not increase systemic circulating levels IL-1β protein.

IL-1β protein measured in the presence of mouse anti-ovine-non-specific IgG1 mAb and mouse anti-ovine-IL-1β mAb in vitro

IL-1β protein was measured on samples containing increasing concentrations of ovine IL-1β protein (7.81 to 1000 pg/ml) in the presence of 0.154 M NaCl, 100 μg/ml of anti-ovine non-specific IgG1 mAb, and 100 μg/ml of anti-ovine-IL-1β mAb (data not shown). The presence of 0.154 M NaCl and 100 μg/ml of anti-ovine non-specific IgG1 mAb did not interfere with the detection of ovine IL-1β protein in the ELISA assay. In contrast, ovine IL-1β protein concentrations remained below the lower limits of detection in the presence of 100 μg/ml of anti-ovine-IL-1β mAb (data not shown).

Anti-IL-1β mAb and IL-1β protein concentrations in fetal brain parenchyma and CSF

Intravenous infusions of anti-ovine-IL-1β mAb resulted in a significant accumulation of anti-IL-1β mAb within the brain parenchyma (39.6 ± 40.5 ng/g brain, mean ± SD, ANOVA: main effects for anti-IL-1β mAb concentration in brain parenchyma in the study groups, $F = 4.92$, $P < 0.03$ and Fig. 2A) and cerebral spinal fluid (0.07 ± 0.05 μg/ml, M ± SD, ANOVA: main effects for anti-ovine-IL-1β mAb concentration in CSF in the study groups, $F = 10.27$, $P < 0.01$, Fig. 2B). Ischemia-related increases in IL-1β concentration measured by ELISA were also attenuated by the anti-IL-1β mAb infusions (Fig. 2C, ANOVA: main effects for IL-1β protein concentration in brain parenchyma for the study groups, $F = 4.63$, $P < 0.03$). However, differences in IL-1β protein concentrations were not detected in CSF between the study groups (Fig. 2D, ANOVA: main effects for IL-1β protein concentration in CSF, $F = 0.95$, $P = 0.41$).

In vitro verification that mouse anti-ovine-non-specific IgG1 and -IL-1β mAb do not alter determinations of IL-1β protein by Western immunoblot

In vitro analysis showed that exogenous mouse anti-ovine-non-specific IgG1 and -IL-1β mAb added to cerebral cortical sections from a placebo-treated ischemic fetus did not change the quantity of IL-1β protein expression detected among sections incubated with supplementary 0.154 M NaCl, mouse IgG1 and anti-IL-1β mAb after incubation for 10 min (ANOVA main effects for IL-1 β expression, $F = 0.55$, $P = 0.58$) or 24 h ($F = 0.99$, $P = 0.40$, data not shown).

IL-1β, TNF-α, and IL-6 protein expression in fetal brain

Ischemia-related increases in IL-1β protein expression were attenuated by the systemic intravenous anti-IL-1β mAb infusions (ANOVA: main effects for IL-1β protein expression for the study groups, $F = 27.82$, $P < 0.001$, Fig. 3). Although increases in TNF-α protein expression

