Published in final edited form as:

Brain Res. 2009 December 15; 1303: 15–25. doi:10.1016/j.brainres.2009.09.086.

Ontogeny and the Effects of Exogenous and Endogenous Glucocorticoids on Tight Junction Protein Expression in Ovine Cerebral Cortices

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Abstract

Maternal glucocorticoid treatment reduces blood-brain permeability early, but not late in fetal development, and pretreatment with glucocorticoids does not affect barrier permeability in newborn lambs. In addition, endogenous increases in plasma cortisol levels are associated with decreases in blood-brain barrier permeability during normal fetal development. Therefore, we tested the hypotheses that development as well as endogenous and exogenous glucocorticoids alters the expression of tight junction proteins in the cerebral cortex of sheep. Cerebral cortices from fetuses at 60%, 70%, and 90% of gestation, newborn and adult sheep were snap frozen after four 6-mg dexamethasone or placebo injections were given over 48-h to the ewes and adult sheep. Lambs were treated similarly with 0.25 mg/kg-dexamethasone or placebo. Tight junction protein expression was measured by Western immunoblot. Claudin-1 was higher ($P<0.05$) in fetuses at 60% of gestation than in newborn and adult sheep. Claudin-5 was higher at 60% than 70% of gestation, and than in newborn and adult sheep. ZO-1 was higher in newborn than adult sheep. ZO-2 was higher at 90% gestation, in newborn and adult sheep than 60% gestation. Claudin-5 was higher in dexamethasone than placebo-treated lambs, and ZO-2 was higher in fetuses of dexamethasone than placebo-treated ewes at 90% gestation. ZO-2 expression demonstrated a direct correlation with increases in plasma cortisol during fetal development. We conclude that claudin-1, claudin-5, ZO-1, and ZO-2 expression exhibit differential developmental regulation, exogenous glucocorticoids regulate claudin-5 and ZO-2 *in vivo* at some, but not all ages, and increases in endogenous fetal glucocorticoids are associated with increases in ZO-2 expression, but not with occludin, claudin-1, claudin-5 or ZO-1 expression in ovine cerebral cortices.

Keywords

adult; blood-brain barrier; fetus; sheep; steroids; tight junction proteins

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1. Introduction

The blood-brain barrier has been shown to be developmentally regulated [24,35,45]. We have previously demonstrated ontogenic decreases in blood-brain barrier permeability with development from 60% of gestation up to maturity in adult sheep [45]. The blood-brain barrier is composed of a continuous layer of cerebrovascular endothelial cells connected by intercellular tight junctions [8]. The tight junctions are composed of transmembrane and associated cytoplasmic proteins [23]. The transmembrane proteins of the claudin family seal the intercellular region between adjacent endothelial cells, whereas occludin, an important support molecule, increases electrical resistance across the barrier and decreases paracellular permeability [23]. The associated cytoplasmic proteins ZO-1 and ZO-2 stabilize tight junctions by connecting them to actin [23].

The protein constituents of the tight junctions exhibit developmental regulation in rodents [24,35]. Hirase et al., demonstrated that very little occludin was present in rat brain endothelial cells at P8, but that the expression increases with age [24]. Nico et al showed that the development of the ZO-1 proteins appeared to parallel the functional development of the barrier in the mouse [35]. In contrast to rodents, in which the development of the blood-brain barrier occurs primarily after birth and similar to our findings in the sheep fetus [45], the human barrier and its tight junction protein complex develop mainly *in utero* [10,11,50]. Claudins and occludin are expressed as early as 14 weeks in human fetuses and are thought to play important early roles in blood-brain barrier development [4,51]. Although we have demonstrated maturational decreases in blood-brain barrier permeability from early in fetal development to adulthood in sheep [45], information is not available regarding the expression of the proteins that compose of the tight junctions in the blood-brain barrier of a precocial species such as the sheep.

Maternally administered antenatal corticosteroids have been widely used to reduce the incidence of respiratory distress syndrome in low birth weight infants [34]. This therapy has also been shown to facilitate the transition from fetal to neonatal life by beneficial effects on multiple other organ systems including the brain [7,20,32,33,36,37,43]. Antenatal glucocorticoid administration has been reported to have an important role in lowering the risk of early-onset and severe intraventricular hemorrhage in premature infants [20,32,33]. These effects might be explained in part by accelerated vascular maturation. We have shown that maternally administered antenatal glucocorticoids reduce blood-brain permeability early, but not late in fetal development, and pretreatment with glucocorticoids does not effect barrier permeability in newborn lambs [46–48]. In addition, endogenous increases in plasma cortisol concentrations are associated with decreases in blood-brain barrier permeability during normal fetal development [47].

Glucocorticoids have been shown to increase the expression of tight junction proteins *in vitro* [19,38]. Dexamethasone up-regulates occludin and ZO-1 in an immortalized endothelial cell line [38] and hydrocortisone up-regulates occludin in immortalized cerebral capillary endothelial cells [19]. The ovine fetus has been widely used to investigate brain development [3,21,47]. The neurodevelopment of the immature ovine brain is similar to that of the premature infant with respect to completion of neurogenesis, onset of cerebral sulcation, and detection of the cortical component of the auditory evoked potentials [3,5,6,13]. However, there have been no reported studies examining the influence of glucocorticoid treatment during development or the effects of endogenous changes in fetal cortisol concentrations on the expression of the proteins components of the tight junction complex *in vivo* during development.

