Genomics of the fetal hypothalamic cellular response to transient hypoxia: endocrine, immune, and metabolic responses

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1Department of Physiology and Functional Genomics, University of Florida College of Medicine, Gainesville, Florida; 2Department of Animal Sciences, University of Florida College of Agriculture and Life Sciences, Gainesville, Florida; 3Department of Physiological Sciences, University of Florida College of Veterinary Medicine, Center for Environmental and Human Toxicology, Gainesville, Florida; and 4Department of Pharmacodynamics, University of Florida College of Pharmacy, Gainesville, Florida

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Wood CE, Rabaglino MB, Chang EI, Denslow N, Keller-Wood M, Richards E. Genomics of the fetal hypothalamic cellular response to transient hypoxia: endocrine, immune, and metabolic responses. Physiol Genomics 45: 521–527, 2013. First published May 7, 2013; doi:10.1152/physiolgenomics.00005.2013.—Fetuses respond to transient hypoxia (a common stressor in utero) with cellular responses that are appropriate for promoting survival of the fetus. The present experiment was performed to identify the acute genomic responses of the fetal hypothalamus to transient hypoxia. Three fetal sheep were exposed to 30 min of hypoxia and hypothalamic mRNA extracted from samples collected 30 min after return to normoxia. These samples were compared with those from four normoxic control fetuses by the Agilent 019921 ovine array. Differentially regulated genes were analyzed by network analysis and by gene ontology analysis, identifying statistically significant overrepresentation of biological processes. Real-time PCR of selected genes supported the validity of the array data. Hypoxia induced increased expression of genes involved in response to oxygen stimulus, RNA splicing, apoptosis, vascular smooth muscle proliferation, and positive regulation of Notch receptor target. Downregulated genes were involved in metabolism, estrogen receptor-mediated immunity, macromolecular complex assembly, S-phase, translation elongation, RNA splicing, protein transport, and posttranscriptional regulation. We conclude that these results emphasize that the cellular response to hypoxia involves reduced metabolism, the involvement of the fetal immune system, and the importance of glucocorticoid signaling.

Material and Methods

Animal procedures. All of the experiments were approved by the University of Florida Institutional Animal Care and Use Committee. All animals were housed in individual pens located in the Animal Care Services Department at the University of Florida. Fetal sheep were chronically catheterized, as described below. The gestational age at the time of experimentation was 124–132 days. The rooms maintained controlled lighting and temperature, and sheep were given food and water ad libitum.

Food was withheld from the pregnant ewes for 24 h before surgery. Surgery and catheter placement for all fetuses was performed with aseptic technique as previously described, with femoral arterial and venous catheters as well as amniotic fluid catheters (29, 45); animals were anesthetized with isoflurane in oxygen. Maternal femoral arterial and venous catheters were implanted chronically, and a catheter (3 mm external diameter, constructed of polyvinylchloride) was implanted into the maternal trachea. At the end of the surgery, antibiotics (750 mg ampicillin) were administered into the amniotic cavity via direct injection. Vascular catheters were exteriorized through the flank of the ewe. The tracheal catheter was exteriorized through a small incision in the side of the neck of the ewe and held in place with an expandable burn dressing (Spandag; Medi-Tech, New York, NY). Postoperative treatment and care were performed as previously described (17, 44). None of the animals in this study showed any signs of postoperative infection.

Experiments, blood and tissue collection, and plasma hormone assays. We studied three fetal sheep subjected to hypoxia (two males, one female) and four fetal sheep subjected to catheterization...
and a similar period of normoxia (two males, two females). Four of the fetal sheep (two in each group) were from twin pregnancies and three from singleton pregnancies. At least 5 days after surgery, ewes were subjected to a 30 min period of ventilatory hypoxia, induced by infusion of gaseous nitrogen into the trachea of the ewe, or 30 min of normoxia (44). A subset of specific gene expression measurements from the control group has been reported previously (44). Blood samples (not reported; 5 ml each, total of 25 ml per experiment) were collected to measure blood gases and plasma hormone concentrations as previously described (29, 42, 44). One hour after the beginning of the experiment, ewes and fetuses were euthanized with an overdose of pentobarbital sodium. Fetal brains were rapidly removed, dissected into distinct regions as previously described (36), snap-frozen in liquid nitrogen, and then stored at −80°C until processed for mRNA. In the present microarray experiment, the mRNA isolated from hypothalamus was analyzed. This region was selected because it controls endocrine function and initiation of parturition in the sheep (25).

