Original Research

Ontogeny of inter-alpha inhibitor proteins in ovine brain and somatic tissues

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Abstract

Inter-alpha inhibitor proteins (IAIPs) found in relatively high concentrations in human plasma are important in inflammation. IAIPs attenuate brain damage in young and adult subjects, decrease during sepsis and necrotizing enterocolitis in premature infants, and attenuate sepsis-related inflammation in newborn rats. Although a few studies have reported adult organ-specific IAIP expression, information is not available on age-dependent IAIP expression. Given evidence suggesting IAIPs attenuate brain damage in young and adult subjects, and inflammation in newborns, we examined IAIP expression in plasma, cerebral cortex (CC), choroid plexus (CP), cerebral spinal fluid (CSF), and somatic organs in fetal, newborn, and adult sheep to determine the endogenous expression patterns of these proteins during development. IAIPs (enzyme-linked immunosorbent assay) were higher in newborn and adult than fetal plasma ($P < 0.05$). Western immunoblot detected 125 kDa *PaI* (Pre-alpha Inhibitor) and 250 kDa *IaI* (Inter-alpha Inhibitor) in plasma, CNS, and somatic organs. *PaI* expression in CC and CP was higher in fetuses than newborns and adults, but *IaI* expression was higher in adults than fetuses and newborns. Both *PaI* and *IaI* were higher in fetal than newborn CSF. IAIPs exhibited organ-specific ontogenic patterns in placenta, liver, heart, and kidney. These results provide evidence for the first time that plasma, brain, placenta, liver, heart, and kidney express IAIPs throughout ovine development and that expression patterns are unique to each organ. Although exact functions of IAIPs in CNS and somatic tissues are not known, their presence in relatively high amounts during development suggests their potential importance in brain and organ development.

Keywords: Brain development, cerebral spinal fluid, inter-alpha inhibitor proteins, ontogeny, sheep

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Introduction

Inter-alpha inhibitor proteins (IAIPs) are a family of structurally related proteins found in mammalian plasma in relatively high concentrations. IAIPs play an important role in inflammation as part of innate immunity, wound healing, and cancer metastasis.[1–3] Major forms found in human plasma are Inter-alpha Inhibitor (*IaI*), which consists of two heavy chains (H1 and H2) and a single light chain, and Pre-alpha Inhibitor (*PaI*), which consists of one heavy (H3) and one light chain. High levels of circulating IAIPs normally found in plasma of adults and newborns, and even in prematurely born infants, suggest that these proteins are important. Moreover, complete absence of IAIPs has not been reported in humans, suggesting that these proteins have important functions in human biology.[1] In premature infants, IAIPs recently have been shown to decrease in association with sepsis and necrotizing enterocolitis.[4–6] In addition, both disorders are associated with increased incidences of brain damage in premature infants.[7,8]

Although the physiological functions of IAIPs remain to be established, current findings suggest that these molecules are part of innate immunity and play a critical role during inflammation. IAIPs have unique immunomodulatory roles during sepsis in neonatal rats.[2,9,10] Bikunin or urinary trypsin inhibitor also has been suggested to be effective in inhibiting premature delivery most likely by suppressing cytokines and other inflammatory mediators.[11–15] Bikunin knockout mice have increased sensitivity to lipopolysaccharide-induced death *in vivo*.[16,17] Bikunin carries a chondroitin sulfate chain to which the heavy chains are covalently linked.[11] The heavy chains can be transferred from IAIPs to hyaluronan molecules and become covalently linked in the extracellular matrix.[11,18] Heavy chains linked to hyaluronan molecules have also been found in inflamed tissues.[18] However, the

physiological role of heavy chains of IAIPs is not known. Taken together, these results suggest that proteins of the IAIP family, including bikunin, are anti-inflammatory agents. These functions potentially explain its beneficial effects in systemic inflammation and suggest that IAIPs could play an important role in inflammation-related disorders during the perinatal period.

IAIP related molecules and mRNA have been detected in neurons, astrocytes, and meningeal cells of the brain.[19–29] Recent work suggests associations between variations in phenotypes of the heavy chain alleles with brain disorders such as schizophrenia and bipolar disease[30] suggesting that IAIPs might be important to normal brain development.

Although there is very little information regarding the effects of these molecules on the brain, bikunin may have neuroprotective properties against the development of stroke-related ischemic injury in adult rats,[31] protect oligodendrocytes from apoptosis, and promote remyelination in an experimental autoimmune encephalitis model in adult rats.[32] The light chain of IAIPs, also called bikunin or urinary trypsin inhibitor or ulinastatin, has recently been shown to have neuroprotective properties in young piglets exposed to hypothermic low-flow cardiopulmonary bypass potentially via its anti-inflammatory properties.[33] Bikunin has also been shown to block the production of inflammatory cytokines during reperfusion after ischemic injury in several somatic organs.[34–38] Nonetheless, there is very little information regarding the expression of these molecules in brain and other organs during development.[20,39]

The choroid plexus (CP) produces cerebral spinal fluid (CSF), which provides physical protection for the brain, and removes brain metabolites via drainage of the CSF. However, studies that are more recent suggest that the CP-CSF system also plays a much more active role in development, homeostasis, and repair of the central nervous system (CNS).[40–42] The CP is a highly specialized tissue, strategically positioned within the ventricles to provide the CNS with variety of biologically active growth factors essential for normal brain development.[41–43] These factors include neurotrophic and angiogenic factors, which are potentially involved in neurogenesis and axonal guidance during CNS development, and in responses to brain injury and during the subsequent repair processes.[44–53] Previous studies reported that many species during development, including human premature infants, have very high CSF protein concentrations that are most likely important for brain development.[54–57] Therefore, proteins found in CSF most likely influence brain development and responses to injury. Very limited information suggests that urinary trypsin inhibitor is present in CSF of adults, particularly in those with brain tumors and in the postoperative state.[58] However, levels of IAIPs have not been previously examined in the CSF of any species during development.

Information is also very limited regarding the organ-specific distribution of these critical molecules, including in brain. In humans, IAIPs were detected in cerebrum, cerebellum, lungs, kidney, liver, colon, skin, and testes.[21] Information is not available regarding the expression of IAIPs in the brain or somatic organs during normal development in any species.

The ovine fetus has been widely used to investigate brain development.[59–61] 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.[59,62,63] Full term in sheep pregnancy is 148 days of gestation. The preterm fetal sheep brain between 94 and 96 days of gestation is comparable to that of the preterm infant between 24 and 28 weeks of gestation, whereas fetal sheep at 135 days of gestation is similar to that of the near-term human infant.[64] In the present study, we obtained samples of brain and somatic organs over a wide range of ages to examine changes in IAIPs expression over a broad developmental window. Although rodents are frequently used to study brain development, the rodent brain is immature at birth[64] and almost completely agyric. In contrast, similar to the nonhuman primate and human brain, the sheep brain develops prenatally and is gyrencephalic.