Given the above considerations, we tested the hypotheses that development as well as endogenous and exogenous glucocorticoids alters the expression of tight junction proteins in

the cerebral cortex of sheep. We examined the fetal sheep at 60%, 70%, and 90% of gestation, newborn lambs, and adult sheep. The rationale for examining the expression of the tight junction proteins in these age groups was that we have shown decreases in barrier permeability over these stages of development [45], and that the barrier was responsive to glucocorticoids in the fetuses at 60% and 70% of gestation, but not at 90% of gestation or in newborn lambs [40,47,48].

2. Results

The study subjects, pre-study treatment, plasma cortisol concentrations, and numbers of sheep in each group, from which the brain samples were obtained, are summarized in Table 1. The plasma cortisol samples were obtained 18 hours after the last dose of dexamethasone or placebo had been given to the fetuses, newborn lambs and adult sheep, and just before the cerebral cortical tissue samples were obtained. As expected the plasma cortisol concentrations were higher ($P<0.05$) in the newborn lambs than the fetal and adult sheep (Table 1, ANOVA, main effect for age group, $F=16.30$, $P<0.001$). There was an overall effect of dexamethasone versus placebo treatment on plasma cortisol concentration (ANOVA, main effect for dexamethasone versus placebo treatment, $F=12.36$, $P<0.01$; Figure 1). However, specific significant differences were not detected ($P>0.05$) by post hoc analyses between the fetuses of the ewes treated with placebo and dexamethasone or the lambs and adult sheep pretreated with placebo and dexamethasone.

There was an overall effect of age on occludin abundance (ANOVA, main effect for age group, $F=6.15$, $P<0.01$; Figure 1). However, specific significant differences were not detected ($P>0.05$) by post hoc analyses among the different age groups. Occludin abundance was not affected by dexamethasone treatment (ANOVA, main effect for placebo versus dexamethasone treatment, $F=1.03$, $P=0.31$; Figure 1).

Claudin-1 protein expression was higher in the fetuses of the placebo treated ewes at 60%, 70%, and 90% of gestation than in the placebo treated newborn and adult sheep (ANOVA, main effect for age group, $F=24.8$, $P<0.001$; Figure 2A). Dexamethasone treatment did not affect claudin-1 expression at any age (ANOVA, main effect for placebo versus dexamethasone treatment, $F=1.28$, $P=0.26$).

Claudin-5 protein expression was higher in the fetuses of the placebo treated ewes at 60% and 90% of gestation than at 70% of gestation; higher in the fetuses of the placebo treated ewes at 60% and 90% of gestation than in the placebo treated newborn sheep; and higher in the fetuses of the placebo treated ewes at 60%, 70%, and 90% of gestation and in the placebo treated newborn than in the placebo treated adult sheep (ANOVA, main effect for age group, $F=39.84$, $P<0.001$; Figure 2B). Claudin-5 expression was higher in the dexamethasone than in the placebo treated newborn sheep (ANOVA, interactions for age by placebo versus dexamethasone treatment, $F=4.24$, $P<0.01$; Figure 2B).

ZO-1 is expressed as two isoforms as a result of alternative RNA splicing differing in the presence of an 80-amino acid region referred to as “motif-a”: the 235 kDa upper band corresponds to the ZO-1a⁺ isoform, and the 225 kDa lower band corresponds to the ZO-1a⁻ isoform. The expression patterns are dynamic and cell specific during development [27,29]. Although we identified two ZO-1 protein bands in some age groups, the densitometry values for the two bands were not sufficiently distinct in all age groups to permit accurate analysis of the separate bands. Hence, the densitometry values of the two ZO-1 bands were combined for analysis. ZO-1 protein expression was higher in the fetuses of the placebo treated ewes at 60% and 90% of gestation and in the placebo treated adult sheep than in the fetuses at 70% of gestation, and higher in the placebo treated newborn sheep than in the fetuses of the placebo

treated ewes at 60%, 70%, and 90% of gestation and in the placebo treated adult sheep (ANOVA, main effect for age group, $F=29.33$, $P<0.001$; Figure 3). Dexamethasone treatment did not affect ZO-1 expression in any age group (ANOVA, main effect for placebo versus dexamethasone treatment, $F=0.28$, $P=0.60$; Figure 3).

We identified three ZO-2 protein bands in the ovine cerebral cortex at all ages. As described in the methods, we used MDCK as a positive control for ZO-2 and confirmed that the ZO-2 bands at 160 kDa were dependent upon incubation with primary antibody. Two isoforms of ZO-2 have previously been described [12]. However, only the ZO-2 A isoform was found to be abundant in human brain [12]. In the ovine cerebral cortex, we identified three ZO-2 bands; each band of the ZO-2 protein was analyzed separately (Figure 4A). There was an overall effect of age on the protein expression of the upper ZO-2 band (ANOVA, main effect for age group, $F=3.96$, $P<0.01$; Figure 4B). However, specific significant differences were not detected ($P>0.05$) by post hoc analyses among the different age groups. Protein expression of the upper ZO-2 band was not affected by dexamethasone treatment in any age group (ANOVA, main effect for placebo versus dexamethasone treatment, $F=0.16$, $P=0.70$; Figure 4B).

