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The frontal cortex IGF system is down regulated in the term, intrauterine growth restricted fetal baboon

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Abstract

Objective—The IGF system exerts systemic and local actions during development. We previously demonstrated that fetal cerebral cortical IGF1 is reduced at 0.5 gestation in our IUGR baboon nonhuman primate model. We hypothesized that by term protein expression of several key IGF system stimulatory peptide pathway components and downstream nutrient signaling effectors of IGF, mammalian target of rapamycin (mTOR) and S6, would decrease, indicating reduced cellular nutrient uptake and protein synthesis.

Design—We fed 7 control baboons *ad libitum* while 6 baboons ate a globally reduced diet (70% of feed eaten by controls) from 0.16 gestation through pregnancy that produces IUGR. Fetuses were removed at Cesarean Section at 0.9 gestation. Frontal cortex sections were stained for IGFI, IGFI, IGFR1, IGFR2, IGFBP2, 3, 5 and 6, and mTOR and ribosomal protein S6 and double stained with NeuN a neuron-specific nuclear antigen.

Results—All proteins stained neuronal cytoplasm except IGFR-1 which showed only glial cell cytoplasmic and blood vessel staining. IUGR fetuses showed decreased frontal cortical immunoreactive IGFI, IGFI, IGFR1, IGFBP2, 5 and 6, and mTOR and S6 ($p < 0.05$). IGFBP3 increased ($p < 0.05$) and IGFR2 was unchanged ($p > 0.05$). There were no differences between male and female fetal brains.

Conclusions—When fetal nutrient availability is decreased, IUGR down regulates the IGF system and its mTOR signaling pathway in the fetal frontal cortex coincident with slowed growth. These findings emphasize the importance of the local tissue IGF system in fetal primate brain development.

Keywords

IGF; IGFI; IGF receptors; binding proteins; mTOR; S6; baboon fetus; IUGR; brain

INTRODUCTION

We have developed a non-human primate model of poor maternal and fetal nutrition in pregnancy in which moderately reduced maternal nutrition, 30% global maternal nutrient reduction, leads to intrauterine growth restriction (IUGR) of about 12% ⁽¹⁾, a similar magnitude of the fetal weight reduction observed at the 10th percentile that defines human IUGR ⁽²⁾. IGF1 in the developing fetal frontal cortex is down regulated at 0.5 gestation (G)

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in this model in association with marked retardation of fetal cortical brain growth⁽³⁾. The IGF system consists of several factors, the ligand peptides IGFI and II, the receptors IGFR1 and R2, and at least six binding proteins that either decrease or enhance IGF peptide availability⁽⁴⁾. Together these components play a central role in development and growth of multiple organs and tissues, especially in the embryonic and fetal periods of life under both normal conditions as well as in response to such challenges as nutrient restriction during fetal life in several species including the baboon⁽⁵⁾.

The IGF system plays a central role in brain development^(6, 7) and is negatively regulated by glucocorticoids⁽⁸⁾ in a manner such that as normal term approaches most tissues switch from proliferation to terminal differentiation⁽⁹⁾. Other factors such as glucose and amino acid levels also regulate the IGF system⁽¹⁰⁾. In turn, the IGF system regulates key nutrient sensing and growth promoting cellular systems such as mammalian target of rapamycin (mTOR) a serine/threonine kinase that controls amino acid uptake, cell growth and metabolism⁽¹¹⁾. mTOR is regulated by many factors - amino acids, glucose, oxygen, energy status and growth factors including the IGF's⁽¹²⁾. mTOR signals through ribosomal protein S6 (S6)⁽¹³⁾. Thus information is needed on changes in the brain IGF system in the setting of impaired fetal growth in IUGR.

We hypothesized that by the end of gestation the frontal cortical IGF system in IUGR fetuses would continue to show widespread signs of dysfunction. We used immunohistochemical approaches to evaluate cellular distribution and quantity of IGFI, IGFI, IGFR1, IGFR2, IGFBP 2, 3, 5, and 6 as well as the downstream IGF signaling regulatory molecules mTOR and S6.