In summary, IAIPs attenuate inflammatory responses during the perinatal period,[9,11–15] decrease during sepsis and necrotizing enterocolitis in premature infants,[5,6] and are important in ischemic and inflammatory related brain and somatic organ damage.[31,32] Given the potential importance of these molecules in perinatal period inflammation, and in brain and somatic organ damage, as an initial approach to understand these critical molecules during development, we examined levels of IAIPs in the plasma, brain, CP, CSF, and somatic organs throughout ovine development with a primary focus on developmental expression patterns of IAIPs in brain.

Materials and methods

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

Animal preparation and experimental design

Plasma, cerebral cortical, CP, CSF, placenta, liver, heart, and kidney tissues samples for the present study were frozen samples obtained from placebo-treated sham-operated control animals from previous studies.[61,65–67] Samples from all age groups were obtained over similar time intervals. Surgical procedures and physiological measures were performed for the former studies.[61,65–68] As described previously in detail,[61,65–67] surgery was performed under ketamine (10 mg/kg) and 1–2% halothane anesthesia in pregnant ewes at 60% (87–90 days), 70% (106–107 days), 90% (135–138 days) of gestation; newborn lambs (4–6 days of age); and adult nonpregnant sheep (3 years of age). The sham-operated control animals from our previous studies were sacrificed without further intervention. Plasma samples were obtained from all animals just before the euthanasia. At the end of the studies, a CSF sample was obtained from the fetal and newborn sheep via a direct puncture of

Table 1 Number of plasma, cerebral cortex, CP, CSF, placental, liver, heart, and kidney samples by age group

Groups	Plasma	Cerebral cortex	Choroid plexus	CSF	Placenta	Liver	Heart	Kidney
60% (87–90 days of gestation)	–	–	5	–	–	–	–	–
70% (106–107 days of gestation)	5	8	–	4	5	6	6	6
90% (135–138 days of gestation)	7	8	5	4	5	6	5	6
Newborn (4–6 days)	4	8	5	5	–	6	5	5
Adult (3 years)	3	3	3	–	–	3	3	3

(–) indicates sample not available.

the allantoic membrane. The sample was inspected for blood contamination and discarded if there was evidence of contamination. CSF samples were not available from the adult sheep. Tissues, plasma, and CSF were snap frozen in liquid nitrogen and remained at –80°C until analysis. The number of animals per group for different organs available for analysis is summarized in Table 1. Although CP samples were not available from fetuses at 70% gestation, samples were available from fetal sheep at 60% gestation.

Competitive enzyme-linked immunosorbent assay (ELISA) to measure IAIPs level in ovine plasma and CSF

IAIPs concentrations were measured by a competitive ELISA in sheep plasma using a polyclonal antibody raised against human IAIPs (R-20 pAb). The polyclonal antibody was generated by immunizing rabbits with highly purified human plasma derived IAIPs.[69] The R-20 pAb cross-reacts with nonhuman IAIPs including sheep and detects both 250 kDa *IaI* and 125 kDa *PaI* molecules in Western immunoblot analysis. Furthermore, this R-20 pAb binds to heavy chains as well as light chain of human IAIPs after enzymatic digestion.[69] Ninety-six-well high-binding microplates Microlon 600 (Greiner Bio-One, Monroe, NC, USA) were coated with purified sheep IAIPs. Sheep IAIPs were purified from sheep serum (Quad Five, Ryegate, MO, USA) by anion-exchange chromatography on a Toyopearl Q-600 C-AR column (Tosoh Bioscience, King of Prussia, PA, USA). Bound IAIPs were eluted with a buffer containing 750 mM NaCl. The purified sheep IAIPs were diluted in 100 mM $NaPO_4$ buffer pH 6.5 and immobilized on the microplates (50 ng/per well) for 1 h at room temperature or overnight at 4°C. Subsequently, the microplate was blocked with 200 µL of 5% nonfat dried milk in phosphate buffered saline (PBS) and 0.05% Tween. Sheep plasma was diluted in PBS and a known amount of purified sheep IAIPs was serially diluted in PBS to establish a standard curve for quantitative analysis of IAIP concentrations in the samples. After 50 µL of samples and serially diluted IAIPs standards were added to the wells, 50 µL of R-20 PAb diluted in 1:1200 in PBS was added to each well. Plates were incubated for 1 h at room temperature and subsequently washed with PBS and 0.05% Tween using automated plate washer (Biotek EL-404, Winooski, VT, USA). The bound R-20 pAb was detected by adding HRP-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. After washing, 100 -µL Enhanced K-Blue TMB substrate (Neogen Corp, Lexington, KY, USA) was added to the wells and the reaction was stopped by adding 100 µL 1 N HCl solution. The absorbance at 450 nm was measured on SpectraMAX Plus microplate reader (Molecular Devices, Sunnyvale, CA, USA). Each sample was tested in triplicate and assays were repeated at least twice on all samples.

Preparation of cytosolic tissue fractions

Cell cytosolic fractions of cerebral cortex (CC), CP, placenta, liver, heart, and kidney for IAIPs were extracted in buffer A (TRIS 10 mM pH 6.8, Sucrose, MgCl) with 1% complete protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Total protein concentrations of the homogenates were determined with a bicinchoninic acid protein assay (BCA, Pierce, Rockford, IL, USA). Aliquots of the extracted samples were stored at –80°C. We examined the cytosolic fraction for this study because the primary antibody R-20 recognized IAIPs only in the cytosol.

Ovine IAIP

To investigate the relationship between *IaI* (250 kDa) and *PaI* (125 kDa) in adult sheep plasma, ovine IAIPs were purified from ovine serum by anion-exchange chromatography. Sheep serum (Quad Five, Ryegate, MT) was obtained and filtered to remove large precipitates and bacteria. A preparative chromatographic system (BioCad, Applied Biosystems) was used for two successive separation steps: a quaternary ion exchange (QA-R) column (Tosoh Bioscience, King of Prussia, PA) and a monolithic CIMmultus DEAE anion exchange column (BIA Separations, Villach, Austria). The wash method involved buffers of varying pH and salt concentrations in order to remove other contaminating serum proteins, thereby obtaining highly pure IAIP.