The protein expression of the middle ZO-2 band was higher in the fetuses of the placebo treated ewes at 90% of gestation than in the fetuses of the placebo treated ewes at 70% of gestation, and higher in the placebo treated newborn lambs and adult sheep than in the fetuses of the placebo treated ewes at 60%, 70% and 90% of gestation (ANOVA, main effect for age group, $F=56.42$, $P<0.001$; Figure 4C). Protein expression of the middle ZO-2 band was higher in the dexamethasone than placebo-treated fetuses at 90% of gestation (ANOVA, main effect for placebo versus dexamethasone treatment, $F=15.08$, $P<0.001$; Figure 4C).

The protein expression of the lower ZO-2 band was higher in the fetuses of the placebo treated ewes at 90% of gestation than those in the fetuses at 60% and 70% of gestation; higher in the placebo treated newborn lambs than in the fetuses of the placebo treated ewes at 60%, 70% and 90% of gestation, and higher in the placebo treated adult sheep than in the fetuses of the placebo treated ewes at 60%, 70%, and 90% of gestation and in the placebo treated newborn sheep (ANOVA, main effect for age group, $F=131.30$, $P<0.001$; Figure 4D). Protein expression of the lower ZO-2 band was higher in the dexamethasone than placebo-treated fetuses at 90% of gestation (ANOVA, placebo versus dexamethasone treatment, $F=16.27$, $P<0.001$; Figure 4D).

In order to examine the potential relationships between tight junction protein expression and endogenous glucocorticoid concentrations during normal fetal development, tight junction protein expression as a ratio to the internal control standard was compared to plasma cortisol concentrations using the least-squares regression analysis in the fetuses of the placebo treated ewes. The tight junction protein expression was also compared to gestational age, because the plasma cortisol concentrations increase with advancing gestation [47,53]. Occludin expression demonstrated a direct correlation with gestational age, but not with the endogenous plasma cortisol concentrations (gestation: $r=0.48$, $n=18$, $P<0.05$; plasma cortisol concentrations: $r=0.21$, $n=18$, $P=0.40$; Data not shown). Claudin-1, claudin-5 and ZO-1 protein expression values did not demonstrate correlations with gestational age or endogenous plasma cortisol concentrations (Claudin-1: gestation: $r=-0.43$, $n=15$, $P=0.11$, plasma cortisol concentrations: $r=-0.44$, $n=15$, $P=0.10$, $P=0.15$; Claudin-5: gestation: $r=0.07$, $n=15$, $P=0.79$; plasma cortisol concentrations: $r=-0.03$, $n=15$, $P=0.92$; ZO-1: gestation: $r=0.19$, $n=14$, $P=0.51$; plasma cortisol concentrations: $r=0.13$, $n=14$, $P=0.64$; Figure 5A). ZO-2 expression demonstrated direct correlations with both gestational age and plasma cortisol concentrations (gestation: $r=0.72$, $n=13$, $P<0.01$; plasma cortisol concentrations: $r=0.68$, $n=13$, $P<0.05$, Figure 5B).

The multiple regression partial correlational analysis was used to compare the relative strength of the association between the increases in gestation and plasma cortisol concentrations and changes in tight junction protein expression. This analysis confirmed that occludin protein expression showed a direct correlation with gestational age ($\beta=0.86$, $P=0.02$), but not with plasma cortisol concentration ($\beta=-0.46$, $P=0.19$). ZO-2 demonstrated direct correlations with both gestational age and plasma cortisol concentrations. However, the relative strengths of association were similar for the gestational age ($\beta=0.27$, $P=0.47$) and the plasma cortisol concentrations ($\beta=0.46$, $P=0.23$).

3. Discussion

We examined the ontogeny, and effects of exogenous and endogenous glucocorticoids on tight junction protein expression in the cerebral cortex of sheep. The ovine fetus has been widely used to investigate the development of the fetal brain [3,21,45]. Further, we have demonstrated ontogenic decreases in blood-brain barrier permeability in ovine fetuses from 60% of gestation up to maturity in adult sheep, that maternal treatment with a glucocorticoids reduced barrier permeability in the fetus early, but not late in fetal development, and that increases endogenous fetal cortisol concentrations are associated with developmental decreases in barrier permeability [40,45–47]. The novel findings of this study are the following: First, claudin-1, claudin-5, ZO-1, and ZO-2 protein expression exhibits differential developmental regulation. Second, treatment with exogenous glucocorticoids regulates claudin-5 and ZO-2 *in vivo* at some, but not all ages. Third, increases in endogenous glucocorticoids are associated with increases in ZO-2 protein expression, but not with changes in occludin, claudin-1, claudin-5 or ZO-1 expression in ovine cerebral cortices.

The tight junctions are composed of a complex array of transmembrane and cytoplasmic proteins [1]. The expression of these proteins is developmentally regulated in rodents [24, 35]. Occludin expression is low in rat brain endothelial cells at postnatal day eight, but increases by day 70 [24] and ZO-1 protein expression increases between fetal days 15 and 19, and after birth in mouse brain microvessels [35]. In contrast to the rodent, recent evidence confirmed that the proteins of the tight junction complex are present very early during human fetal brain development [2,4,51,52]. The developmental pattern of tight junction protein expression in human fetuses and premature infants is somewhat controversial because two studies reported maturational changes in the expression of these proteins [2,51], whereas, one did not find similar changes [4]. The primary endothelial tight junction molecules identified so far in the developing human brain include occludin, claudin-5, JAM-1 and ZO-1 [2,4,51,52]. Occludin and claudin-5 are expressed in the primary vessels of the telencephalon by the 12th week of gestation and show dramatic changes by mid gestation [51]. Human endothelial tight junction development begins earlier and proceeds faster than in other vertebrate species such as rodents [24,35].