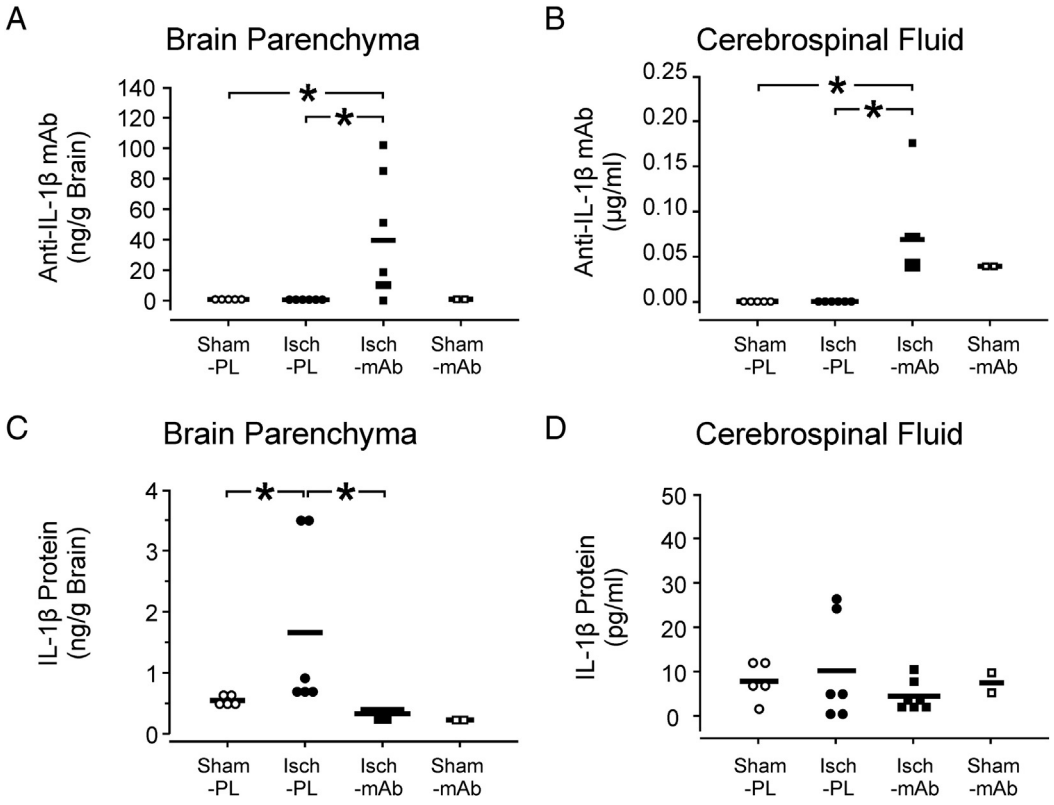

Fig. 2. Anti-IL-1β mAb and IL-1β protein concentrations in brain parenchyma and CSF by study group. Scatter grams of anti-IL-1β mAbs within the brain parenchyma (A) and CSF (B) plotted on the Y-axis in ng/g brain tissue and μg/ml, respectively for the Sham-PL (open circles, n = 5), Isch-PL (closed circles, n = 6), Isch-mAb (closed squares, n = 7), and Sham-mAb (open squares, n = 2) groups on the X-axis. IL-1β protein concentrations in the brain parenchyma (C) and CSF (D) plotted on the Y-axis in ng/g brain and μg/ml, respectively. Group designations as for A and B, $^{*}P < 0.05$.

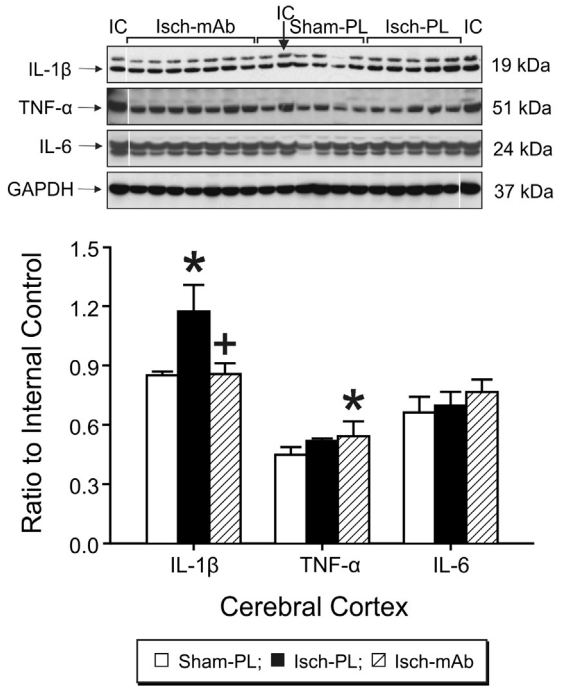

Fig. 3. Protein expression of IL-1β, TNF-α, and IL-6 in fetal brain measured by Western-immunoblot. Top panel shows the expression of an internal control standard protein (IC), and Western immunoblots for IL-1β, TNF-α, and IL-6 protein expressions in the Isch-mAb, Sham-PL, and Isch-PL groups. GAPDH is shown as a loading control. The lower panel shows bar graphs for IL-1β, TNF-α, and IL-6 protein expression plotted on the Y-axis as ratios to the IC proteins. $^{*}P < 0.001$ versus Sham-PL group. $^{+}P < 0.001$ versus Isch-PL group.

were not observed 24 h after ischemia in the fetal sheep brain, intravenous anti-IL-1β mAb infusions were associated with a small but statistically significant increase in TNF-α protein expression (ANOVA: main effects for TNF-α protein expression, $F = 4.65$, $P < 0.03$, Fig. 3). Changes were not observed in the expression of IL-6 protein between the study groups (ANOVA: main effects for IL-6 protein, $F = 3.45$, $P = 0.06$, Fig. 3).

Regional brain blood volume measurements

Table 2 contains blood volume values in the brain regions of the fetal sheep measured with ^{99m}Tc-labeled red blood cells. Regional brain blood volume values were higher in the cerebral cortex (ANOVA:

Table 2
Brain blood volume measured with ^{99m}Tc labeled ovine red blood cells.