MATERIALS AND METHODS

Animal care and maintenance

All procedures were approved by the Texas Biomedical Research Institute (TBRI) Institutional Animal Care and Use Committee and conducted in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Baboons were held in independently housed groups each consisting of 16 females and one male. Each group was housed in a cage that was 3.5 m high with a floor area of 37 m². Details of housing structure and environmental enrichment provided have been published elsewhere⁽¹⁴⁾. Maternal morphometric measurements were made prior to pregnancy to ensure homogeneity of females in the two groups. Each group of 16 females was initially housed with a vasectomized male to establish a stable social group. Once a day prior to feeding, all baboons were run into individual feeding cages. Once in individual cages they were fed Purina Monkey Diet 5038 (Purina, St Louis, MO, USA), standard biscuits. At the start of the feeding period, each baboon was given 60 biscuits in the feeding tray. At the end of the 2-h feeding period after the baboons had returned to the group cage, the biscuits remaining in the tray and on the floor of the cage and in the pan were counted. Food consumption of animals, their weights and health status were recorded daily. At the end of a 30-day period of adaptation to the feeding system, a fertile male was introduced. All baboons were fed *ad libitum* until 30 days gestation (dG – term 184 dG) when control (CTR) baboons continued to feed *ad libitum* and nutrient restricted baboons were fed 70% of feed consumed by controls on a weight-adjusted basis. Water was continuously available in the feeding cage at several locations in the group housing. Pregnancy was dated by following sex skin swelling and confirmed at 30 dG by ultrasonography. We studied a total of 13 baboon fetuses, 7 CTR (3males and 4 females) and 6 on reduced nutrition (3 males and 3 females).

Caesarean sections were performed at 165dG (0.9G) under isoflurane anesthesia (2%, 2.0 l/min) to obtain the fetus and placenta. Techniques used and postoperative maintenance have been previously described in detail ⁽¹⁵⁾. Analgesia was provided with buprenorphine hydrochloride at 0.015 mg/kg/day during 3 postoperative days (Buprenex® Injectable, Reckitt Benckiser Health Care (UK) Ltd, Hull, UK). Fetal morphometrics were obtained at the time of caesarean section. In addition, fetal brains were collected and cut in half longitudinally. One half quickly dissected with areas/nuclei immediately snap frozen in liquid nitrogen and stored at -80 °C. The other brain half was fixed in formalin and embedded in paraffin for histological analyses.

Immunocytochemical analyses

Immunohistochemical methods have been described in detail ⁽⁵⁾ Immunohistochemistry was conducted on 7 fetuses of control mothers and 6 IUGR fetuses. Sources and final dilutions of the primary antibodies for the proteins and peptides examined are presented in Table 1. Immunohistochemical methods were performed as previously reported ⁽¹⁶⁾. Data presented are for fraction stained as that measure most closely reflects the numbers of neurons expressing the peptides.

The location of the brain sections studied is shown in Figure 1. Precise physical separation of the cortex and underlying white matter is very difficult to perform. Figure 1A shows that it is always possible that the final specimens analyzed will contain varying portions of cortex and underlying white matter which contains some of the proteins under study in the cortex. The white matter was not part of this investigation. Inclusion of any white matter in a sample taken for a western will thus be a complicating factor when the same protein exists in both areas but in different quantities. As a result, it is our conclusion that in complex, heterogeneous tissues such as the brain, immunohistochemical quantification of protein provides better information and fewer confounds than western analysis. Since the present study focused on the cortex we wished to avoid contamination by variable amounts of subcortical white matter and therefore used the dual strengths of immunohistochemistry, its ability to evaluate both distribution and quantity of the specific proteins studied.

Measurement of plasma cortisol

Cortisol was measured using an Immulite 1000 analyzer with kits from Diagnostic Products Corporation (Los Angeles, CA, USA) as previously described in detail ⁽¹⁷⁾.