Similar to the human, the brain of the ovine fetus develops primarily before birth [14]. Similar to findings in the human, we found that occludin, claudin-1, and claudin-5, ZO-1 and ZO-2 were expressed early in fetal life and throughout ovine development. The patterns of expression varied among the tight junction proteins with development, such that the transmembrane protein expression appeared higher in the fetuses than in the newborn and adult sheep. In contrast, the expression of the accessory cytoplasmic proteins such as expression of ZO-1 appeared highest in newborn sheep, and expression of ZO-2 appeared higher in the newborn and adult sheep than in the fetuses. Therefore, there appears to be differential regulation in the expression of the proteins constituting the tight junction complex in the ovine cerebral cortex during development.

It is important to point out that the patterns of change, which we observed in the tight junction protein expression, did not necessarily reflect the ontogenic changes in barrier permeability that we have reported [45]. Blood-brain barrier permeability of the cerebral cortex was similar at 60% and 90% of gestation and higher at these fetal ages than in newborn and adult sheep [45]. Whereas, the pattern of tight junction protein expression did not increase consistently after birth as would have been expected from the changes in barrier permeability [45]. Therefore, the lack of concordance between the patterns of change in barrier permeability [45] and expression of tight junction proteins is most likely because the amount of protein is not the only determinant of the barrier permeability properties *in vivo*. Rather, the proteins of the tight junction complex most likely work in concert to form an effective barrier [1]. Nonetheless, the expression of the ZO-1 and ZO-2 cytoplasmic adaptor proteins tended to increase after birth, which is consistent with the lower barrier permeability in the newborn and adult sheep [45]. ZO-1 and ZO-2 are important components of the tight junction. They are peripheral scaffolding proteins that appear to organize the transmembrane proteins and couple them to other cytoplasmic proteins and actin microfilaments. This interaction is critical to tight junction stability and function because ZO-1 dissociation from the junctional complex results in increased permeability [17,23]. ZO-2 coprecipitates with ZO-1 and binds to the structural constituents of the tight junction [23]. Therefore, it remains possible that the increases in the ZO-1 and ZO-2 expression after birth could contribute to the decreases in permeability that we reported in newborn lambs and adult sheep [45].

In this study, we measured tight junction protein expression only in the cerebral cortex because we had samples available from this region of the brain in all of the age and treatment groups from our former studies. However, inspection of the regional K_i values measured with α -aminoisobutyric acid in our former work [45] suggests that the cerebral cortex exhibits the smallest differences in the K_i between the fetuses at 60% of gestation and the adult sheep. Whereas, the more caudal brain regions, including the cerebellum, medulla and spinal cord, exhibit relatively larger developmental decreases in permeability between the fetuses at 60% of gestation and adult sheep. These findings suggest that the cerebral cortex represents a relatively privileged site because of its unique neuronal composition and function. Therefore, if we had been able to measure the tight junction protein expression of the more caudal brain structures, the maturational differences in protein expression could have been accentuated. Nonetheless, the early expression of the tight junction proteins that we observed in the ovine cerebral cortex is consistent with the very precocious barrier differentiation in the human fetal cerebral cortex [51,52].

We have shown that maternally administered exogenous antenatal glucocorticoids reduces blood-brain permeability at 60%, 70%, and 80% of gestation, but not at 90% of gestation and that postnatal glucocorticoid treatment does not reduce barrier permeability in lambs [40,46–48]. The site of action of glucocorticoids on the blood-brain barrier in the fetus [40,46,47] is most likely on the capillary endothelial cells because physiological hydrocortisone concentrations have been shown to increase trans-endothelial resistance and decrease permeability in cerebral capillary endothelial cells [25]. The main structures responsible for the action of glucocorticoids are most likely the intercellular tight junctions of cerebrovascular endothelium in the fetal blood-brain barrier because glucocorticoids *in vitro* up-regulate endothelial cell expression of several tight junction proteins [16–18,28,38]. Even though glucocorticoids are widely used to treat women at risk for premature labor [34] and to treat premature infants with chronic lung disease [54], there have been no previous studies examining the influence of this treatment on the regulation of tight junction proteins *in-vivo* in the brain of the fetus and newborn, except for our recent work [31]. We have shown that treatment with exogenous glucocorticoids increased claudin-5 in newborn lambs and ZO-2 expression in fetuses at 90% of gestation *in vivo*, but did not affect the expression of the other tight junction proteins at any other age group. Similar to findings in a model of the human

blood-brain barrier [18], the increases in tight junction proteins in the fetal and newborn cerebral cortex that we found suggests that the tight junctions are molecular targets of glucocorticoids after treatment of the mother or newborn.