Brain Regions (μl/g brain)	Sham-PL (N = 5)	Isch-PL (N = 6)	Isch-mAb (N = 7)
Cerebral cortex	23.1 ± 5.8	37.4 ± 11.0*	22.2 ± 10.8+
White matter	12.4 ± 4.9	26.5 ± 2.8	15.7 ± 15.6
Caudate nucleus	15.6 ± 1.1	21.6 ± 6.7	13.5 ± 10.9
Hippocampus	17.1 ± 3.1	33.4 ± 17.9*	17.5 ± 5.7+
Cerebellum	35.6 ± 13.9	51.1 ± 10.5*	27.4 ± 7.0+
Thalamus	16.5 ± 5.8	21.2 ± 9.7	15.8 ± 9.1
Superior colliculus	17.1 ± 3.2	24.4 ± 6.3	17.0 ± 7.1
Inferior colliculus	25.9 ± 12.5	38.3 ± 20.0	21.0 ± 15.6
Pons	32.9 ± 20.8	40.8 ± 26.0	22.9 ± 8.3
Medulla	25.3 ± 11.7	55.8 ± 35.2*	14.0 ± 8.0+
Spinal cord	22.5 ± 7.9	33.4 ± 16.3	26.1 ± 15.6

Values are means ± SD; n = number of animals. Sham-PL is sham control placebo treated, Isch-PL is ischemic-placebo, and Isch-mAb is ischemic anti-IL-1β antibody treated group.

* $P < 0.05$ vs. sham operated control group.

+ $P < 0.05$ vs. Isch-PL group.

main effects for groups, $F = 4.57$, $P < 0.03$), hippocampus ($F = 4.25$, $P < 0.04$), cerebellum ($F = 8.58$, $P < 0.01$), and medulla ($F = 6.21$, $P < 0.02$) in the placebo-treated ischemic than in placebo-treated sham and anti-IL-1β mAb-treated ischemic groups. These findings suggest that ischemia–reperfusion is associated with increases in blood volume in some brain regions and that systemic infusion of anti-IL-1β mAb reverses the ischemia-related increases in regional brain vascular spaces.

BBB permeability (K_i) measurements

The regional brain K_i values differed significantly across brain regions among the placebo-treated sham, placebo-treated ischemic, and anti-IL-1β mAb-treated ischemic groups (ANOVA: main effects for groups across brain regions, $F = 4.66$, $P < 0.03$, Fig. 4). The K_i values were higher across brain regions in the placebo-treated ischemic compared with the placebo-treated sham group (ANOVA: main effects for group across brain regions, $F = 7.83$, $P < 0.03$, Fig. 4). K_i values were lower across the brain regions of the anti-IL-1β mAb-treated ischemic compared with the placebo-treated ischemic group (ANOVA: main effects for group across brain regions, $F = 5.37$, $P < 0.05$). In addition, K_i values did not differ across the brain regions of the placebo-treated sham and anti-IL-1β mAb-treated ischemic treated groups (ANOVA: main effects for group across brain regions, $F = 0.49$, $P = 0.49$). K_i values also did not differ across the brain regions of the placebo-treated sham and anti-IL-1β mAb-treated sham groups (ANOVA: main effects for group across brain regions, $F = 0.50$, $P = 0.51$). Therefore, systemic infusions of anti-IL-1β mAb attenuate ischemia-related increases in regional BBB permeability in the fetal sheep brain.

The K_i values also exhibited significant heterogeneity among the brain regions within the fetuses of the placebo-treated sham (ANOVA: main effect for region, $F = 24.4$, $P < 0.001$), placebo-treated ischemic (ANOVA: $F = 16.7$, $P < 0.001$) and anti-IL-1β mAb-treated ischemic groups (ANOVA: $F = 34.9$, $P < 0.001$). Therefore, the anti-IL-1β mAb treatment did not attenuate the regional heterogeneity observed in the sham control or after ischemia in the fetal sheep brain.

The cerebral cortical K_i values demonstrated an inverse linear correlation with the concentration of anti-IL-1β mAb within in the cerebral cortical parenchyma ($r = -0.65$, $n = 13$, $P < 0.02$, Fig. 5).

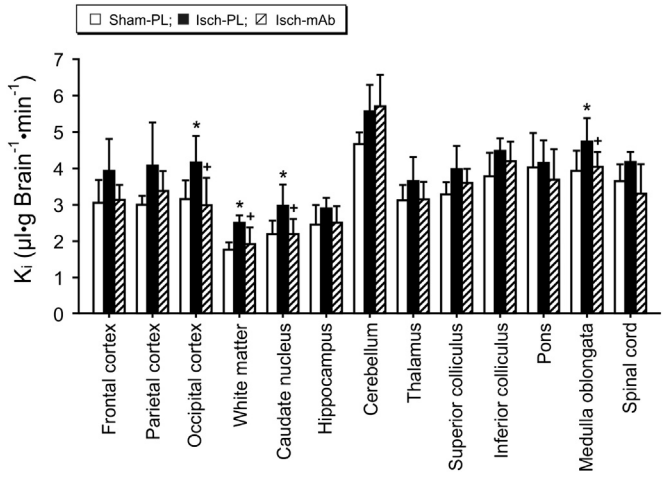

Fig. 4. Blood-to-brain transfer constants (K_i) in brain regions by study group. Blood-to-brain transfer constants (K_i) measured with AIB plotted on the Y-axis in the Sham-PL (open bars, n = 5), Isch-PL (closed bars, n = 6), and Isch-mAb (hatched bars, n = 7) plotted for brain regions on the X-axis. Sham-PL versus Isch-PL across brain regions (ANOVA: $F = 7.83$, $P < 0.03$); Isch-PL versus Isch-mAb across brain regions (ANOVA: $F = 5.37$, $P < 0.05$). Values are mean ± SD. *$P < 0.05$ vs. sham-PL group, $^{+}P < 0.05$ vs. Isch-PL group.