Western immunoblot detection and quantification of proteins

A total of 15–50 µg protein of total protein per well (CC: 50 µg, CP: 15 µg, CSF: 22.5 µL, plasma: 1 µL from 1:100 dilution; placenta: 30 µg, liver: 50 µg, heart: 50 µg, and kidney: 50 µg) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and polyvinyl diflouride membrane (SDS-PAGE) and transferred onto polyvinyl diflouride (PVDF) membranes (0.2 µm, Bio-Rad Laboratories, Hercules, CA) using a semi-dry technique. Membranes were each incubated with a rabbit polyclonal

antibody against human IAIPs (R-20 pAb, ProThera Biologics, East Providence, RI, USA) at a dilution of 1:5000. The immunoblots were incubated in primary antibody overnight at 4°C. Peroxidase-labeled secondary antibody goat anti-rabbit (Alpha Diagnostic, San Antonio, TX, USA) was incubated for 1 h at room temperature in a dilution of 1:10,000. Binding of the secondary antibody was detected with enhanced chemiluminescence (ECL plus, Western Blotting Detection reagents, Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) before exposure to autoradiography film (Daigger, Vernon Hills, IL, USA).

Experimental samples were normalized to a reference protein standard that was obtained from a homogenate protein pool from the tissues of a single adult sheep. For the purpose of this report, we refer to these samples as internal control (IC) samples. As we have previously described,[70–73] these samples served as an IC for quality of loading, transfer of the samples, normalization of the densitometric values, and to permit accurate comparisons among the different immunoblots.[70,71,74] The use of IC is unique to our laboratory and allows us to compare large groups of animals over a large number of different immunoblots. We developed this methodology because investigation of a large number of housekeeping proteins showed that they all exhibited significant variations during ovine development mitigating their use as housekeeping proteins. The experimental protein autoradiographic densitometrical values were expressed as a ratio to the IC, thus facilitating normalized comparisons among different groups and multiple immunoblots. When this methodology was used within a single age group (newborn), the method correlates well with values that were normalized as ratios to β-actin.[70]

Each immunoblot included samples from the four groups and three IC samples. The IC samples were included in three lanes, as the first, middle, and last samples on each immunoblot. We calculated a coefficient of variation for the IC samples on each immunoblot. The values for the experimental samples were accepted as valid only if the percent coefficient of variation for the IC samples was less than 20% on the immunoblot. Human plasma-derived IAIPs served as a positive control (PC) for all immunoblots to ascertain that the antibody correctly identified the ovine proteins. Molecular weight standards (Bio-Rad Laboratories, Hercules, CA, USA) were included in each immunoblot. The rabbit polyclonal anti-IAIP (R-20 pAb) detected IAIPs bands at 125 and 250 kDa (*PaI* and *IaI*) in all organs. Uniformity in inter-lane loading was also established by Coomassie blue (Sigma, St. Louis, MO, USA) staining of the polyacrylamide gels and uniformity of transfer to the polyvinylidene difluoride membranes was confirmed by Ponceau S staining (Sigma, St. Louis, MO, USA).

Samples from all animals and age groups were placed on each immunoblot to permit identical standard conditions among all of the tissue samples from the different age groups. However, in order to facilitate graphic clarity, to clarify the age group comparisons for each tissue, and to place the immunoblots above the bar graphs on the same figures, we selected the one specific immunoblot that most closely represented the mean expression value for each specific age group for the bar graph value of each tissue. Illustrations displaying the entire gels in the figures would have not adequately emphasized differences among the various age groups with respect to the IAIP expression.

Densitometric analysis

Band intensities were analyzed with a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA). All experimental samples were normalized to the respective average of the three IC samples. However, the band intensities were expressed as integrated optical density (IOD) units for CP and CSF as we did not have adult CP or CSF. The final values represented averages of the densitometry values obtained from the different immunoblots (plasma, $n = 2$; CC, $n = 8$; CP, $n = 5$; CSF, $n = 2$; placenta, $n = 5$; liver, $n = 5$; heart, $n = 5$; kidney, $n = 5$) and were presented as a ratio to the IC sample except for CP and CSF.

Statistical analysis

All results were expressed as standard error of the mean ($\pm$SEM). Two-way analysis of variance (ANOVA) was used to compare the differences among the groups. The factors were age group (fetuses at 60%/70%, 90% of gestation, newborn, and adult) and protein expression (125 kDa *PaI* and 250 kDa *IaI*). When a significant difference was detected by ANOVA, the Fisher's least significant difference test was used to further describe the statistically significant differences among the groups. $P < 0.05$ was considered statistically significant.

Results

IAIPs detection by SDS-PAGE, ELISA, and Western immunoblot in ovine plasma

The SDS-PAGE and Western immunoblot illustrate that ovine IAIPs contain two major proteins, *IaI* and *PaI* with the purity of 85–90% (Figure 1). The Western immunoblot (Figure 2) comparing adult human (Figure 2(a)) with adult ovine plasma (Figure 2(b)) illustrates that both human and ovine plasma contain the *IaI* (250 kDa) and the *PaI* (125 kDa) with similar apparent molecular weights.

IAIPs detected by competitive sheep ELISA in ovine plasma were lower ($P < 0.05$) in the fetuses at 70 and 90% gestation than in the newborn lambs and lower in the fetuses at 90% of gestation than in adult sheep (Figure 3). The IAIPs were detected as 125 kDa *PaI* and 250 kDa *IaI* bands in ovine plasma by Western immunoblot (Figure 4). The expression of 125 kDa *PaI* did not differ among the age groups. In contrast, the expression of 250 kDa *IaI* was lower in the fetuses at 70 and 90% gestation and in the newborn lambs than in the adult sheep.

IAIPs detection by Western immunoblot in CC, CP, and CSF

IAIPs were detected in CC, CP, and CSF as 125 kDa *PaI* and 250 kDa *IaI* protein bands by Western immunoblot using the specific antibody against IAIPs (Figures 5 to 8). The expression of *PaI* was higher in the CC in fetuses at 70% of

