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Developmental changes in the distribution of corticosteroid-binding globulin in fetal and newborn sheep tissues

Вивчено зміни імуногістохімічної локалізації кортикостероїдзв'язувального глобуліну (КЗГ) у тканинах плодів різного віку та новонароджених ягнят. Зразки тканин для досліджень брали на 63–64, 100–103, 125–128 і 142–144-му добу гестаційного розвитку або через дві доби після народження. У печінці імунореактивний КЗГ (ір-КЗГ) виявлявся в гепатоцитах. Інтенсивність КЗГ-забарвлення була найвищою на 63–64-му добу гестації, а потім поступово зменшувалася до незначного рівня у новонароджених ягнят. У нирках ір-КЗГ вибірково накопичувався в епітелії проксимальних і дистальних звивистих трубочок. Його кількість протягом розвитку змінювалась аналогічно до змін у печінці. У легенях і підшлунковій залозі плодів спостерігалося значне збільшення ір-КЗГ наприкінці вагітності. В легенях плодів ір-КЗГ виявлявся в респіраторному епітелії третинних бронхів, бронхіол і термінальних бронхіол, тоді як альвеоли й інші структурні елементи легенів були КЗГ-імунонегативними. У підшлунковій залозі ір-КЗГ-забарвлення було асоційовано з ацинарними клітинами, а острівці Лангерганса не містили ір-КЗГ упродовж усієї вагітності. Динаміка ір-КЗГ не повторювала відомий трифазний профіль концентрації КЗГ у плазмі крові плодів овець протягом гестаційного періоду. Це вказує на існування незалежних клітинних механізмів, що регулюють вміст КЗГ у тканинах. Особливості внутрішньоорганного розподілу та змін вмісту ір-КЗГ свідчать, що внутрішньоклітинний КЗГ може регулювати концентрацію біодоступного кортизолу у тканинах овець під час внутрішньоутробного розвитку та у перші дні після народження.

INTRODUCTION

Corticosteroids are important contributory factors to fetal organ maturation during gestation, as well as to parturition [6, 12, 16, 23, 24]. Biological availability of glucocorticoids to tissues depends mainly upon interaction with their high-affinity plasma protein, corticosteroid-binding globulin (CBG) [22]. In fetal sheep,the prepartum increase in plasma CBG concentration seems to be a component of activation of hypothalamo-pituitary-adrenal axis as one of the triggers for the onset of parturition [3].

It has been supposed that CBG may participate in steroid delivery to target tissues [18]. In addition,the expression of the CBG gene was found not only in the liver,which primarily produces CBG, but in some other organs of fetal mouse [19] and rabbit [21]. Accordingly to our preliminary immunohistochemical observations, immunoreactive CBG (irCBG) is present in the fetal sheep liver,kidney and lung [6].

In the present study we have examined immunohistochemical localization of irCBG in the fetal sheep liver, lung, pancreas and kidney at different gestation ages as well as in newborn lambs. CBG concentration in the fetal sheep circulation is known to be significantly higher at early and late gestation as compared with mid-gestation, and declines rapidly after birth [1, 2]. Presumably, developmental changes in the fetal tissue distribution of CBG occurs over gestation.

METHODS

Animals and tissues. Tissues for this study were obtained from fetuses (4–5 at each gestation age of days 63–64, 100–103, 125–128 and 143–144) of mixed-breed sheeps with time-dated pregnancy (term 145–147 days) and from 2 lambs at postnatal day 2. Pregnant animals and newborn lambs were killed by an overdose of Euthanyl (MTC Pharmaceuticals, Cambridge, Ontario, Canada). The fetuses were delivered by cesarean section and killed by Euthanyl via cardiac puncture. The lung, pancreas, kidney and liver tissues were fixed in 4 % paraformaldehyde – 0.2 % glutaraldehyde,then washed in phosphate buffered saline (0.01 M ; pH 7.45) and stored in 70 % ethanol before embedding them in paraffin wax. The paraffin blocks were sectioned at 5 мм for immunohistochemistry,and two sections per slide have been placed.

Immunohistochemistry. Polyclonal primary CBG antibody was raised in rabbits against purified ovine CBG. Tissue sections were deparaffinized,incubated in 0.3 % H_2O_2, then with 10 % normal goat serum followed by CBG antibody at a dilution of 1:1000 (20 hrs at 4°C), and stained for irCBG by avidin-biotin-peroxidase method using the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine. Sections were counterstained with Carazzi·s hematoxylin, dehidrated and mounted with Permount.