Our findings confirm that exogenous glucocorticoids given in doses similar to those used in the clinical setting affect the regulation of claudin-5 and ZO-2 in the cerebral cortex of sheep at some, but not all ages. These increases are of particular importance because ZO-2 helps to stabilize transmembrane tight junction proteins [23], whereas claudin-5 increases trans-endothelial electrical resistance across the blood-brain barrier and decreases permeability of the barrier [1]. However, the effects of exogenous glucocorticoids that we have observed on claudin-5 and ZO-2 differ from our findings of their effects on barrier permeability in the fetus and newborn [47,48]. Although we observed an increase in claudin-5 in the lambs, the same dose of dexamethasone does not affect barrier permeability in the newborn lambs [48]. Likewise, although maternal glucocorticoid treatment at 90% of gestation resulted in increases in ZO-2 expression, the same treatment does not affect barrier permeability at this fetal age [47]. Similarly, we did not find glucocorticoid-related changes in brain water content in fetal sheep at 90% of gestation or in the newborn lambs [42,48]. Therefore, there is a lack of concordance between the effects of glucocorticoids on barrier function and brain water content and the effects on individual tight junction proteins in our current report. These findings are consistent with current concepts that there are many mechanisms by which glucocorticoids regulate the barrier [28].

The molecular basis of the tight junction protein regulation by maternal and neonatal glucocorticoid treatment cannot be determined by our studies. However, glucocorticoids have been reported to reinforce the barrier via signal transduction pathways originating from stimulation of the endothelial intracellular glucocorticoid receptor in the brain microvasculature, which controls the expression of glucocorticoid target genes and their corresponding tight junction proteins [15–19,28]. Glucocorticoids have been shown to regulate specific response elements on the promoters of both occludin and claudin-5 genes [9,22]. Therefore, it remains possible that similar mechanisms were responsible for the increased claudin-5 expression in the cerebral cortex in the lambs. However, similar mechanisms have not been reported regarding the molecular regulation of ZO-2.

Evidence in adult subjects suggests that the blood-brain barrier is under endogenous hormonal control [30]. The pituitary-adrenal cortical axis matures during fetal development and cortisol concentrations increase particularly in the latter part of gestation [53]. We have shown that endogenous increases in cortisol concentrations are associated with decreases in blood-brain barrier permeability during normal fetal development [47]. These findings suggest that the blood-brain barrier is also hormonally responsive in the fetus and that glucocorticoids are important in regulation of barrier maturation [47]. Our current findings further support the contention that increases in endogenous fetal glucocorticoids during development are associated with increases in the expression of ZO-2 in the fetal cerebral cortex.

Maternal glucocorticoid therapy is widely used to treat pregnant women in premature labor. The relatively low dose treatment regimen that was given to the ewes and lambs in our study was similar to those used in clinical settings. Our findings may be interpreted to suggest that these extensively used treatments affect some of the molecular properties of the microvasculature in the cerebral cortex of the fetus and newborn. We speculate that maternal treatment with glucocorticoids could improve the vascular integrity in the premature human fetus, thereby potentially providing protection from brain injury such as intraventricular hemorrhage [32,33].

In summary, claudin-1, claudin-5, ZO-1, and ZO-2 expression exhibit differential developmental regulation, exogenous glucocorticoids regulate claudin-5 and ZO-2 *in vivo* at some, but not all ages, and increases in endogenous fetal glucocorticoids are associated with increases ZO-2 expression in ovine cerebral cortices.

4. Material and Methods

The present study was conducted after approval by the Institutional Animal Care and Use Committees of Brown University and Women and Infants' Hospital of Rhode Island and according to the National Institutes of Health Guidelines for use of experimental animals.

Animal Preparation and Experimental Design

All of the plasma and cerebral cortical samples for the present study were obtained from animals that were previously used to examine the effects of glucocorticoid pretreatment on blood-brain barrier permeability and on Aquaporin 4 water channels in the ovine cerebral cortex [39,40,47,48]. The surgical procedures and physiological measures were preformed for the former studies [39,40,47,48]. As previously described in detail [40,44], surgery was performed under ketamine (10 mg/kg) and 1%–2% halothane or isoflurane anesthesia in pregnant ewes at 60% (87–90 days; n=17), 70% (106–107 days; n=11) and 90% (135–138 days; n=16) of gestation, newborn lambs (4–6 days, n=13) and adult sheep (3 years; n=7). Catheters were placed for the previous studies in the brachial vein and the thoracic aorta via a brachial artery in the fetal, newborn, and adult non-pregnant sheep [39,40,47,48].

After 2–7 days of recovery from surgery, the pregnant ewes at each gestational age were given a 6 mg intramuscular injection of dexamethasone (Fujisawa, Deerfield, IL, U.S.A., concentration=4 mg/ml, 1.5 ml was given to each ewe) or placebo (0.9% NaCl, 1.5 ml) every 12 hours over 48 hours. Eighteen hours after the last injections had been administered to the ewes and just before the brain samples were obtained, plasma for arterial cortisol concentrations was obtained from the fetuses as previously described [40,47].

Pregnant ewes were anesthetized with pentobarbital (15–20 mg/kg). The fetal sheep were delivered by hysterotomy and then the ewes were euthanized with pentobarbital (200 mg/kg). Cerebral cortical samples were obtained from the fetuses and snap-frozen 18 hours after the last dose of dexamethasone or placebo was given to the ewes. We selected this brain region because the cerebral cortex represents the largest and most important region of the brain and we had frozen samples available from the our previous studies [39,40,47,48].