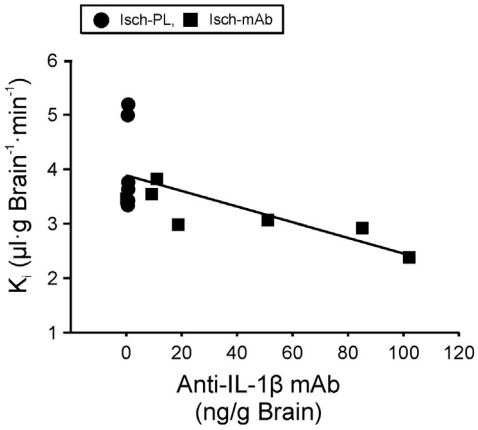

Fig. 5. K_i values measured with AIB in the cerebral cortex of the Isch-PL (solid circles, n = 6), and Isch-mAb (solid squares, n = 7) fetuses plotted against the anti-IL-1β mAb concentrations in the cerebral cortex. The least squares linear regression analysis was used to compare the K_i values to the anti-IL-1β mAb concentrations in the cerebral cortex, $r = -0.65$, $n = 13$, $P < 0.02$.

Tight junction protein expression

We selected three brain regions (cerebral cortex, caudate nucleus, and medulla oblongata) which exhibited significant differences in K_i values among the placebo-treated sham, placebo-treated ischemic and anti-IL-1β mAb-treated ischemic groups, to measure the expression of TJ proteins. In addition, we also examined the expression of TJ proteins in the cerebellum in order to examine a region in which K_i values did not differ between groups. There were no differences in occludin, claudin-1, claudin-5, ZO-1 or ZO-2 protein expressions in the parietal cortex among the placebo-treated sham, placebo-treated ischemic and anti-IL-1β mAb-treated ischemic groups (Fig. 6A). In the caudate nucleus, claudin-1 protein expression was higher (ANOVA: main effect for group, $F = 5.9$, $P < 0.02$, Fig. 6B) and ZO-2 lower (ANOVA: main effect for group, $F = 40.78$, $P < 0.001$) in the placebo-treated ischemic and anti-IL-1β mAb-treated ischemic compared with the placebo-treated sham group. In the cerebellum, ZO-2 protein expression was lower in the placebo-treated ischemic and anti-IL-1β mAb-treated ischemic groups than the placebo-treated sham group (ANOVA: main effect for group, $F = 10.54$, $P < 0.01$, Fig. 6C). In the medulla oblongata, occludin protein expression was lower in the anti-IL-1β mAb-treated ischemic than in the placebo-treated sham group (ANOVA: main effect for group, $F = 6.35$, $P < 0.02$, Fig. 6D) and ZO-1 was lower in the anti-IL-1β mAb-treated ischemic than in the placebo-treated ischemic group (ANOVA: main effect for group, $F = 6.15$, $P < 0.02$). Therefore, although anti-IL-1β mAb treatment attenuated the ischemia-related increases in BBB permeability determined by K_i in the brain, major changes in the expression of the tight junction proteins were not associated with changes in BBB permeability.

Figs. 7A–C contains a schematic representation of the results of our study. In the placebo sham group, the BBB is relatively impermeable (Fig. 7A). Ischemia in the placebo-treated group results in increases in BBB permeability in some brain regions, which is associated with increases in cerebral cortical concentrations of IL-1β protein (Fig. 7B). Systemic anti-IL-1β mAb infusions increase anti-IL-1β mAb levels in the systemic circulation and brain parenchyma, and are associated with decreases in IL-1β and a small increase in TNF-α in the brain parenchyma, and with decreases in regional BBB permeability (Fig. 7C).