Each batch of tissue that was processed included sections of fetal liver as positive control. The specificity of CBG immunostaining was verifierd by the negative control experiments in which nonimmune rabbit serum (1:1000), antibody dilution buffer or primary antibody preabsorbed with purified ovine CBG (final CBG concentration of 20 nM, overnight incubation at 4°C) substituted primary antibody.

Stained sections were examined using a Leitz Aristoplan microscope. For quantification of the intensity of staining a scoring system was established: 0 – immunonegative, 1 – pale, 2 – good, 3 – very good, 4 – very intense staining.

Data analysis. Immunostaining scores were expressed as the mean ± S.E.M. for each experimental group and analysed using Student's t-test and Mann-Whitney nonparametric test. Significant differences were set at $P<0.05$.

RESULTS

irCBG in the liver. In the fetal liver, irCBG was localized to the hepatocytes through the course of gestation (Fig. 1). It was not associated with haematopoetic cells. The intensity of staining was highest in the liver tissue of 63–64-days-old fetuses. It appeared to be significantly less since 100–103 gestation days, and remain at low level in preterm lambs (Table). In newborn lamb liver, irCBG was almost undetectable.

irCBG in the lung. The lung tissue was CBG-immunonegative in 63–64-days-old fetuses. irCBG appears as very pale staining by gestation age of 100–103 days and remains to be very weak up to preterm period of time, when its quantity increases drastically (Fig. 2, Table).

The distribution of irCBG in the fetal sheep lung was limited by respiratory epithelial cells of tertiary bronchi, bronchioles and terminal bronchioles. Respiratory bronchioles, alveolar sacs, and other lung tissues did not contain irCBG.

irCBG in the pancreas. At 63–64 gestation days, irCBG was almost undetectable in the pancreas. The intensity of CBG-staining slightly increased through the course of gestation. Dramatic increase of irCBG quantity was revealed in preterm fetuses (Fig.3, Table) that was comparable with that in the lung tissue.

In the fetal pancreas, irCBG presence was associated with the acinary cell cytoplasm. Specific immunostaining seems mostly to be basally located within exocrine cells. Langerhans islets were immunonegative.

irCBG in the kidney. irCBG in the fetal kidney was localized to the epithelium of proximal and distal convoluted tubules (Fig.4). At gestation days of 100–103, immunopositive granules in the convoluted tubules look more

Intensity of immunohistochemical staining for corticosteroid-binding globulin in fetal sheep and newborn lamb tissues*

Tissue	Gestation age				
	63–64d	100–103d	125–128d	142–144d	Newborn
Liver	2.3±0.12	1.0±0.20 P<0.001	1.0±0,00 P<0.001	0.9±0.13 P<0.001	0.5
Lung	0	0.8±0.17 P<0.01	0.7±0.20 P<0.01	2.3±0.33 P<0.001	-
Pancreas	0.6±0.12	0.8±0.14 P>0.05	1.1±0.12 P<0.05	2.4±0.31 P<0.05	-
Kidney	1.5±0.00 P>0.05	1.1±0.24 P>0.05	1.1±0.19 P<0.05	0.7±0.33	0.25

* Values are scores expressed as means ± S.E.M. for 4–5 fetuses or means for 2 newborns.
P presents statistical significance vs group of 63-64 gestation days.

condenced than that of days 63–64. Although good staining one can see in the loop of Henle, collecting tubules and ducts, and some other tissues of the renal medulla, this is also observed in negative controls with the rabbit nonimmune serum or preabsorbed CBG antibody thereby demonstrating nonspesific staining. irCBG was not present in glomeruli.

The highest level of irCBG during the fetal life has been found at an earlier stage of gestation (Table). It is getting weaker along with the kidney development, and very close to background staining in newborn lambs.

DISCUSSION

The results of this study demonstrate the presence of irCBG and its ontogeny in the liver and extrahepatic sites of the fetal sheep. Two main questions arise from this observations: 1) What is the origin of intracellular CBG? and 2) What is possible role of intracellular CBG in fetal growth and physiology? Certainly, immunohistochemistry does not permit differentiation of alternative possibilities, however,some speculations can be proposed.