Statistical analysis

We studied frontal cortex immunoreactive proteins in 7 CTR fetuses (3 males and 4 females) and 6 IUGR (3 males and 3 females). An initial analysis was performed to determine if there were any differences by sex of fetus for all variables. The only difference in response to IUGR by sex was in the female fetuses in which IGF2R was reduced by IUGR but not male fetuses and when pooled there was no overall difference. Thus molecular and morphometric data were pooled and reported as mean $\pm$ SEM. Analysis throughout was by the two-sided Student's t test.

RESULTS

Maternal and fetal morphometrics at 0.9 gestation

Table 2 provides the maternal and fetal morphometrics.. While these group fetal weights did not differ significantly by sex or treatment due to group sizes, fetal weights in the different groups studied fell within the ranges previously published with this degree of maternal food

reduction which showed significant IUGR for both male and female fetuses of mothers on reduced nutrition ⁽¹⁾. We therefore refer to the fetuses in the treated group as IUGR fetuses.

There were no differences in fetal organ weights by sex or treatment or evidence of hypoxemia in the IUGR fetuses (Table 2).

Fetal plasma cortisol

Fetal cortisol was 202 ± 32.4 ng/ml (CTR, n=7,) and 276 ± 26.6 ng/ml. (IUGR n=6) ($p < 0.07$). There were no differences between sexes in the two groups.

Figure 2A shows the histological distribution within the frontal cortex of the ten peptides and signaling molecules studied. To confirm the localization of the various peptides, sections were double stained for NeuroN a neuron marker. All proteins and peptides stained the cytoplasm of neurons except IGFR-1, which showed cytoplasmic staining only in glial cells.

Figure 2B shows a summary of the data for all the animals for the ten peptides and proteins. The only protein which increased was IGFBP3 which acts to retain IGF-1. Of the other nine proteins and peptides, six were significantly reduced ($p < 0.05$) and two – IGF2 and mTOR for which the p value for the decreases was $p < 0.1$. IGFR2 was unchanged.

DISCUSSION

The IGF system is a major regulator of fetal organ growth and development ⁽⁴⁾. IGFI and II are produced widely by fetal tissues and act as autocrine, paracrine and endocrine regulators. The IGF system includes the IGFI and IGFI peptides, receptors and binding proteins. IGFI and II, the ligands responsible for IGF's cellular biological actions, have structural similarities to insulin and regulate metabolism, mitogenesis and differentiation in fetal and placental tissues and inhibit apoptosis ⁽¹⁸⁾. They act as cell cycle progression factors, increasing DNA and protein synthesis and cell differentiation. IGFR1 and IGFR2, are structurally unrelated cell surface ligand receptors. IGFBP1 to 6, are associated binding proteins, which alter access of IGFI and II to their receptors as well as their biological half lives.

In support of a local brain action for the IGF system Dudek et al. provide evidence that IGFI functions as a classical survival factor for neurons ⁽¹⁹⁾. IGFI has pleiotropic effects on the brain in both pre- and postnatal animals including: protection of neurons from apoptosis ^(20–22), promotion of neuronal glucose utilization during development ⁽²³⁾, vascularization and remodeling of brain blood vessels ^(24, 25). Circulating fetal and neonatal IGFI concentrations are reported to be independent of pituitary growth hormone ⁽²⁶⁾, but there is some controversy in animal studies ⁽²⁷⁾. In humans, IGFI levels in umbilical cord blood show a good correlation with size of the fetus at birth ⁽²⁸⁾.

The IGF binding proteins are generally considered to retain IGFI and II, increasing their half-life and restricting their activity. The possible exception is IGFBP1. We were unable to find suitable antibodies for IGFBP1 and 4 despite trying multiple commercial sources. IGFBP2, IGFBP3 and IGFBP5, inhibit IGF1 and II induced cell proliferation ⁽²⁹⁾. IGFBP2 levels are inversely related to the rate of catch up growth in small for gestational age babies ⁽³⁰⁾. Our finding of decreased IGFBP2, IGFBP5, and IGFBP6 would indicate generalized cellular changes directed at making IGFs more available to the cell and may thus represent compensation for the decreased IGF. However the rise in IGFBP3 runs counter to that conclusion.