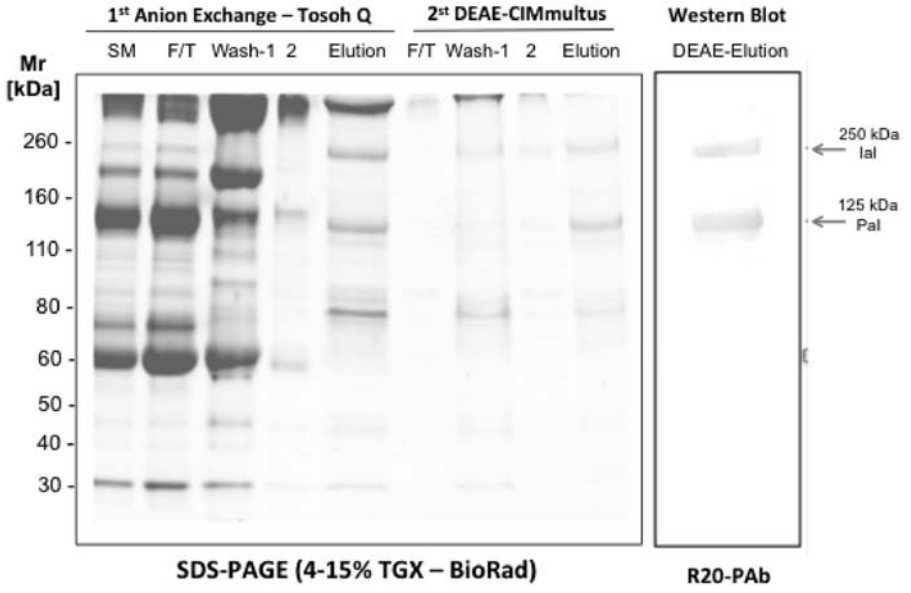

Figure 1 SDS-PAGE illustrating purification of ovine IAIPs and Western immunoblot of the purified ovine IAIPs illustrate that ovine IAIPs consist of two major proteins, the *Ial* (250 kDa) and the *Pal* (125 kDa). *Ial*: Inter alpha Inhibitor; *Pal*: Pre alpha Inhibitor.

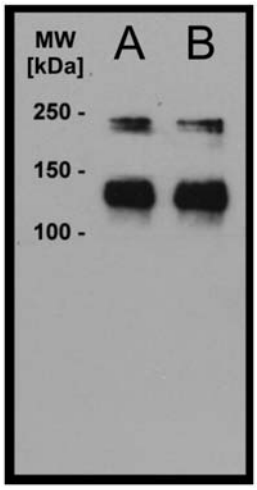

Figure 2 Western immunoblot comparing adult human (a) with adult ovine plasma (b) shows that both human and ovine plasma contain the IAIPs (*Ial* and *Pal*) protein moieties at similar apparent molecular weights (MW)

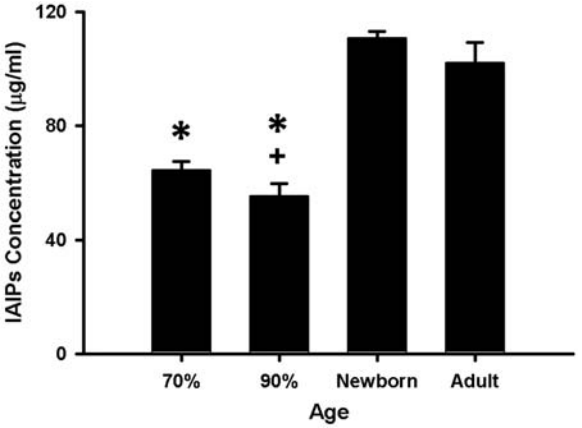

Figure 3 Plasma concentrations of IAIPs plotted for the different age groups. Fetuses at 70 and 90% gestation have significantly lower plasma IAIP concentrations than newborn lambs, $^{*}P < 0.05$ versus newborn lambs, and fetuses at 90% gestation have significantly lower plasma IAIP concentrations than the adult sheep, $^{+}P < 0.05$ versus adult sheep. Values are mean ± SEM. IAIP: inter-alpha inhibitor protein

gestation than in the newborn lambs and higher in the adult sheep than in the newborn lambs (Figure 5(a)). The 250 kDa *IaI* expression was lower in the CC in the fetuses at 90% gestation and in the newborn lambs than in the adult sheep (Figure 5(b)).

Figure 6 shows a representative Western immunoblot for cerebral cortical IAIP expression in order to illustrate the appearance of original immunoblots. The immunoblot contains two representative animals for each age group. The expression of the 125 kDa *PaI* and 250 kDa *IaI* proteins is shown for the ovine cerebral cortical samples in the fetuses at 70 and 90% of gestation and in the newborn and adult sheep. Two types of standards were used on all immunoblots. The human plasma-derived IAIPs were used as PCs in all immunoblots to be certain that we identified the correct IAIP bands in each ovine plasma, tissue, and CSF sample. As described previously,[70–73,75] the IC protein standard was also used on each immunoblot; this sample protein had been obtained from the single adult sheep brain and was used for normalization for all of the tissues and age groups.

The expression of the *PaI* in CP was lower in newborn lambs than in the fetuses at 60 and 90% of gestation and in

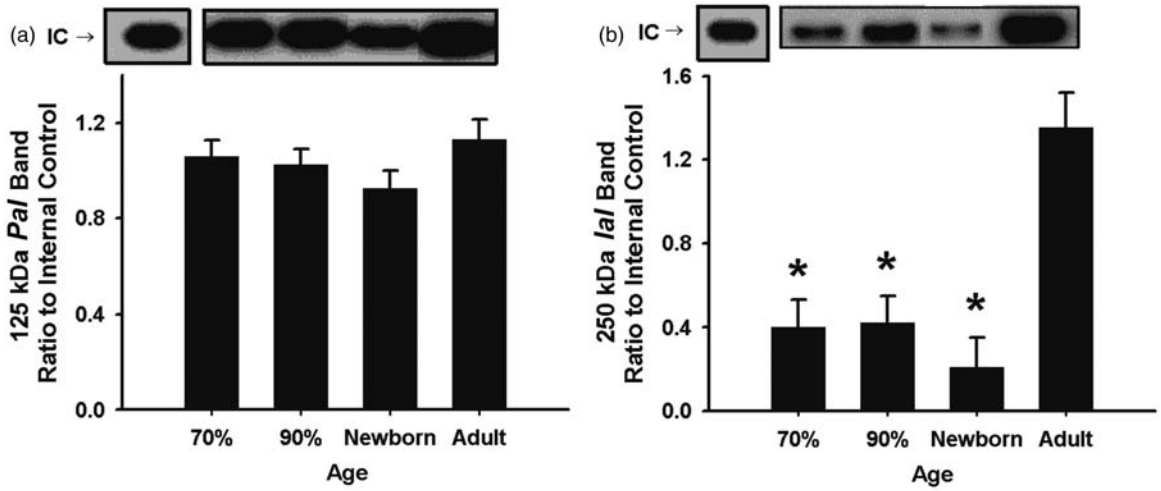

Figure 4 125 kDa *PaI* and 250 kDa *IaI* expression in ovine plasma plotted as the ratio to the IC standard for the different age groups. (a) 125 kDa *PaI*: Expressions did not differ among the different age groups. (b) 250 kDa *IaI*: Fetuses at 70 and 90% and newborn lambs have significantly lower expression than the adult sheep. *$P < 0.05$ versus adult sheep. IC designates the band for IC standard. Values are mean ± SEM