Arterial and venous catheters were placed via the femoral vessels into the 2-day-old lambs, using 0.5%–1% isoflurane for anesthesia. After twenty-four hours of recovery from surgery, lambs were randomly assigned to placebo (0.9% NaCl) or 0.25 mg/kg dexamethasone per dose. The 0.25 mg/kg dose of dexamethasone was selected as an estimate of the amount of dexamethasone that most likely reached the fetuses after antenatal treatment of the ewes [41]. This estimate takes into consideration that if the ewe receives 6 mg of dexamethasone, the fetus will receive approximately 0.25 mg/kg per dose [41]. The lambs were given four intramuscular injections of dexamethasone or placebo 12 h apart on days 3 and 4 of life. Samples from the cerebral cortices of adult sheep were also obtained from adult non-pregnant sheep similarly exposed to 6 mg dexamethasone or placebo. Samples from the newborn lambs and adult sheep were obtained 18 h after the last dose of dexamethasone or placebo had been given. The cerebral cortical samples in this study remained at –80°C until analysis.

Total plasma cortisol concentrations were measured using the Clinical Assays™ GammaCoat™ Cortisol ¹²⁵I-radioimmunoassay (DiaSorin, Stillwater, MN) as previously described [40]. The GammaCoat™ antiserum exhibits 100% cross reactivity with cortisol. The

observed coefficient of variation for inter- and intra-assay precision was 10.1 and 7.9% respectively.

Western Immunoblot Detection and Quantification of Occludin, Claudin-1, Claudin-5 and ZO-1, and ZO-2

The expression of occludin, claudin-1, claudin-5, ZO-1 and ZO-2 was measured by Western immunoblot as previously described [31]. Transmembrane proteins (occludin, claudin-1, and claudin-5) from the frontal cerebral cortical samples were separated from soluble proteins by homogenization in Triton/Deoxycholate/SDS (100 mM NaCl, 1% Triton X, 0.5 Sodium Deoxycholate, 0.2% SDS, 2 mM EDTA, 1 mM benzamidine) with 1% of complete protease inhibitor cocktail (Sigma, St. Louis, MO). The solutions were then placed on ice for 20 minutes, and centrifuged at 1.4×10^4 rpm for 30 minutes. Urea buffer (6 M urea, 150 mM NaCl, 5 mM MgCl_2 , 5 mM EGTA, 10 mM Tris, pH 8.0, 1% TX-100) with 1% of complete protease inhibitor cocktail was used to extract the cytoplasmic proteins, ZO-1 and ZO-2. Protein concentrations of the homogenates were determined by a bicinchoninic acid protein assay (BCA, Pierce, Rockford, IL). Extracted samples were then aliquoted and stored at -80°C .

Aliquots containing 50 μg of protein in 50 μl of solution were loaded on SDS-polyacrylamide gel. Ten percent polyacrylamide gels were used for occludin, 16% for claudin-1 and claudin-5, and 7% for ZO-1 and ZO-2. The gels were then immunoblotted to polyvinylidene difluoride membranes (PVDF, 0.2 μm , Bio-Rad Laboratories, Hercules, CA) using a semi-dry technique. The membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBST with 0.1% of Tween-20) for 1 h at room temperature, washed three times in TBST for 10 min per wash, and incubated overnight at 4°C with the appropriate primary antibody solution. The proteins were probed with the following primary antibodies: Occludin with monoclonal mouse anti-occludin (Zymed, South San Francisco, CA) at a dilution of 1:5000, claudin-1 with polyclonal rabbit anti-claudin-1 (Zymed) at a dilution of 1:5000, claudin-5 with monoclonal mouse anti-claudin-5 (Zymed) at a dilution of 1:5000, ZO-1 with monoclonal mouse anti-ZO-1 (Zymed) at a dilution 1:5000, and ZO-2 with polyclonal rabbit anti-ZO-2 (Zymed) at a dilution of 1:5000. The immunoblots were then washed in three times in TBST for 10 minutes per wash and then incubated for 1 h at room temperature with goat anti-mouse secondary antibodies (Zymed) at a dilution of 1:10,000 for occludin, claudin-5 and ZO-1 and goat anti-rabbit secondary antibodies (Zymed) at a dilution of 1:10,000 for claudin-1 and ZO-2. After incubation with the secondary antibodies, the immunoblots were again washed four times in TBST for 10 minutes per wash. Binding of the secondary antibody was detected with enhanced chemiluminescence (ECL-plus) Western blotting detection reagents (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) before exposure to autoradiography film (Daigger, Vernon Hills, IL).

All experimental samples were normalized to a reference protein standard that had been obtained from a homogenate protein pool from the cerebral cortex of a single fetal or adult sheep. As we have previously described, these samples served as the internal control reference standard for quality control for loading, transfer, verification of potential internal variability across the gel, and for normalization of the cerebral cortical densitometric values to permit accurate comparisons against a single internal control standard among the different groups and immunoblots [26,31,39]. The same internal control sample was used for each specific tight junction protein on all of the immunoblots, thereby minimizing potential differences among the immunoblots due to loading, transfer, and the autoradiographic densitometry measurements. The experimental cerebral cortical tight junction protein autoradiographic densitometry values were expressed as a ratio to the internal control reference standards, thus facilitating normalized comparisons among the different age and treatment groups. The internal control reference standards for occludin, ZO-1, and ZO-2 were obtained from the cerebral cortex of an adult sheep. Whereas, the internal control reference standards for claudin-1 and

claudin-5 were obtained from the cerebral cortex of a non-experimental fetal sheep brain. The fetal cerebral cortex was used as the internal control reference standard for claudin-1 and claudin-5, because the signal from the adult cerebral cortical samples was not sufficiently robust to serve as an internal control standard for these proteins. For the purpose of this report, we refer to the internal control reference standards from the fetal or adult sheep cerebral cortex hereafter as the internal control samples.