Liver. The liver is the major site of CBG biosynthesis in human and all animal species have been examined to date [11]. irCBG in fetal sheep hepatocytes is likely of local source, as there is no transplacental transfer of maternal plasma CBG into fetal compartment [3]. The greatest content of irCBG in early gestation liver (63–64 days) matches high level of corticosteroid-binding capacity (CBC) of ovine fetal plasma [1]. Surprisingly, low intensity of irCBG staining in the liver tissue that we observed at late pregnancy did not correlate with prepartum increase in fetal plasma CBC and hepatic CBG mRNA levels [1, 2, 5]. Presumably, it is attributed to accelerated release of newely synthesized proteins from liver into the circulation as fetal liver maturation occurs. Negligible presence of irCBG in the liver of newborn lambs corresponds with a significant fall in CBG biosynthesis and its plasma level at this age. Similarly, both irCBG and CBG mRNA were undetectable in the neonatal mouse liver [20].

Liver is known to be one of the most important target organs for glucocorticoid hormones. We suppose that low intracellular CBG quantitiy in prepartum and newborn lambs could contribute to greater availability of corticosteroids for liver tissue, where they induce accumulation of glycogen and cause other metabolic changes as a component of the fetus preparation for labor stress and early postnatal adaptation for a new environment.

Lung. We were unable to detect irCBG at pseudoglandular stage of fetal sheep lung development (63-64 days) despite high level of

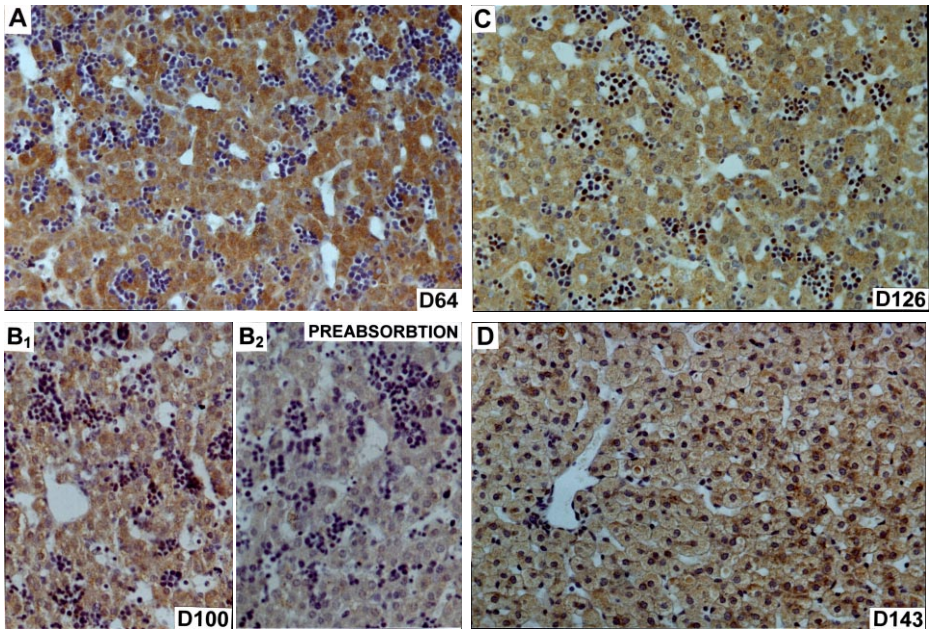

Fig. 1. Immunohistochemical staining for corticosteroid-binding globulin (CBG) in the liver of sheep fetuses (Panels A–D). Gestation age (days) is presented in the bottom of panels. irCBG is localized to the hepatocytes. Panel B_2 shows the result of CBG staining when the primary antibody was preabsorbed with purified ovine CBG (20 nM), in a tissue section to B_1. Magnification: Panels A–D, × 375