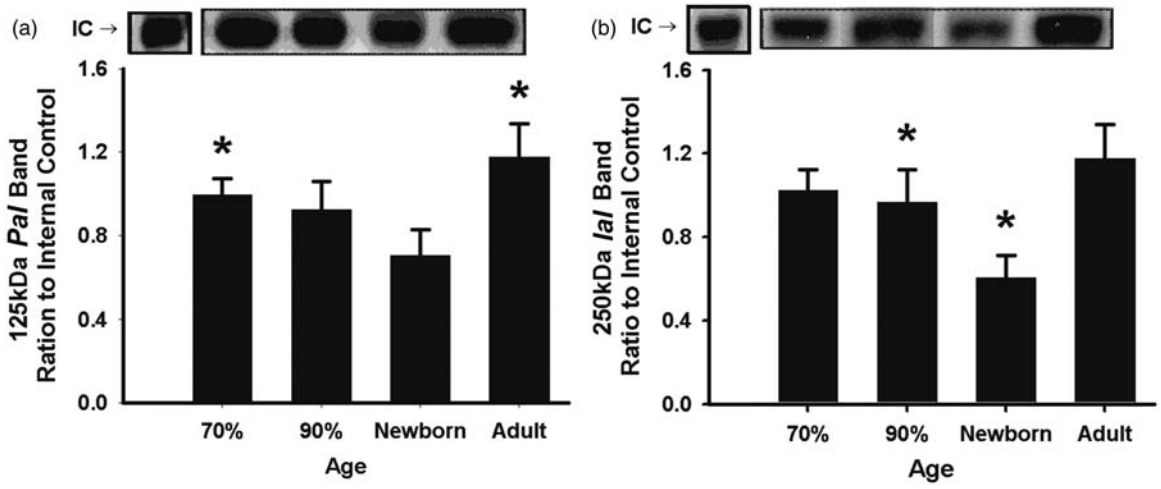

Figure 5 125 kDa *PaI* and 250 kDa *IaI* expression in ovine cerebral cortex plotted as the ratio to the IC standard for the different age groups. (a) 125 kDa *PaI*: Expression was higher in fetuses at 70% of gestation and in the adult sheep than in the newborn lambs. *$P < 0.05$ versus newborn lambs. (b) 250 kDa *IaI*: Expression was lower in fetuses at 90% of gestation and in newborn lambs than in the adult sheep. *$P < 0.05$ versus adult sheep. IC designates the band for IC standard. Values are mean ± SEM

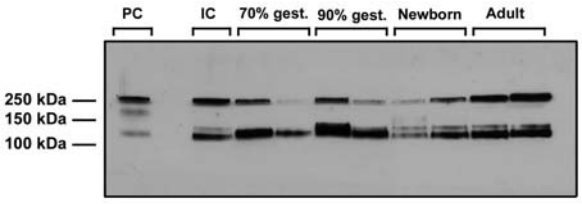

Figure 6 125 kDa *PaI* and 250 kDa *IaI* expression in ovine cerebral cortex at 70 and 90% of gestation, newborn, and adult. Human plasma-derived *Inter-alpha Inhibitor* proteins used as a PC. IC standard protein derived from the adult cerebral cortex as described in the ''Materials and methods'' section. IC: internal control; PC: positive control

the adult sheep (Figure 7(a)), and *IaI* expression was higher in the fetuses at 60% of gestation and in the adult sheep than in the newborn lambs, but lower in the fetuses at 60 and 90% gestation than in the adult sheep (Figure 7(b)). The expression of the *PaI* and *IaI* in CSF was higher in fetuses at 70 and 90% of gestation than in the newborn lambs (Figure 8(a) and (b)). The IAIPs levels in the CSF were below the limit of detection by the sheep-specific ELISA. In summary, the CC, CP, and CSF, each exhibit distinct patterns of expression for the 125 kDa *PaI* and 250 kDa *IaI*. However, both molecules appear lower in the newborn lambs than in the fetuses at 70 and 90% of gestation in CP and CSF. We do not know the pattern of expression in adult sheep, as we did not have samples from the adult sheep.

Table 2 contains the expression of the 125 kDa *PaI* and 250 kDa *IaI* in the different somatic organs by age group as ratio to the IC proteins. IAIPs were also detected as *PaI* and *IaI* in placenta, liver, heart, and kidney in fetal, newborn, and adult sheep. In placenta, the *PaI* and *IaI* expressions did not differ significantly between the fetuses at 70 and 90% of gestation. In the liver, *PaI* was higher in fetuses at 70 and

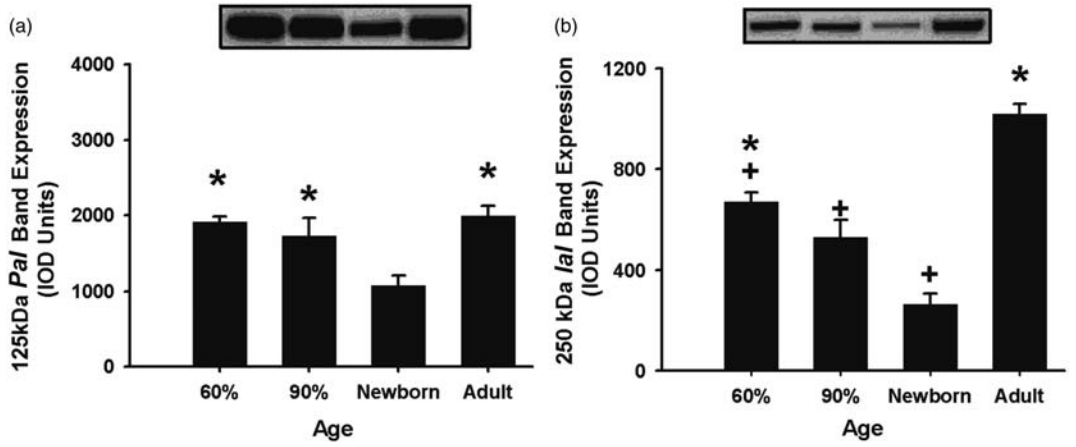

Figure 7 125 kDa *Pal* and 250 kDa *Ial* expression plotted as arbitrary integrated optical density (IOD) units in the choroid plexus for the different age groups. (a) 125 kDa *Pal*: Expression is higher in the fetuses at 60 and 90% gestation and in the adult sheep than in the newborn lambs, $^{*}P < 0.05$ versus newborn lambs. (b) 250 kDa *Ial*: Expression band was higher in fetuses at 60% gestation and in the adult sheep than in the newborn lambs and lower in fetuses at 60 and 90% gestation and newborn lambs than in the adult sheep. $^{*}P < 0.05$ versus newborn lambs, $^{+}P < 0.05$ versus adult sheep. Values are mean ± SEM