Each immunoblot included two samples from each age and treatment group (dexamethasone or placebo) and three internal control samples. The internal control samples were included in three equally spaced lanes on each immunoblot. We calculated a coefficient of variation for the internal control samples on each immunoblot. The values for the experimental samples were accepted as valid only if the percent coefficient of variation for the internal control samples was less than 20% on the immunoblot. The final values represented an average of the densitometry values obtained from the different immunoblots. We have previously shown that this method correlates well with values that have been normalized as ratios to β -actin [26]. Uniformity in inter-lane loading was also established by Coomassie blue (Sigma, St. Louis, MO) staining of the polyacrylamide gels, and uniformity of transfer to the PVDF membranes confirmed by Ponceau S (Sigma, St. Louis, MO) [49].

Densitometric Analysis

Band intensities were analyzed with Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD). The experimental densitometry values were normalized to the average of the three internal control samples on each immunoblot. We identified two ZO-1 protein bands in some age groups. Initially, each band of the ZO-1 protein was analyzed separately. However, the densitometry values for the two ZO-1 bands were not sufficiently distinct in all age groups to allow for accurate differentiation between the bands or to allow for separate band analyses. Therefore, the densitometry values for the two ZO-1 bands were combined in the final analysis. In contrast, three ZO-2 bands were detected and analyzed separately. However, when the ZO-2 protein expression values were compared to plasma cortisol concentrations and gestational age using the least-squares method, a composite value for the three ZO-2 bands was used.

Samples from each experiment were analyzed on at least three Western immunoblots, and the final values represented an average of the densitometry values obtained from the different immunoblots. Our laboratory has previously used similar normalization techniques to examine the ontogeny and effects of corticosteroid pretreatment on Aquaporin water channels in the ovine cerebral cortex [39], on NaK-ATPase alpha and beta subunits [26] and on the effect of maternal treatment with glucocorticoids on tight junction protein expression in the cerebral cortex of the ovine fetus with and without exposure to *in utero* cerebral ischemia [31].

As previously described [31], Madin-Darby canine kidney cell lysate (MDCK, Canine Kidney Carcinoma, BD Biosciences, San Diego, CA) was used as positive controls for occludin, claudin-1, ZO-1 and ZO-2 and rat lung for claudin-5. Further, the presence of claudin-1 in ovine fetal and adult cerebral cortex was confirmed by using MDCK and adult rat liver as positive controls along with the experimental adult and fetal cerebral cortical samples. The anti-claudin-1 antibody (Zymed, South San Francisco, CA) accurately detected the presence of 23 kDa bands in the MDCK cells, rat liver, adult, and fetal cerebral cortex. Western immunoblots performed without the primary antibodies were used to rule out non-specific binding. Detection of the occludin, claudin-1, claudin-5, ZO-1, and ZO-2 bands at 65, 23, 23, 225, and 160 kDa, respectively, was dependent on incubation with primary antibody, omission of which resulted in the absence of this signal.

Statistical Analysis

Results were expressed as means $\pm$ standard deviation. Two-way analysis of variance (ANOVA) was used to determine the effects of development and dexamethasone treatment on occludin, claudin-1, claudin-5, ZO-1, and ZO-2 protein expression in the cerebral cortices of sheep. The factors were treatment (placebo or dexamethasone) and age (fetuses at 60%, 70%, and 90% of gestation, newborn, and adult sheep). When the results from a two-way analysis of variance were statistically significant, the Newman-Keuls test was used to detect specific differences among age and treatment groups.

The occludin, claudin-1, claudin-5, ZO-1, and ZO-2 protein expression values were compared to plasma cortisol concentrations using the least-squares regression analysis. The occludin, claudin-1, claudin-5, ZO-1 and ZO-2 protein expression values were also compared with gestational age, because plasma cortisol concentrations increase during gestation [47,53]. In this analysis, we only included the fetuses of the placebo treated ewes, because we have previously shown that increases in gestation and endogenous cortisol concentrations were associated with ontogenic decreases in barrier permeability during normal fetal development [47]. Multiple regression partial correlational analyses were also used to compare the relative strengths of the associations between increases in gestation and plasma cortisol concentrations with the changes in protein expression during fetal development. Differences are considered statistically significant at $P < 0.05$.

Acknowledgments

Supported by NIH R01-HD34618 and 1R01-HD-057100

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Figure 1.

Occludin protein expression. Panel A shows a representative Western immunoblot of occludin protein expression in the cerebral cortex of the fetuses at 60%, 70%, and 90% of gestation of the placebo (P) and dexamethasone (D) treated ewes and of the placebo and dexamethasone treated newborn and adult sheep.

Panel B illustrates the occludin protein expression plotted as a ratio of the experimental densitometry values to the internal control values in the cerebral cortex of the fetuses at 60%, 70%, and 90% of gestation, newborn and adult sheep. Open bars represent fetuses of the placebo ewes and closed bars the fetuses of the dexamethasone treated ewes. Bar designations are identical for the newborn and adult sheep.

Figure 2.