Discussion

The overall objectives of the current study were to examine the ability of systemically infused anti-IL-1β mAb to enter the fetal brain after ischemia and to attenuate ischemia-related increases in BBB

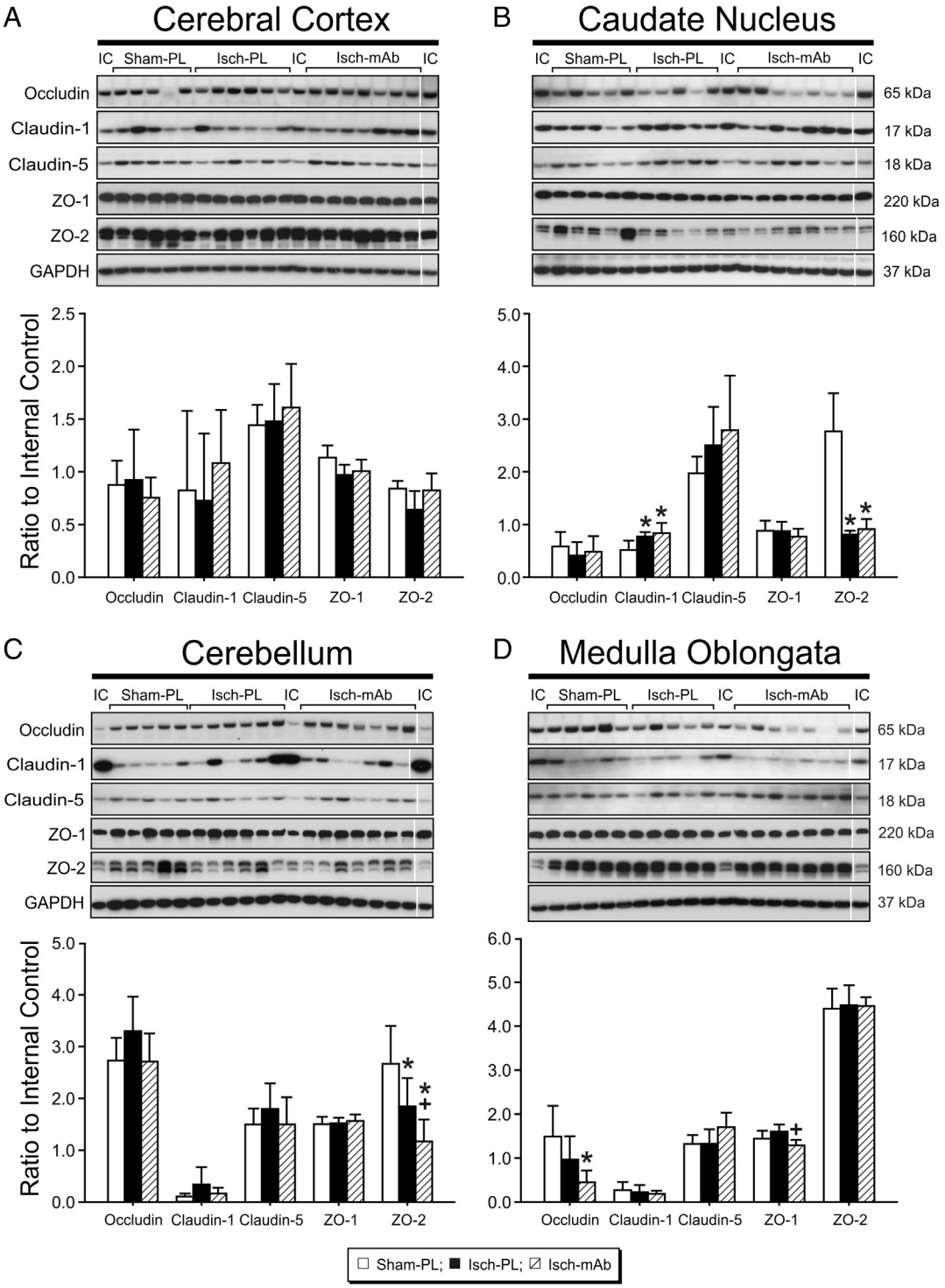

Fig. 6. Tight junction protein expression in the cerebral cortex, caudate nucleus, cerebellum, and medulla oblongata by study group. Expression of occludin, claudin-1, claudin-5, ZO-1, and ZO-2 proteins in the Sham-PL (open bars, n = 5), Isch-PL (closed bars, n = 5–6), Isch-mAb (hatched bars, n = 7) groups in cerebral cortex (A), caudate nucleus (B), cerebellum (C), and medulla oblongata (D) measured by Western immunoblot. Immunoblots shown above the bar graphs. IC indicates the internal control standard protein derived from the adult cerebral cortex. GAPDH is also shown as a loading control. Values are expressed as mean ± SD. $^{*}P < 0.05$ vs. Sham-PL, $^{+}P < 0.05$ vs. Isch-PL group.

permeability in the ovine fetus (Chen et al., 2012). The novel findings of the current study were as follows. First, after exposure to ischemia and intravenous infusions of anti-IL-1β mAb, cerebral cortical anti-IL-1β mAb levels increased. Second, intravenous anti-IL-1β mAb infusions attenuate ischemia-related increases in the concentration and expression of IL-1β protein in fetal brain parenchyma. Third, the anti-IL-1β mAb infusions reduce ischemia–reperfusion related increases in BBB permeability across brain regions, but do not have a major impact upon the expression of TJ proteins in the fetal brain.