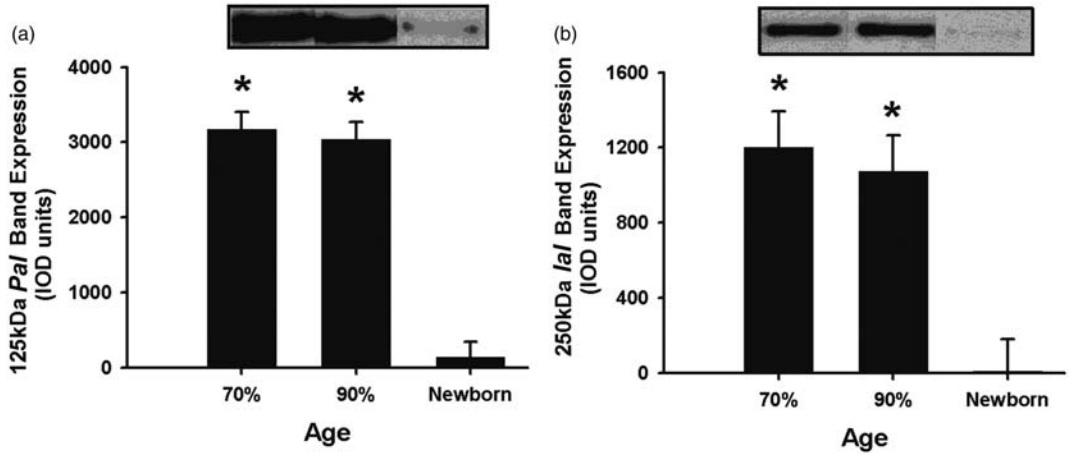

Figure 8 125 kDa *Pal* and 250 kDa *Ial* expression plotted as arbitrary IOD units in CSF for the fetuses at 70 and 90% of gestation and newborn lambs. (a) 125 kDa *Pal* and (b) 250 kDa *Ial*: Expressions were higher in fetuses at 70 and 90% gestation than in newborn lambs. $^{*}P < 0.05$ versus newborn lambs. Values are mean ± SEM

Table 2 IAIP values in somatic organs by age group as ratio to the internal control protein standard

Organ	Age			
	70%	90%	Newborn	Adult
Placenta				
125 kDa *Pal*	0.73 ± 0.21	1.51 ± 0.21	–	–
250 kDa *Ial*	1.27 ± 0.43	1.28 ± 0.43	–	–
Liver				
125 kDa *Pal*	0.99 ± 0.11*	0.92 ± 0.11*	0.71 ± 0.11	1.18 ± 0.18
250 kDa *Ial*	0.29 ± 0.07†	0.47 ± 0.07†	0.14 ± 0.07†	0.77 ± 0.10
Heart				
125 kDa *Pal*	1.64 ± 0.10	1.30 ± 0.09	1.07 ± 0.11	1.55 ± 0.13
250 kDa *Ial*	3.83 ± 0.91*	3.68 ± 0.83*	1.67 ± 1.01	2.10 ± 1.17
Kidney				
125 kDa *Pal*	1.36 ± 0.10	0.87 ± 0.10†‡	1.16 ± 0.10	1.37 ± 0.15
250 kDa *Ial*	0.53 ± 0.13†	0.38 ± 0.13†	0.34 ± 0.13†	1.1 ± 0.18

Ial: Inter alpha Inhibitor; *Pal*: Pre alpha Inhibitor.
Values are mean ± SEM, expressed as ratio to internal control. (–) value not available.
$^{*}P < 0.05$ versus newborn, †$P < 0.05$ versus adult, ‡$P < 0.05$ versus fetuses at 70% of gestation.

90% gestation than the newborn lambs and lower in the newborn lambs than in the adult sheep. *IaI* expression was lower in the fetuses at 70 and 90% of gestation and in the newborn lambs than in the adult sheep and higher in the fetuses at 90% of gestation than in the newborn lambs. In the heart, the expression of *PaI* did not differ among the groups. In contrast, *IaI* was higher in the fetuses at 70 and 90% of gestation than in the newborn lambs. In the kidney, the *PaI* band expression was lower in fetuses at 90% gestation than in the fetuses at 70% of gestation and in the adult sheep, but the expression of *IaI* was lower in fetuses at 70 and 90% of gestation and in the newborn lambs than in the adult sheep.

Inspection of Figures 5, 7, and 8 compared with Figure 4 suggests that the pattern of expression of IAIPs in the brain, CP, and CSF differed from those in the plasma for the same age groups. We had brain vascular volume measurements available from our previous experiments determined by using intravenous [^{3}H]-sucrose (MW 344) in the fetuses at 70% gestation[76] and [^{3}H]-polyethylene glycol (MW 1000) in the fetuses at 90% gestation, newborn, and adult sheep.[61,77] Based upon these vascular volume measurements, we were able to calculate the estimated mean IAIP concentrations contained within the brain vasculature per gram brain tissue (Table 3). Inspection of Table 3 suggests that the amount of IAIPs contained within the brain vasculature represented a relatively small amount of the total IAIPs contained within the brain parenchyma.

Table 4 schematically summarizes the 125 kDa *PaI* and 250 kDa *IaI* expression for each organ in the fetuses at 60 or 70% and 90% of gestation, newborn lambs compared with the adult sheep.

Discussion

The main purpose of our study was to examine the expression of IAIPs in the brain and to compare changes in brain with those in plasma and somatic organs of sheep during development as an initial approach to understanding these critical molecules during development. The presence of IAIPs was identified for the first time in plasma, CC, CP, liver, heart, and kidney from early in fetal life and through the neonatal period up to maturity in adult sheep, and in the placenta and CSF during fetal life as both the 125 kDa *PaI* and 250 kDa *IaI*. The findings of our study are novel because to the best of our knowledge previous work has not reported distributions of IAIPs in the brain and somatic organs during a wide span of development in any species. The major findings of this study were as follows. (1) The level of IAIPs increases in plasma after birth. (2) The 125 kDa *PaI* expression was higher in the adult and fetal than in newborn lamb cerebral cortices, but the 250 kDa *IaI* expression was higher in adult than fetal and newborn cerebral cortices. (3) The expression of *PaI* and *IaI* in CP was highest in the adult sheep. (4) *PaI* and *IaI* were high in CSF of fetal sheep and very low in newborn lambs after birth. (5) IAIPs exhibit ontogenic patterns of expression specific to each molecular species and organ. The presence of both molecules of IAIPs with organ-specific patterns of expression during ovine development may be interpreted to suggest that these proteins have important immunomodulatory[1,2] functions during organ development.