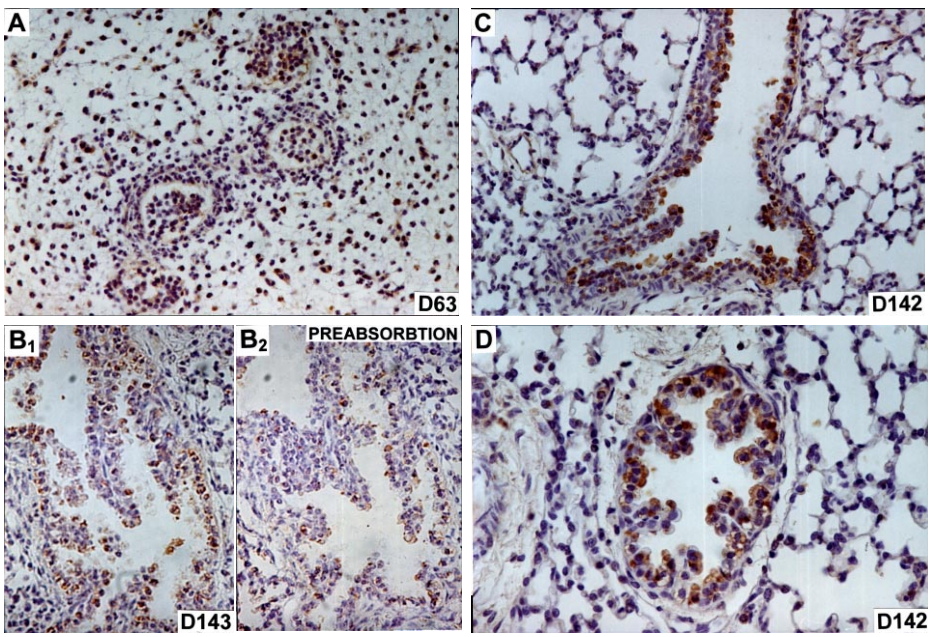

Fig. 2. Immunohistochemical staining for corticosteroid-binding globulin (CBG) in the lung of sheep fetuses (Panels A–D). Gestation age (days) is presented in the bottom of panels. Large amount of irCBG is present in terminal bronchioles (Panel C) and bronchioles (Panel D) at day 142 of gestation. Panel B_2 shows the result of CBG staining when the primary antibody was preabsorbed with purified ovine CBG (20 nM), in a consecutive tissue section to B_1. Magnification: Panels A–D, × 600; B_1, B_2, C, × 375

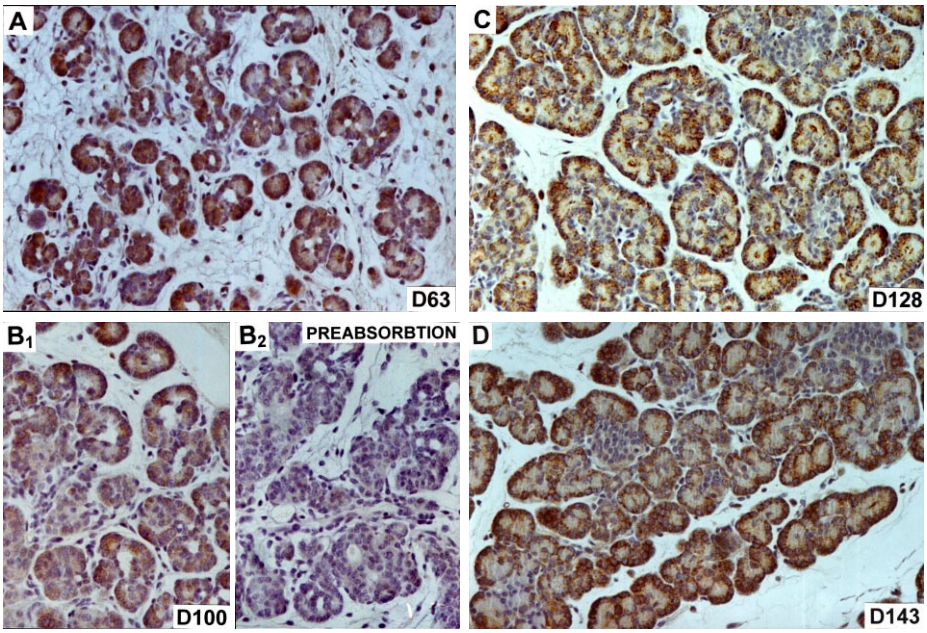

Fig. 3. Immunohistochemical staining for corticosteroid-binding globulin (CBG) in the pancreas of sheep fetuses (Panels A–D). Gestation age (days) is presented in the bottom of panels. Large amount of irCBG is present in exocrine cells at day 143 of gestation (Panel D). Panel B_2 shows the result of CBG staining when the primary antibody was preabsorbed with purified ovine CBG (20 nM), in a consecutive tissue section to B_1. Magnification: Panels A– D, × 375