Claudin-1 and claudin-5 expression. Panel A shows a representative Western immunoblot of claudin-1 protein expression in the cerebral cortex of the fetuses at 60%, 70%, and 90% of gestation of the placebo and dexamethasone treated ewes and of the placebo and dexamethasone treated newborn and adult sheep. Designation legends as for figure 1. The bar graph illustrates the claudin-1 protein expression plotted as described for occludin in figure 1 in the cerebral cortex of the fetuses at 60%, 70%, and 90% of gestation, newborn, and adult sheep. Bar legends as for figure 1. ^a $P < 0.05$ versus fetuses of the placebo treated ewes at 60% of gestation; ^b $P < 0.05$ versus fetuses of the placebo treated ewes at 70% of gestation; ^c $P < 0.05$ versus fetuses of the placebo treated ewes at 90% of gestation.

Panel B shows a representative Western immunoblot of claudin-5 protein expression in the cerebral cortex of the fetuses at 60%, 70%, and 90% of gestation of the placebo and dexamethasone treated ewes and of the placebo and dexamethasone treated newborn and adult sheep. Designation legends as for figure 1. Bar graph illustrates the claudin-5 protein plotted as described for occludin in figure 1. Bar legends as for figure 1. ^a P<0.05 versus fetuses of the placebo treated ewes at 60% of gestation; ^b P<0.05 versus fetuses of the placebo treated ewes at 90% of gestation; ^c P<0.05 versus fetuses of the placebo treated ewes at 70% of gestation; ^d P<0.05 versus placebo treated newborn lambs.

Figure 3.

ZO-1 protein expression. Panel A shows a representative Western immunoblot of ZO-1 protein expression in the cerebral cortex of the fetuses at 60%, 70%, and 90% of gestation of the placebo and dexamethasone treated ewes and of the placebo and dexamethasone treated newborn and adult sheep. Designation legends as for figure 1. Note that two bands were apparent in some but not all of the age groups.

Panel B illustrates the ZO-1 protein expression plotted as for occludin in figure 1 in the cerebral cortex of the fetuses at 60%, 70%, and 90% of gestation, newborn and adult sheep. Bar designations as for figure 1. ^a $P < 0.05$ versus fetuses of the placebo treated ewes at 60% of gestation; ^b $P < 0.05$ versus fetuses of the placebo treated ewes at 70% of gestation; ^c $P < 0.05$ versus fetuses of the placebo treated ewes at 90% of gestation; ^d $P < 0.05$ versus placebo treated newborn lambs.

Figure 4.

ZO-2 protein expression. Panel A shows a representative Western immunoblot of ZO-2 protein expression in the cerebral cortex of the fetuses at 60%, 70%, and 90% of gestation of the placebo and dexamethasone treated ewes and of the placebo and dexamethasone treated newborn and adult sheep. Designation legends as for figure 1. Note three bands of varying expression intensity are present in all age groups.

Panel B illustrates the ZO-2 protein expression of the upper band plotted as described for occludin in figure 1 in the cerebral cortex of the fetuses at 60%, 70%, and 90% of gestation, newborn, and adult sheep. Bar legends as for figure 1.

Panel C illustrates the ZO-2 protein expression of the middle band plotted as described for occludin in figure 1 in the cerebral cortex of the fetuses at 60%, 70%, and 90% of gestation, newborn, and adult sheep. Bar legends as for figure 1. ^a $P < 0.05$ versus fetuses of the placebo treated ewes at 70% of gestation; ^b $P < 0.05$ versus fetuses of the placebo treated ewes at 60% of gestation; ^c $P < 0.05$ versus fetuses of the placebo treated ewes at 90% of gestation.

Panel D illustrates the ZO-2 protein expression of the lower band plotted as described for occludin in figure 1 in the cerebral cortex of the fetuses at 60%, 70%, and 90% of gestation, newborn and adult sheep. Bar legends as for figure 1. ^a $P < 0.05$ versus fetuses of the placebo treated ewes at 60% of gestation; ^b $P < 0.05$ versus fetuses of the placebo treated ewes at 70% of gestation; ^c $P < 0.05$ versus placebo treated newborn lambs; ^d $P < 0.05$ versus fetuses of the placebo treated ewes at 90% of gestation.

Figure 5.

Panel A shows ZO-1 protein expression in the cerebral cortex of the fetuses of the placebo treated ewes at 60%, 70%, and 90% of gestation plotted against gestational age and plasma cortisol concentrations.

Panel B shows ZO-2 protein expression in the cerebral cortex of the fetuses of the placebo treated ewes at 60%, 70%, and 90% of gestation plotted against gestational age and plasma cortisol concentrations. A composite value for the three ZO-2 bands was used for the correlational analyses.

Table 1

Study groups, pre-study treatment, plasma cortisol concentrations, and numbers of sheep.

Subject	Pre-study Treatment	Plasma Cortisol nmol/l	N
Fetus at 60% gestation	Placebo	20.3±3.96 *	8
	Dexamethasone	17.1±3.69	9
Fetus at 70% gestation	Placebo	24.3± 5.24 *	5
	Dexamethasone	16.9±1.97	6
Fetus at 90% gestation	Placebo	35.6±5.54 *	6
	Dexamethasone	38.1±26.99	10
Newborn lambs	Placebo	171.0±82.14	6
	Dexamethasone	25.1±15.31	7
Adult sheep	Placebo	4.9±1.87 *	3
	Dexamethasone	0.6±0.07	4

Values are M±SD;

* P<0.001 versus placebo treated newborn lambs.