The level of activity of the IGF system is regulated by nutrient availability. In the presence of reduced nutrients, growth is slowed. Fetal hepatic phosphoenolpyruvate carboxykinase, the rate limiting enzyme in gluconeogenesis is increased in this model at 0.9G⁽³¹⁾. This change is likely responsible for observation that fetal plasma glucose levels are normal⁽³¹⁾ even in the face of reduced glucose availability. We have previously reported that plasma concentrations of five amino acids (asparagine, taurine, methionine, leucine, and ornithine) were reduced in the same set of IUGR fetuses compared with same CTR fetuses evaluated in this study⁽¹⁾. This reduction probably reflects both decreased amino acid delivery to the fetus and increased gluconeogenesis especially by the fetal liver⁽³¹⁾. Thus the reduced plasma amino acid availability may play a role in the decreased activity of the IGF system observed.

IGF signals in part through the mTOR system, considered by many investigators to represent a major cellular nutrient-sensing pathway. In response to nutrient deprivation protein synthesis is inhibited by decreased activity in the mTOR signalling pathway⁽³²⁾. The serine/threonine kinase mTOR integrates signals from nutrients, growth factors and energy availability to control amino acid uptake, cell growth and metabolism^(11, 33). mTOR is regulated by a wide range of factors including growth factors. In a study in the fetal mouse brain, in contrast to our findings, maternal under nutrition increased mTOR at the mRNA level⁽³⁴⁾. This would seem counterproductive at a time when growth is slowing. The observation – only at the mRNA level and in an altricial species, shows the need for studies in precocial species such as non-human primates. In the IUGR fetal baboons studied here, mTOR protein and its downstream signaling molecule S6 were both reduced, indicating a significant down regulation of the drive to cellular growth and protein synthesis. While there is good evidence for a decrease in placental mTOR activity in human IUGR⁽³⁵⁾ as well as animal models of IUGR such as rats fed a low protein diet⁽³⁶⁾ there are no data in any fetal tissue in nonhuman primates. The evidence provided here clearly shows that the decrease in IGF activity is accompanied by decreased downstream signalling pathways regulated by the IGF system.

The IGFbps are sensitive to the steroid environment in pregnancy and the changes observed may reflect steroid changes. In the present study the increase in cortisol in IUGR fetuses did not quite reach statistical significance. In a larger group of fetuses whose mothers were exposed to the same level of nutrient reduction, the difference between CTR and IUGR fetal cortisol reached a significance level of 0.03⁽¹⁾. There is abundant evidence that glucocorticoids inhibit the fetal IGF system⁽³⁷⁾. Increased glucocorticoid production is a key component of the mammalian organism's strategy to conserve energy for survival rather than growth in response to several different forms of stress. With regard to the developing nervous system, IGF1 expression in the neonatal rat brain is decreased by dexamethasone⁽³⁸⁾. Dexamethasone also down-regulates the IGF-system in glial cells⁽³⁹⁾. The glucocorticoid effect may be specific to IGF1 since glucocorticoids regulate IGF1 but not IGFII in human fetal chondrocytes *in vitro*⁽⁸⁾. Thus IGF system changes during IUGR induced by decreased fetal nutrition may in part reflect the increased activity of the fetal adrenal axis that we have shown in this model^(1, 40).

Conclusions

The changes in the brain IGF system observed in the presence of IUGR resulting from decreased fetal nutrient supply appear to be directed at slowing brain growth so as not to exceed the available supply of nutrients. While this down-regulation of the IGF system can be an effective short-term strategy, it carries the risk of long-term impairment of brain growth and function. We have shown in this model that cognitive function is impaired at 3 years of age⁽⁴¹⁾. Further studies are needed to evaluate progression in the neonatal period

and possible mechanisms of preventing unwanted outcomes by interventions both during pregnancy and neonatal life.

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Figure 1.

A) Location of brain sections studied by histology: asu- arcuate sulcus upper limb; asl – arcuate sulcus lower limb; ps – principal sulcus B) Diagrammatic representation to show that removal of sections of brain for western analysis can result in study of different proportions of cortical grey matter (GM) and underlying white matter (WM).