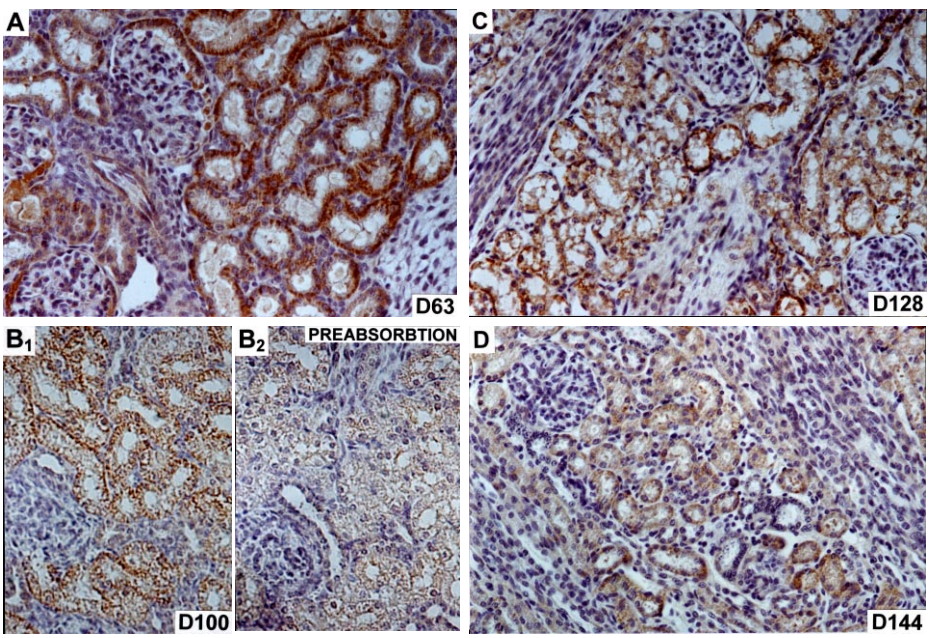

Fig. 4. Immunohistochemical staining for corticosteroid-binding globulin (CBG) in the kidney of sheep fetuses (Panels A–D). Gestation age (days) is presented in the bottom of panels. irCBG is localized to convoluted tubules where its largest amount is present at day 63 of gestation (Panel A). Panel B_2 shows the result of CBG staining when the primary antibody was preabsorbed with purified ovine CBG (20 nM), in a consecutive tissue section to B_1. Magnification: Panels A–D, × 375

circulating CBG at that time. Only negligible abundance of irCBG could be observed later, at canalicular stage. By contrast, at saccular stage, in correlation with preterm increase in plasma CBG level, developing lung displayed remarkable abundance of irCBG in small population of cells within fetal lung, namely, in respiratory epithelium of airways. If this is of blood plasma or amniotic fluid origin, there should be a mechanism for selective uptake of CBG and its internalization. Another possible source of irCBG in respiratory epithelial cells is its local synthesis. Although CBG mRNA has not been detected by northern blotting in fetal and adult sheep lung [5], it perhaps might be found in small clusters of lung cells using in situ hybridisation. In support of this assumption, CBG mRNA has been identified by this method in cells lining the basement membrane of bronchiolar epithelium in adult rat [9], and CBG cDNA has been isolated from a human lung cDNA library [10].

Glucocorticoids are the best known of the hormones affecting maturation of the lung surfactant and thereby increasing the size of the alveoli before term. So the absence of irCBG in developing alveolar tissue is not surprising, otherwise this protein could attenuate cortisol-induced acceleration of glycerophospholipid biosynthesis in type II pneumocytes. As for respiratory epithelium of bronchi and bronchioles, intracellular CBG seems to protect them from excess of cortisol rather than delivers it.

Pancreas. Developmental changes in irCBG amount in fetal sheep exocrine pancreas follows a pattern similar to that in the lung. Biosynthesis of CBG in ovine pancreas has not been studied. However, there is an evidence for colocalization of irCBG and its mRNA in the exocrine cells of the fetal mouse pancreas [19]. If this is so in the fetal sheep exocrine pancreas, then dual derivation of pancreatic CBG can be speculated as of systemic circulation and local synthesis.

Glucocorticoids were shown to modulate in vitro development of the embrionic rat exocrine pancreas [17]. Intracellular accumulation of CBG a few days before parturition seems to be important for transition of fetal exocrine pancreas to high functional activity in postnatal life.

