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The effects of betamethasone on allopregnanolone concentrations and brain development in preterm fetal sheep

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ABSTRACT

Background: The risk of preterm delivery often means that the fetus will be exposed to exogenous synthetic glucocorticoids to accelerate fetal lung maturation, but effects on other organs, particularly the brain, are not understood. The neurosteroid allopregnanolone (AP) is a $GABA_A$ receptor agonist that influences fetal brain development, suppresses CNS activity, and has neuroprotective properties. In this study we determined the impact of maternal glucocorticoid (betamethasone) administration on brain development and AP synthesis in preterm fetal sheep.

Methods: Pregnant ewes underwent surgery at 105 days gestation (term ~147) for implantation of fetal catheters. Ewes received either betamethasone (BM; 11.4 mg; $n = 10$) or vehicle (saline; $n = 5$) by i.m injection on days five (BM1) and six (BM2) following surgery. Five fetuses of the BM treated ewes received an intra-carotid artery infusion of alfaxalone (20 mg) over 48 h commencing 30 min prior to BM1. All animals were euthanased on day 7 (48 h after BM1), and the fetal brains collected to determine AP concentrations and histopathology.

Results: BM significantly reduced AP levels in the fetal brain (cortex, periventricular white matter, striatum, hippocampus, cerebellum) and placental cotyledons, and also in fetal plasma without altering progesterone concentrations. There was a significant decrease in the number of myelinating cells (MBP-positive) in subcortical white matter, but no change to total oligodendrocyte (Olig-2 positive) number. Co-administration of the AP analog alfaxalone with BM prevented this change in MBP expression.

Conclusions: BM, given at a dose clinically prescribed to accelerate lung maturation, adversely affects neurosteroid levels in the preterm fetal brain, and affects the maturational profile of white matter development; these effects were mitigated by the co-administration of alfaxolone.

1. Introduction

In human pregnancy, the endogenous neurosteroid allopregnanolone (AP) is present in relatively high concentrations in both the maternal and fetal circulations at the time of birth (Bicikova et al., 2002). AP is a $GABA_A$ receptor agonist that, due to its role in suppressing fetal central nervous system (CNS) activity and reducing excitotoxicity, is considered a neuroprotective, neuroactive steroid (Yawno et al., 2007). Indeed, we have shown that AP moderates apoptosis and enhances neuronal cell proliferation in the late gestation fetal brain, consistent with it having a role in normal brain development (Yawno et al., 2009). In pregnancy, the precursors for AP synthesis, such as progesterone and pregnenolone, are primarily derived from the placenta (Crossley et al., 1997). AP concentrations in fetal plasma and brain increase as pregnancy progresses, but then decrease abruptly at birth with loss of the placental circuit (Nguyen et al., 2003). Accordingly, because of premature birth results in premature separation from the placenta, babies born preterm are deficient in AP during an important phase of brain development, an effect that can be mitigated by the administration of progesterone to the neonate, at least in preterm guinea pigs (Kelleher et al., 2013).

Administration of synthetic glucocorticoids to women in anticipation of preterm birth is now standard clinical practice to advance fetal lung maturation, and world-wide it has resulted in reduced neonatal mortality and morbidity (NIH Consensus Statement, 1994). However, a number of potentially adverse non-pulmonary effects of antenatal glucocorticoids have been described (Miller

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and Wallace, 2013), including reduction of fetal growth (Miller et al., 2007, 2012; Sutherland et al., 2012), reduced fetal brain weight (Huang et al., 1999), and reduced myelination within the fetal brain (Dunlop et al., 1997; Huang et al., 2001; Antonow-Schlorke et al., 2009). In human pregnancies, for example, birth weight is reduced for babies born more than 7 days after single (Murphy et al., 2012) or multiple (Wapner et al., 2007; Crowther et al., 2011) courses of maternal glucocorticoid treatment. In mice, a single prenatal dose of betamethasone (BM) impaired performance of the offspring in behavioral tests (Rayburn et al., 1998). In children, recent work shows an association between antenatal glucocorticoids and altered brain development, including a thinner cortex and increased vulnerability to mental health problems in children at 6–10 years of age (Hirvikoski et al., 2007; Davis et al., 2013).