Recent studies have shown relatively high levels of circulating IAIPs are normally present in adult human plasma[2,78] and even in plasma of premature infants.[4–6] Our findings during ovine development extend these

Table 3 Estimated mean inter-alpha inhibitor proteins concentration per gram brain tissue (μg/g)

	70% Gestation	90% Gestation	Newborn	Adults
Mean blood volume (μL/g)	41	35	22	17
Mean plasma IAIP conc. (μg/mL)	64	55	111	102
Estimated mean IAIP conc. (μg/g)	2.6	1.9	2.4	1.7

Table 4 Schematic summary of 125 kDa *PaI* and 250 kDa *IaI* protein expression compared with adult values for each tissue by age

	125 kDa *PaI* expression			250 kDa *IaI* expression		
Organ	Age 60%/70%	90%	Newborn	Age 60%/70%	90%	Newborn
Plasma	↔	↔	↔	↓	↓	↓
Brain	↔	↔	↓	↔	↓	↓
CP	↔	↔	↓	↓	↓	↓
Liver	↔	↔	↔	↓	↓	↓
Heart	↔	↔	↔	↔	↔	↔
Kidney	↔	↓	↔	↓	↓	↓

↑ indicates significant increase compared to adult, ↓ indicates significant decrease compared to adult, ↔ indicates no change compared with adult values. CSF not listed because adult samples were not available.

observations in human plasma and suggest that the concentrations of IAIPs, measured by ELISA, increase markedly after birth. In addition, the expression of the IAIP-related molecules, which contribute to the total amount of IAIPs measured by ELISA, differs with respect to their expression during development such that the expression of the 125 kDa *PaI* is similar at all ages, but that of the 250 kDa *IaI* increases markedly after birth. This finding suggests that it is the 250 kDa *IaI* moiety that contributes to the high levels of total IAIPs observed in adult sheep plasma. However, although the level of the total IAIPs (measured by ELISA) is high in the newborn lambs, the expression of the 250 kDa *IaI* appears low in the lambs, suggesting that 125 kDa *PaI* moiety is more likely to contribute to the relatively high levels of the total IAIP protein after birth.

IAIPs and related proteins have previously been localized in various tissues in adult rodents and humans, including cerebrum and cerebellum, lung, liver, intestines, colon, kidney, bladder, testes, and skin.[21,79–81] IAIPs also have been shown to exhibit a specific distribution within the brains of mice and rats with localization primarily in CC, hippocampus, and hypothalamus.[82] Unfortunately, we only had residual cerebral cortical and CP samples from our previous studies,[61,65–67] so that we cannot comment on the amounts of IAIPs expressed in other brain regions. However, in the CC, we observed different ontogenic patterns for the 125 kDa *PaI* and 250 kDa *IaI*, such that the former was higher in the fetuses at 70% of gestation and in adult sheep than in newborns, but the later higher in the adult sheep than in the newborns and fetuses at 90% of gestation. Although we cannot comment upon the distribution of IAIPs in other brain regions, identify the localization of IAIPs to specific cell types, or identify the biological functions of IAIPs from our study, others have reported that IAIPs are most likely produced within the neurons and/or astrocytes in the murine brain, because intense immunoreactivities were localized to neuronal processes.[28,82]

Inflammation plays a key role in many CNS disorders.[83] There is now evidence to suggest that bidirectional communications between the CNS and periphery could contribute to acute and chronic CNS disorders.[83] Increased levels of IAIPs in ovine plasma and CNS tissue during development could be related to the importance of these molecules in systemic and CNS inflammatory and immunological responses.[1,2] Recent evidence suggests that bikunin, the light chain of IAIPs, reduces oxidative stress, early inflammation, and endothelial activation in the forebrain of rats[84]; reduces ischemia–reperfusion-related delayed neuronal apoptosis in gerbils[85]; protects against white matter demyelination and oligodendrocytes from apoptosis; and promotes remyelination in a model of experimental autoimmune encephalomyelitis.[32] In addition, bikunin attenuates polymorphonuclear neutrophil infiltration and decreases infarct volume in ischemic-reperfusion injury in the brain of adult rats.[31] Moreover, endogenous bikunin appears to be directly involved in repair process of injured neurons,[31] and protease inhibitors derived from neuronal cells function as regulators of neurite regeneration and outgrowth.[86] A recent study also suggests that urinary trypsin inhibitors have neuroprotective properties in young piglets exposed to hypothermic low-flow cardiopulmonary bypass potentially via its anti-inflammatory properties.[33] Hence, IAIPs appear to have a variety of important neuroprotective effects in several animal models. Therefore, based upon our findings identifying the presence of IAIPs in relatively large amounts throughout ovine development, we speculate that these molecules could potentially represent endogenous anti-inflammatory molecules with neuroprotective properties and/or be important to brain development.

The patterns of IAIP expression in the CP were somewhat similar to those of the CC during development. CSF is produced as an ultrafiltrate of plasma by the CP and from drainage of interstitial fluid from CNS tissues. Approximately 80% of the total amount of protein in CSF originates from blood with the remaining 20% originating directly from the CNS.[87] CSF in adults has much lower protein concentrations than plasma due to restricted entry of blood-derived components through the blood–CSF barrier.[41] Most abundant proteins in plasma are also elevated in CSF with exception of proteins forming large complexes, and, consequently, exhibiting very low diffusion rates into CSF.[41] High concentrations of protein have been previously reported in the immature CSF of fetal sheep[56] and in newborn and preterm infants with levels several times higher than those in adults.[88] The higher protein concentrations in fetal CSF are most likely a result of local production by CP rather than immaturity of the blood–brain or blood–CSF barriers because the blood–brain and blood–CSF barriers form very early during development in the fetus.[41,55,77,88]

The high levels of both the 125 kDa *PaI* and 250 kDa *IaI* molecules expressed in CSF in the fetal sheep, which decrease after birth, are consistent with findings of elevated levels of other proteins during gestation in several other species including rodents, pigs, rabbits, chickens, and in premature infants.[55,88–92] Although initially it was thought that elevated protein concentrations in CSF simply reflected an immature leaky blood–brain barrier to proteins during development, more recent information suggests that elevated CSF proteins in the fetus and newborn most likely have important roles for brain growth and development.[41] The protein composition of CSF in the early stages of fetal development is very complex. The majority of proteins are low molecular weight proteins such as albumin, alpha-fetoprotein, transferrin, lipoproteins, etc. the concentrations of which show significant variations during different stages of development.[55,93] These protein fractions most likely represent molecules that have important biological functions including growth factors and cytokines, which could influence the development of neuroepithelial cells.[41,89,94]