Kidney. In developing fetal sheep kidney, irCBG was present exclusively in convoluted tubules. Likely to observations in fetal mouse kidney [19], in this study specific CBG immunostaining was not localized to the collecting tubules, glomeruli and other regions of the kidney. Epithelial cells of convoluted tubules comprise more than 20 % of the entire kidney tissue, however CBG mRNA has not been found in samples of total kidney RNA neither from sheep [5] nor from mouse fetuses [19]. irCBG in the fetal sheep kidney most likely derives from blood or glomerular filtrate that presumably is mediated by membrane CBG receptors have been identified on the luminal surface of rat renal tubules [14]. This mechanism probably forms developmental profile of irCBG amount in fetal kidney that does not follow preterm increase of CBG level in blood circulation.

Renal tubular function in utero begins at first trimester of pregnancy. Although cortisol depresses proximal sodium reabsorption, it also enchances this process in the distal nephron of fetal sheeps aged 125 to 135 days, so that there is no change in total tubular reabsorption [13]. However, younger fetuses (111–120 days) demonstrated natriuresis in response to administration of cortisol [25]. In our study, the greatest amount of irCBG in renal tubules was observed in young fetal sheeps (63–64 days). Its putative physiological role could be protecting renal tubules from rise of circulating cortisol at early gestation. This protection would be important during all fetal life and extended to renal effects of circulating progesterone thus preventing binding both steroids to mineralocorticoid receptors. It would be of a great interest to compare spatial and temporal distribution of irCBG in fetal sheep kidney with that of 11β-hydroxysteroid dehydrogenase. This enzime is syn-

thesized in ovine kidney during fetal and neonatal development [26] and it is considered as a key factor for tissue sensitivity for aldosterone [8]. 11β-hydroxysteroid dehydrogenase type 2 activity increased in late gestation (after day 85) in the fetal sheep kidney [15]. In the light of considerable importance of glucocorticoid hormones for the maturation of the sodium pump in rat kidney near term [7], the negligible quantity of irCBG in preterm fetal sheep kidney is supposed to allow greater bioavailability of cortisol.

Based on the fact that many tissues contain binding sites for plasma CBG, this protein was suggested to facilitate glucocorticoid delivery to target cells [18]. As for developing lung and kidney, our findings of irCBG distribution in those organs do not support «the delivery hypothesis». Indeed, irCBG has not been found in alveolar tissue that demands much glucocorticoids, meanwhile it was present in renal tubules that need protection from excess of circulating cortisol.

In conclusion, the results of this study demonstrate discrete distribution of irCBG in fetal sheep tissues and its developmental changes throughout gestation. Taken together this observations support the suggestion that intracellular CBG may regulate bioavailable cortisol concentrations in developing fetal tissues.

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DEVELOPMENTAL CHANGES IN THE DISTRIBUTION OF CORTICOSTEROID-BINDING GLOBULIN IN FETAL AND NEWBORN SHEEP TISSUES

Developmental changes in immunohistochemical localization of corticosteroid-binding globulin (CBG) in fetal and newborn sheep tissues were studied. Tissue samples have been harvested at days 63–64, 100–103, 125–128 and 142–144 of gestation or 2 postnatal days. In the liver, immunoreactive CBG (irCBG) has been identified in hepatocytes. The intensity of CBG staining was highest at 63–64th gestation days and then was lowered gradually down to negligible level in newborn lambs. Within kidney, irCBG was selectively localized to the epithelium of proximal and distal convoluted tubules. Its amount in the course of development followed a pattern similar to that in the liver. By contrast, fetal sheep lung and pancreas demonstrated noticeable rise of irCBG late in gestation. irCBG has been detected in respiratory epithelium of tertiary bronchi, bronchioles and terminal bronchioles, meanwhile alveoli and other lung tissues were CBG-immunonegative. In the pancreas, irCBG staining was associated with acinary cells, whereas Langerhans islets contained no irCBG at all examined stages of pregnancy. Developmental changes in irCBG did not follow reported triphasic profile of fetal sheep plasma CBG concentrations thereby showing the existence of independent cellular mechanisms regulating CBG level in the tissues. Peculiarities of intraorgan distribution and developmental changes in irCBG suggest that intracellular CBG may regulate bioavailable cortisol concentrations in the sheep tissues during fetal and early postnatal life.

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