Synthetic glucocorticoid therapy also affects neurosteroid synthesis, reducing plasma AP concentrations in adult humans (Genazzani et al., 1998), and decreasing 5α-reductase type 2 expression, a key enzyme in AP production, in the guinea pig placenta resulting in effects on the fetal brain (McKendry et al., 2010). Given the importance of neurosteroids for the normal growth and functional development of the brain, we wished to determine whether maternal administration of BM could alter neurosteroid synthesis in the preterm fetal sheep brain, particularly in white matter since this could render these brain regions vulnerable to hypoxic-ischemic damage. To determine if any effects of BM administration were due to loss of AP in the brain, we also determined if the co-administration of alfaxalone, a synthetic form of AP, could mitigate any such effects.

2. Materials and methods

2.1. Animals and surgical preparation

All experimental procedures received prior approval from the relevant institutional animal ethics committee and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Fifteen twin-bearing ewes of known gestational age were used; only one fetus per twin was utilized for this study. During the study, ewes were kept in individual cages with free access to food and water under a 12-h light/dark cycle (lights on, 07:00 h). As previously described (Sutherland et al., 2012), surgery was performed under isoflurane (Isoflo; Abbott, Sydney, New South Wales, Australia) general anesthesia at 105–110 days gestation (term is ~147 days gestation) for implantation of polyvinyl catheters (Dural Plastics, Silverwater, Australia) into a jugular vein of the ewe, and into a fetal brachial artery, femoral artery, and the amniotic sac. The fetus was then returned to the uterus, the catheters were exteriorized through an incision in the ewe's flank, the abdominal incisions repaired, and the ewe was recovered from anesthesia. Antibiotics were given for 3 days post-surgery by maternal intravenous (i.v.) injection (0.1 mg/kg oxytetracycline; Engemycin©, MSA Animal Health, New Zealand), and also into the amniotic sac (250 mg ampicillin; Austrapen, CSL Ltd, Parkville, Australia).

2.2. Experimental design

Fetal arterial blood samples were taken daily following surgery for analysis of fetal oxygenation and acid-base status (ABL700 blood gas analyzer, Radiometer, Copenhagen, Demark). On day 5 following surgery, ten ewes (5 male and 5 female fetuses) received 11.4 mg BM by intramuscular (i.m.) injection (BM1; Celestone Chronodose; Schering Plough, Sydney, Australia), followed by a second dose of 11.4 mg BM (BM2) 24 h later. This dosing regimen reflects the clinical use of BM (Wallace and Baker, 1999; Robertson et al., 2009). Control ewes ($n = 5$; 3 females and 2 males) received an equal volume (3 ml) of saline by intramuscular injection. The suitability of sheep as a model for studying the effects of glucocorticoids on the fetus is supported by measurements of fetal plasma betamethasone concentrations with similar values and rates of clearance in humans (Ballard et al., 1975; Berry et al., 1997). Thirty minutes prior to BM1, five fetuses (3 females and 2 males) in the BM group received synthetic AP (Alfaxalone; 5α-pregnane-3β-ol,11,20-dione; obtained as the commercial formulation Alfaxan-CD RTU; Jurox Pty. Ltd., Rutherford, New South Wales, Australia) as a bolus injection of 10 mg alfaxalone in 1 ml given into the fetal brachial artery, followed by infusion at 2.5 mg/kg/h for 48 h as previously reported (Yawno et al., 2009). Fetal arterial blood samples were collected before each BM dose and at 6 and 12 h after BM1 and BM2 for measurement of SaO_2, PaO_2, $PaCO_2$, glucose, lactate, AP and progesterone. Fetal mean arterial blood pressure (MAP) and heart rate (HR) was calculated by taking a mean of the data over 30 s epochs from 12 h before BM1 (basal) until 12 h after the BM2 treatment.

On day 7, all animals were killed by maternal i.v. injection of pentobarbitone (Lethabarb; Virbac, Peakhurst, Australia), and the fetal brain removed immediately and divided sagittally along the midline. The right hemisphere was placed in a custom-made mold shaped to fit the fetal sheep brain, and coronal sections were obtained by cutting through the hemisphere at 0.5 cm intervals. These slices were fixed by immersion in 4% paraformaldehyde (ProSci Tech, Thuringowa, QLD, Australia) for 48 h, prior to embedding them in paraffin and subsequently obtaining 10 μm microtome sections for histological analysis. The left side of the brain was divided coronally as above, with micro-dissected samples of periventricular white matter (PVWM), cortical gray matter, striatum, hippocampus and the cerebellum frozen in liquid nitrogen and stored at −70 °C for analysis of neurosteroid content. We did not attempt to dissect subcortical white matter (SCWM) and the corpus callosum for biochemical analysis due to the difficulty of obtaining tissue completely free of contamination from the adjacent, non-white matter regions. Whole placental cotyledons were removed, divided into full-thickness smaller sections, and then frozen in liquid nitrogen and stored at −70 °C for analysis of neurosteroid content. Cotyledons from the alfaxalone + BM group were not available for analysis.