Immunohistochemical approaches allow exact evaluation of different zones. C) Six pictures were taken per slide from the areas in red and three adjacent slides were studied. SMA – supplementary motor area; cis – cingulate sulcus. Diagram redrawn from “Atlas of the Rhesus Monkey Brain in stereotaxic coordinates” by Kadharbatha S. Saleem, Nikos K. Logothetis Academic Press Elsevier” 2007

Figure 2.

A) Immunohistochemical staining of the ten proteins and peptides – Rows 1 and 4) in control fetuses (C), IGFR1-C; top insert shows IGF-R1 positive staining in glial cell cytoplasm. Bottom insert shows IGF-R1 positive staining in cytoplasm of capillary epithelial cells. (BV= blood vessels). Rows 2 and 5) in IUGR fetuses of nutrient restricted mothers. Calibration bar applies to all photomicrographs. Rows 3 and 6, double staining for each peptide with NeuN to determine neuronal distribution. B) Summary of fraction stained for the ten peptides. Data are mean $\pm$ SEM, CTR fetuses of *ad libitum* fed mothers (closed histogram, n=7), IUGR fetuses of mothers fed 70% of CTR (open histogram, n=6) * $p < 0.05$; # $p < 0.1$.

Table 1

Antibody antigens, final dilutions and sources used for immunohistochemical staining.

Antigen	Source	Cat #	final dilution
IGFI	Lab Vision	RB-9240	1:80
IGFII	R & D	AF-292-NA	1:50
IGFR1	Chemicon	MAB1120	1:50
IGFR2	Santa Cruz	sc-11410	1:50
mTOR	Abcam	ab51089	1:1000
S6	Cell Signalling	2317	1:500
IGFBP2	Santa Cruz	sc-6001	1:100
IGFBP3	Santa Cruz	sc-9028	1:250
IGFBP5	Santa Cruz	sc-6006	1:50
IGFBP6	Santa Cruz	sc-6007	1:50
Neu N	AVES	NUN	1:100

Table 2

Maternal and fetal morphometrics. There were no differences between any of the groups.

	CONTROL			IUGR		
	Total N=7	Male N=3	Female N=4	Total N=6	Male N=3	Female N=3
MATERNAL MORPHOMETRICS						
Pre-pregnancy weight (Kg)	15.0 ± 0.8	13.7 ± 0.6	16 ± 2.4	14.9 ± 0.4	15.1 ± 0.4	14.7 ± 0.7
FETAL MORPHOMETRICS						
Fetal weight (g)	744.4 ± 31.6	784.3 ± 46.2	714.5 ± 83.2	668.5 ± 33.4	711 ± 55.2	626.0 ± 27.0
Brain weight (g)	0.26 ± 0.0	0.27 ± 0.0	0.25 ± 0.1	0.24 ± 0.02	0.21 ± 0.02	0.28 ± 0.01
Heart weight (g)	77.2 ± 3.1	82.3 ± 4.5	73.3 ± 6.9	77.7 ± 2.5	77.6 ± 4.9	77.8 ± 2.6
Total Kidney weight (g)	4.43 ± 0.4	4.58 ± 0.4	4.31 ± 1.3	3.52 ± 0.1	3.54 ± 0.1	3.50 ± 0.1
Liver weight (g)	3.45 ± 0.2	3.78 ± 0.5	3.2 ± 0.2	3.56 ± 0.3	3.6 ± 0.2	3.51 ± 0.6
Lung weight (g)	20.5 ± 0.4	20.0 ± 0.7	20.8 ± 1.2	18.0 ± 1.4	18.8 ± 2.9	17.2 ± 0.6
Hemoglobin (g/dl)	13.6 ± 0.4	14.2 ± 0.7	13.6 ± 0.2	13.7 ± 0.7	13.9 ± 0.5	14.5 ± 0.2
Hematocrit (%)	41.4 ± 1.4	43.2 ± 1.1	41.0 ± 0.8	41.6 ± 2.2	41.5 ± 1.7	44.9 ± 0.8