In this study, we achieved near-complete cessation of carotid arterial blood flow to the fetal brain for 30 min and attenuations in ECoG amplitude confirming that the ischemic insults were comparable in the placebo- and anti-IL-1β mAb-treated ischemic groups. After ischemia, the values for ECoG, biochemical, heart rate, and mean arterial blood

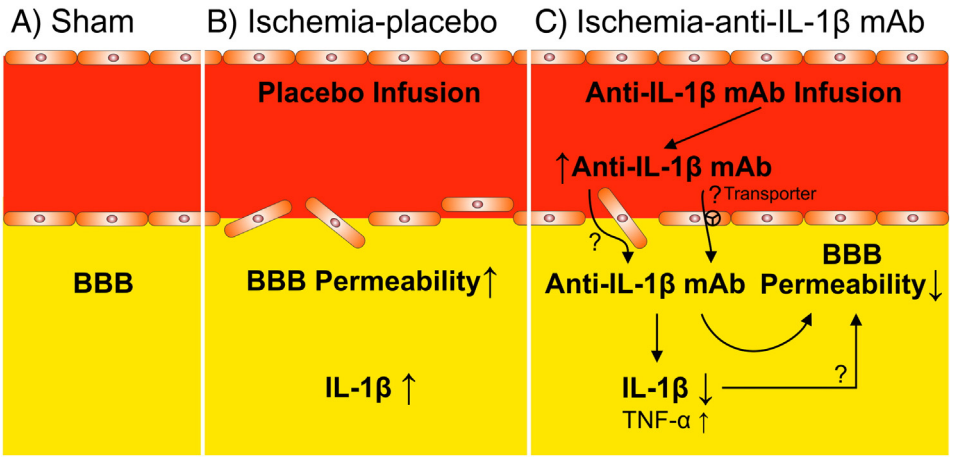

Fig. 7. Schematic representation of the results. In the sham group, the BBB is relatively impermeable (A). Ischemia in the placebo-treated group results in increases in BBB permeability in some brain regions, which is associated with increases in cerebral cortical concentrations of IL-1β protein (B). Systemic anti-IL-1β mAb infusions increase anti-IL-1β mAb levels in the systemic circulation and brain parenchyma, and are associated with decreases in IL-1β and a small increase in TNF-α in the brain parenchyma, and decreases in regional BBB permeability (C).

pressure did not differ from the baseline values between or within the placebo-treated sham, placebo-treated ischemic and anti-IL-1β mAb-treated ischemic groups. Therefore, it appears that mAb treatment after exposure to ischemic brain injury did not have major adverse effects upon systemic biochemical and metabolic homeostasis in fetal sheep.

Consistent with our previous findings showing increases in the expression of IL-1β protein 48 and 72 h after ischemia in the ovine fetal brain (Sadowska et al., 2012), we confirmed that both the expression and concentrations of IL-1β protein increase 24 h after ischemia (Figs. 2C and 3). Intravenous infusions of anti-IL-1β mAb resulted in significant mAb uptake into fetal parietal cortex after ischemia. Elevated anti-IL-1β mAb levels (Fig. 2A) were associated with attenuation in ischemia-related increases in IL-1β concentration (Fig. 2C) and expression (Fig. 3) strongly suggesting that anti-IL-1β mAb infusions have important specific biological effects on IL-1β after ischemia in fetal brain. Changes in the quantity of IL-1β in brain could reflect reduced transfer, binding and/or removal of endogenous IL-1β cytokine. However, given that this is a model of pure ischemia and there was no change in circulating levels of IL-1β levels, we speculate that the decreases in the expression and concentration of IL-1β result from removal of the endogenous IL-1β protein.

Anti-IL-1β mAb administration after ischemia resulted in *small*, but significant, increases in TNF-α protein expression in the cerebral cortex. Therefore, the changes observed with mAb treatment could be a result of direct inhibition of mAb on IL-1β and/or related in part to the increases in TNF-α after mAb treatment. However, in view of the fact that inhibiting endogenous TNF-α is neuroprotective after ischemic brain injury (Barone et al., 1997; Yang et al., 1999), the increases in cerebral cortical TNF-α could represent a potential untoward side effect of the anti-IL-1β mAb treatment. Therefore, we speculate that combined inhibition of TNF-α (Yang et al., 1999) and IL-1β could potentially have enhanced protective affects on ischemia-related BBB dysfunction because inhibition of each cytokine alone attenuates injury to the BBB. On the other hand, although IL-6 expression is highly dependent upon IL-1 signaling in the brain (Thorns et al., 2002), mAb treatment was not associated with significant changes in IL-6 expression (Fig. 3).