The ontogenic patterns of protein concentration in fetal CSF have been studied in several species. In the chick and sheep, CSF protein concentrations increase during the late fetal period and decrease just before birth.[55,95,96] In contrast, a similar decrease does not occur until after birth in rats,[54] suggesting that phylogenic differences play a role in the pattern of protein expression in CSF during maturation. A large proportion of brain development in the sheep occurs before birth,[97,98] but the majority of the rodent brain growth occurs after birth.[97] Differences between patterns of protein concentrations in sheep and rodent

CSF most likely reflect maturational differences in brain development among species.[97]

IAIPs have been previously detected in human CSF in patients with brain tumors and inflammatory diseases, but their levels were not affected by systemic levels.[58] Numerous studies suggest that the high concentration of protein in fetal CSF is not due to simple diffusion from plasma, rather there are specific developmentally regulated transfer mechanisms in the CP.[43,99–105] Inspection of Figures 3 and 8 suggests that this phenomenon is true in sheep as the IAIP levels in CSF are higher in the fetuses than in newborn lambs, but the plasma IAIPs levels are higher in newborn lambs than in fetuses. Therefore, we speculate that the presence of relatively high levels of IAIPs in fetal CSF is probably due to local synthesis by the CP and brain parenchyma during critical periods of brain development, and that these molecules in CSF could be important in brain growth in the fetus.

In the present study, endogenous IAIPs were detected for the first time during the development in the sheep CNS. Furthermore, they were detected in relatively high amounts in the CC and CP at all stages of development and in the CSF during fetal life. However, expression in CC, CP, and CSF decreased in newborn lambs after delivery. Although we cannot discern from our study, the reason that the levels of IAPs decreased dramatically after birth, we speculate that the stress of delivery along with endogenous hormonal changes could have affected the CSF levels of IAIPs after birth. The levels in CC and CP increased again in adult sheep, most likely related to the importance of these proteins in innate immunity. Even though we cannot be certain of the physiological significance of our findings of high IAIPs levels in ovine brain, CP, and CSF during development, we speculate that these molecules represent important factors for brain development.

Similar to our findings in the brain, we have shown for the first time that these immunomodulatory proteins are present in somatic organs and in the placenta, and that they exhibit distinct molecular weight and organ-specific patterns of developmental regulation in liver, heart, kidney, and placenta. Based upon our findings that IAIPs have unique patterns of expression in brain, CP, CSF, placenta, liver, heart, and kidney, which differ from those observed in sheep plasma, it appears unlikely that the serum IAIP levels could account for the organ-specific patterns of expression. Moreover, the brain vascular IAIP levels do not appear to be major contributors to the patterns of IAIP expression within the brain (Table 3). We speculate that although the exact functions of IAIPs are not known, their presence in large amounts with organ-specific variations during development raises the possibility that they represent endogenous anti-inflammatory molecules with organ-specific differential production or modulation during development.

Although we detected the expression of both IAIP proteins in the placenta, we have not measured the potential maternal to fetal transfer of IAIP proteins across the placenta, which would be of great interest for future studies. It would be feasible to radiolabel IAIP proteins and measure their kinetics across the placenta.[106] The type of the placenta could affect protein transfer from the maternal to fetal compartments. Moreover, the transfer of other proteins such as maternal antibodies varies dramatically among species during the gestation.[107,108] In carnivores, some mammals, and rodents, the placenta transfer of antibody can occur from mother to fetus.[107,108] In contrast, the placenta of ruminants such as bovine and ovine species forms a barrier between maternal and fetal circulations that impedes the transfer of antibodies.[109,110] In these species, most of the antibodies are delivered to the newborn via maternal milk. In the human, IAIP levels in newborns do not appear to be dependent upon maternal levels potentially suggesting that fetal levels could be independent of maternal levels.[4] However, the relationship between maternal to fetal transfer of IAIP proteins awaits further investigation.

There are several limitations to our study. We did not have CSF samples from adult sheep available and, consequently could not compare adult values with those from the fetuses and newborn lambs. However, IAIPs are not detectable in CSF from healthy adult humans (Y.-P. Lim 2014, un-published personal communications), but are increased in the presence of inflammation and tumors.[58] We also did not have samples properly saved from our previous studies to determine the specific immunohistochemical location of IAIPs within the brain and we did not have tissue available from other brain regions. Consequently, we cannot comment upon the cellular localization of IAIPs or on their expression in other brain regions. The identity of the ovine IAIPs in this study is based on the reactivity of R-20 pAb that was raised against human IAIPs and was cross-reactive with ovine IAIPs. This antibody has been shown to react against heavy chains and light chain molecules of human IAIPs.[69] Although the composition of the specific molecular moieties of IAIP has not been characterized in sheep, identification of the molecular moieties of ovine IAIPs and their functional characteristics are beyond the scope of the current study. Finally, we only measured protein levels of IAIPs in the brain and somatic tissues, which is also dependent on the protein turnover rate. Although quantitative real-time-polymerase chain reaction (qRT-PCR) could more accurately reflect the IAIP tissue measurements than differences in the protein expression patterns, we did attempt to develop qRT-PCR measures for ovine IAIPs, but ovine IAIPs have not been cloned and, hence, the ovine primers were not available. We attempted to match available *bovine* IAIPs primers with the ovine genome to create several different potential ovine primers. However, only one of the primers gave us a signal consistent throughout the different tissue types. Unfortunately, this primer yielded a much larger sequence than initially anticipated. Thus, we speculated that the recognized sequence was the ovine IAIPs. Unfortunately, although we attempted TOPO A cloning, the procedure was not successful. We consider it important to pursue this area of research in future work. Nonetheless, it should be emphasized that our work is important, as there are no previous studies addressing the quantitative distribution of IAIPs among different tissue types in a single species over this wide developmental time span.

We conclude that IAIPs exhibit specific patterns of expression in the CNS and somatic organs of sheep during development. Although exact functions of IAIP are not known in CNS and somatic tissues, their presence in relatively high amounts suggests their importance to brain and organ development.

Author contributions: MSS designed the study, performed the analysis of the tissues, analyzed and interpreted the data, and wrote the initial draft of the manuscript; GBS contributed to the experimental design and assisted with the analysis of the tissues and interpretation of the data; SWT contributed to the experimental design and assisted with the analysis of the tissues and interpretation of the data; Y-PL purified the IAIPs and developed and supervised the ELISA for IAIPs and Western immunoblot analysis, developed and provided the reagents specific to these studies; BSS supervised the study design, all tissue and statistical analysis, interpretation of the data, and revised the manuscript.

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