2.3. AP radioimmunoassay (RIA)

AP was extracted from fetal plasma, brain and placental tissue by modification of the method of Barbaccia et al. (1992), as previously described (Yawno et al., 2007). Recovery of AP was 81.0 ± 4.0%, and assay results were corrected for this extraction loss in the final calculation of AP concentrations. AP was quantified with an in-house assay using a polyclonal antibody (Agrisera, Sweden). The assay limit of detection for AP was 52.7 ± 2.7 pg/tube ($n = 3$) and the intra- and inter-assay coefficients of variance were 8% and 10%, respectively.

2.4. Progesterone radioimmunoassay (RIA)

Progesterone was extracted from fetal plasma by modification of the method of Barbaccia et al. (1992). Progesterone was quantified using a commercially available kit (Cayman Progesterone EIA Kit; Cayman Chemical Company, MI, USA). The limit of detection for progesterone was 10 pg/ml.

2.5. Histopathology

Myelin Basic Protein (MBP) was used for estimation of the number of mature, myelin-producing oligodendrocytes and axonal myelination, and *Oligodendrocyte transcription factor 2 (Olig-2)*, a marker for oligodendrocytes at all stages of their lineage (Jakovcevski et al., 2009), was used for estimation of the total oligodendrocyte number. *Cyclic-nucleotide-3`-phosphodiesterase (CNPase)* was used for the analysis of the density of myelin sheaths in oligodendrocytes (immature and mature) and Schwann cells. *NEUronal Nuclei, clone A60 (NeuN)* was used to identify neuronal nuclei. MBP, Olig-2, CNPase and NeuN were identified using rabbit anti-human myelin basic protein (1:500; Millipore, MA, USA), rabbit anti-Olig 2 (1:1000; Millipore, MA, USA), mouse anti-CNPase (1:200; Sigma–Aldrich, USA) and mouse anti-NeuN (1:200; Millipore Corporation, USA), respectively. Apoptotic cell death was identified with anti-Capase-3 Active (1:1000; R & D Systems, Minneapolis, USA). Antibodies were diluted in phosphate buffered saline (PBS) solution (0.1 mol/L, pH 7.4). All sections were treated with a secondary antibody (1:200; biotinylated anti-rabbit or anti-mouse IgG antibody; Vector Laboratories, Burlingame, CA, USA) and immunolabeling was visualized using 3,3′-diaminobenzidine (DAB; Pierce Biotechnology, Rockford, IL, USA). Positive and negative control sections were included in each run. Sections were viewed at 400× magnification using a light microscope (Olympus BX-41, Japan), cell bodies were counted and axonal density was measured using ImageJ software (ver. A1.45b, National Institutes of Health, USA). Cells were counted by MM who was blinded to the treatment. Three fields of view were analyzed in each section, and two sections of each brain region were examined. The average value for each fetus was obtained from these six fields of view, and the results were averaged across all animals ($n = 5$) in each group.

2.6. Statistical analysis

Data are shown as mean ± standard error. Statistical analysis was performed with SigmaStat software (Systat Software, San Jose, CA, USA) and/or GraphPad Prism (GraphPad Software, San Diego, CA, USA). One-way ANOVA was used to analyze fetal AP levels and histological data, with post-hoc Least Significant Difference as required. Differences were considered significant at $P \leq 0.05$.

3. Results

BM administration significantly reduced fetal weight from 2.0 ± 0.1 kg to 1.6 ± 1.1 kg by day 7 after surgery (i.e., 115 ± 1.0 days gestation) but had no effect on brain weight so that the fetal brain-to-body weight ratio was significantly increased (Fig. 1). Co-administration of alfaxalone with BM prevented the fall in body weight (1.9 ± 0.2 kg) and resulted in a normal brain-to-body weight ratio (Fig. 1).

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