We also identified increases in anti-IL-1β mAb in CSF of fetal sheep after the mAb infusions and ischemia (Fig. 2B). CSF provides physical protection for the brain and removes brain metabolites via CSF drainage. Recent information suggests that growth factors and other factors within the CSF could play an important role in development, homeostasis, and repair of the central nervous system (Johanson et al., 2011). In numerous species including human premature infants, protein CSF concentrations are very high during development suggesting their importance to support brain growth and development (Johanson et al., 2011). Moreover, the choroid plexus, ependymal cells of blood–CSF, and fetal CSF–brain barriers could be damaged by perinatal hypoxic–ischemic brain injury (Rothstein and Levison, 2002). Hence, the anti-IL-1β mAb that accumulated in CSF in the mAb infused ischemic group could reflect blood–CSF and CSF–brain barrier damage facilitating entry of mAb from plasma and brain interstitial fluid into CSF. The increase in CSF mAb could also facilitate entry the mAb into brain via extracellular pathways (Banks, 2004) and enhance repair processes in the CNS after injury.

Increases in the regional brain vascular spaces after ischemia were reversed by treatment with anti-IL-1β mAb (Table 2). Cerebral blood volume has been previously reported to be, normal, increased, or decreased after acute brain ischemia, which appears to depend upon the severity of hypoperfusion (Hatazawa et al., 1999). However, there is no information available on changes in vascular volume after ischemia in fetal subjects treated with anti-IL-1β antibody infusions. Our findings suggests that IL-1β is involved in the increases in regional vascular volumes after ischemia in the fetal brain and is consistent with previous work showing increases in cerebral blood volume after transient ischemic–reperfusion injury along with influences of IL-1β on cerebral blood flow (Maher et al., 2003). However, the mechanism(s) by which mAb treatment diminishes the ischemia-related increases in vascular space and whether the regional vascular space changes are beneficial to the brain after ischemia require further investigation. Nonetheless, the regional differences in blood volume do not account for ischemia–reperfusion or treatment-related BBB permeability changes because the calculation of K_i accounts for the residual AIB remaining in the vascular space at the end of the studies (Blasberg et al., 1983; Stonestreet et al., 1996).

Most drugs with CNS activity either enter the brain by diffusing across the BBB or are transported by carrier systems across membranes (Banks, 2004). Substances that cannot cross the BBB are largely excluded from the CNS (Banks, 2004). A challenge to develop neuroprotective agents has been exclusion of drugs from the brain by the BBB (Banks, 2004). Our findings demonstrate that systemic anti-IL-1β mAb infusions after ischemia result in anti-IL-1β mAb penetration into brain, diminish ischemia-related increases in IL-1β protein, and attenuate ischemia-related increases in BBB permeability in brain including cerebral cortical regions (Fig. 4). These findings suggest that anti-IL-1β mAb penetration into brain exerts important biological effects on the brain parenchyma. Furthermore, our findings also suggest that IL-1β protein contributes to impaired BBB function after ischemia and that infusions

of anti-IL-1β mAb have protective effects on ischemia-related alterations in BBB function in the fetus. However, the results also imply that IL-1β protein is not the only factor contributing to BBB dysfunction after ischemia because the anti-IL-1β mAb infusions do not return BBB function to control levels in all brain regions (Fig. 4). Alternatively, systemic infusions of mAb do not affect all brain regions to the same degree. Nonetheless, the findings that anti-IL-1β mAbs attenuate increases in BBB permeability after ischemia are novel because previous work has not examined the capability of a selective neutralizing mAb for IL-1β protein to attenuate BBB dysfunction after ischemia in any species or age group. In the current study, we administered the mAb shortly after the onset of reperfusion to determine if the anti-IL-1β mAb was efficacious in reducing ischemia-related increases in BBB permeability. In future studies, it would be of great interest to determine whether a more clinically relevant delayed treatment paradigm of 2 to 6 h would also be effective in attenuating the ischemia related increases in BBB permeability in the fetus.

Little information is available regarding mechanisms underlying regional differences in BBB vulnerability to ischemic insults in the developing brain (Chen et al., 2012). However, our current findings are consistent with our earlier work in the fetus showing regional variations in BBB permeability under basal conditions (Stonestreet et al., 1996), and after stress related conditions such as ventilation (Stonestreet et al., 2000a), osmolar stress (Stonestreet et al., 2006, 2012) and in response to maternal treatment with glucocorticoids (Sadowska et al., 2006; Stonestreet et al., 2000b). The reasons for the regional BBB heterogeneity under basal conditions and in response to stress including ischemia are most likely multifactorial (Chen et al., 2012; Stonestreet et al., 1996, 2000b, 2006, 2012). They probably include regional differences in brain metabolism, differences in the various components of the neurovascular unit (Abbott et al., 2010; Sadowska et al., 2009), and potentially differences in local regional vascular cytokines/growth factors. Inspection of Fig. 4, suggests that ischemia is associated with differences in the regional BBB responses and that treatment with anti-IL-1β antibody does not attenuate the increases in ischemia-related BBB permeability to an equivalent extent in all brain regions. Potential mechanisms for the regional heterogeneity could include relative differences in regional BBB maturation (Stonestreet et al., 1996), and regional variations in metabolism in response to ischemia and/or antibody treatment. However, other possibilities could include responses of IL-1β and/or other cytokines within the regional vasculature to ischemia, and/or differences in their concentrations within different brain parenchymal regions, and the possibility that the anti-IL-1β antibody exhibits regional differences in its penetration into the vasculature and/or parenchyma.

Our findings are also consistent with reports suggesting that systemic antibodies can enter the brain and have therapeutic effects in adult subjects (Banks, 2004; Banks et al., 2002, 2005; Kozlowski et al., 1992). IgG has been shown to enter the brain after closed head-injury (Lu et al., 2001), under hypertonic conditions (Rapoport, 2000), during hypertension (Kuang et al., 2004), and in models of Alzheimer's disease (Banks et al., 2002, 2005). In addition, related work has also shown that infusions of anti-TNF-α mAb reduce brain injury and enhance cerebral blood flow after stroke in adult rats (Lavine et al., 1998) and that BBB disruptions are attenuated by intraventricular injections of neutralizing anti-TNF-α mAb (Yang et al., 1999). These findings combined with our results in fetal sheep suggest that both neutralizing anti-TNF-α and -IL-1β mAb attenuate ischemia-related increases in BBB permeability.

The infusions of cytokine neutralizing antibodies could be protective by one of several mechanisms. One possibility is that they could enter the brain via extracellular pathways (Banks, 2004). A second possibility is that although antibodies are relatively large molecules, which might not penetrate the intact BBB easily, ischemia could facilitate the entry of antibodies across the impaired BBB in the ovine fetus (Chen et al., 2012). Under these circumstances, molecules even as large as antibodies could enter the brain and inhibit the effects of pro-inflammatory cytokines (Figs. 2A, C and 3). The neutralizing anti-IL-1β mAb could also have protected the BBB by inhibiting pro-inflammatory cytokines generated during ischemia–reperfusion and/or inhibiting ischemia-related increases in endothelial derived cytokines that could then leak into the brain via a damaged BBB (Stanimirovic and Satoh, 2000).

The mechanisms by which increases in IL-1β potentially contribute to BBB dysfunction after ischemia cannot be discerned by our studies. However, IL-1β has been shown to potentiate the action of bradykinin, which increases BBB permeability (Hu and Fraser, 1997). In addition, over-expression of IL-1β released from astrocytes and microglia could up-regulate the expression of endothelial and neutrophil adhesion molecules and reactive oxygen species in the cerebral vasculature, subsequently escalating leukocyte recruitment across the BBB (Shaftel et al., 2007). Increased leukocyte recruitment could also increase BBB permeability (Shaftel et al., 2007). Furthermore, changes in the molecular composition of TJs could also have accounted for the ability of anti-IL-1β mAbs to attenuate increases in BBB permeability after ischemia (Yamasaki et al., 1995). Bolton et al. showed that IL-1β increased BBB permeability via decreases in occludin and ZO-1 TJ proteins (Bolton et al., 1998). However, we did not observe major changes in TJ proteins after ischemia and anti-IL-1β mAbs treatment. Consequently, we cannot conclude that ischemia-related increases in endogenous IL-1β protein influenced the expression of TJ protein expression in fetal brain. Numerous different components of the neurovascular unit control the integrity of the BBB including TJ proteins, microvascular interactions of the endothelium with extracellular matrix, and neural elements including astrocyte end-feet, pericytes, and neurons (Hawkins and Davis, 2005). Therefore, the exact mechanism(s) by which IL-1β affects the BBB after ischemia in the developing brain require further investigation. Although we have not examined the effects of anti-IL-1β mAb on measures of neuronal injury in the current study, it would be of interest in future studies to determine the effects of this mAb on caspase activity and/or DNA fragmentation.

Conclusion

The pro-inflammatory cytokine IL-1β contributes to BBB dysfunction after ischemic injury in the fetus. Systemic infusions of anti-IL-1β mAb results in penetration of mAb into the fetal brain and has biological effects that include diminishing the ischemia-related endogenous up regulation of IL-1β protein and increases in BBB permeability in the fetal brain. We speculate that treatment of human neonates with anti-IL-1β mAb is feasible as Canakinumab, a fully humanized anti-IL-1β antibody, is currently in use and showing favorable efficacy in pediatric clinical trials for inflammatory related disorders (Kuemmerle-Deschner et al., 2011; Ruperto et al., 2012). Thus, intravenous anti-IL-1β mAb treatment of preterm and full term infants facing adverse perinatal events may be a promising therapeutic strategy.

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