RNA extraction and preparation. RNA was extracted from the hypothalamus with TRIzol (Invitrogen, Carlsbad, CA), followed by RNaseasy+ kits with on-column DNase treatment (QiAzo1, Valencia, CA). The RNA concentration was determined with a Nanodrop spectrophotometer (ND-1000, ThermoFisher, Wilmington, DE), and the integrity of the RNA was measured with an Agilent Bioanalyzer, 2100 model. RNA integrity number values ranged from 7.4 to 8.4. We labeled 500 ng of the DNase-treated RNA with Cyanine 3 (Cy3) CTP with the Agilent Quick Amp kit (5190-0442, New Castle, DE) according to their methodology, purified with the Qiagen RNeasy kit (Valencia, CA) according to Agilent’s revision of the Qiagen protocol as shown in the Quick Amp kit protocol except that the microcentrifugation was performed at room temperature instead of 4°C. The resulting labeled cRNA was analyzed with the NanoDrop spectrophotometer, and the specific activities and the yields of the cRNAs were calculated; these ranged from 9.0 to 12.8 pmol Cy3/μg RNA and from 4.29 to 10.38 μg, respectively. The labeled cRNA was stored at −80°C until use. Microarray hybridization, washing, and scanning were performed as previously described (31).

Statistical analysis. Transcript levels were normalized to the chip median and log-transformed, to obtain more power in discovering differences between groups and compensate for systematic differences between the arrays. To identify the genes that were differentially regulated (DR) between the treated and control fetuses, the normalized and transformed intensities were analyzed by one-way ANOVA (P < 0.05). All the statistical procedures were carried out using JMP Genomics 5 software (SAS Institute, Cary, NC). All of the microarray data have been uploaded to the Gene Expression Omnibus database (accession number pending).

Functional annotation. We used functional annotation of the genes as a useful tool to categorize the genes into classes, as previously described (31). Also as in our previous study, the platform number is GPL14112 (Agilent-019921 Sheep Gene expression microarray 8 15K, G4813A). Complete lists of these up- and downregulated genes are reported in Supplemental Data.1 Shown in Fig. 1, right, is the dendrogram of significant DR genes in the hypothalamus of the following plug-ins: GeneMania and BINGO. GeneMania was used to infer network data (41). The set of functional association data between genes was downloaded from the Homo sapiens database. The network was inferred for both upregulated and downregulated genes (treatment vs. control). BINGO was run to determine which biological processes were statistically overrepresented in the set of genes corresponding to the identified cluster (23). The statistical test employed was the hypergeometric test (equivalent to the Fisher test), with a threshold P value of 0.05, after correction by the Bonferroni method (2, 3).

Quantitative real-time PCR. Aliquots of mRNA were converted to cDNA with a High Capacity cDNA Archive kit by the methodology recommended by the kit manufacturer (Applied Biosystems, Foster City, CA). The newly synthesized cDNA was stored at −20°C until quantitative real-time PCR was performed. A total of nine genes (Table 1) were selected because of their inclusion in or relevance to the significant clusters of DR genes. Probes and primers were designed with Primer Express software (Applied Biosystems). Sequences and primer concentrations for primers and probes and accession numbers are reported in Table 1. Amplification reactions for all primer or probe and primer pairs had efficiencies >95%. The abundance of β-actin mRNA was determined in each sample, using primers and VIC Taqman probe designed from the ovine β-actin sequence and Taqman RT-PCR master mix (Applied Biosystems). With the exception of CRH (6FAM-labeled Taqman probe), all other genes were measured using Sybr green. All samples were run in triplicate for each gene and for β-actin. Relative mRNA expression of each gene was calculated by determining change in threshold cycle (ΔCt) between the mean Ct for each gene and the mean Ct for β-actin mRNA from the same sample. The effect of hypoxia on each gene was compared by Student’s t-test using the ΔCt values. For all statistical analyses, the criterion for achieving statistical significance was P < 0.05.

RESULTS

Ventilatory hypoxia. Fetal hypoxia, caused by infusion of nitrogen into the trachea of the pregnant ewe, decreased fetal arterial PaO2, from 19 ± 2 to 10 ± 2 mmHg at 5 min to 9 ± 1 mmHg at 30 min (P < 0.001). Apparent changes in PaCO2, from 54 ± 3 to 51 ± 2 mmHg between 5 and 30 min were not statistically significant (P = 0.169). Values of pHi increased from 7.38 ± 0.01 to 7.40 ± 0.01 at 5 min, but returned to 7.38 ± 0.01 by the end of the 3 min period of hypoxia (P = 0.001). Values of PaO2, PaCO2, and pHi in the control group were 19 ± 1 mmHg, 48 ± 2 mmHg, and 7.39 ± 0.01, respectively. Hypoxia increased expression of 541 genes and decreased expression of 837 genes in the fetal hypothalamus. Complete lists of these up- and downregulated genes are reported in Supplemental Data.1

Table 1. Primer sequences used in real-time PCR validation of selected up- and downregulated genes

<table>
<thead>
<tr>
<th>Official Symbol</th>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
<td>TTCCAGAATCTGCGGAGGGG</td>
<td>AGACACTGTCCTCAGGTCAGTC</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin releasing hormone</td>
<td>TCCTCACTTTCTGCTGATCTCT</td>
<td>GAGCTGTCCTGCGCACTGTA</td>
</tr>
<tr>
<td>CRHBP</td>
<td>corticotropin releasing hormone binding protein</td>
<td>ATCTCCGGCCATCCTGAGTCTG</td>
<td>GGCATGTCGCTCTGTAG</td>
</tr>
<tr>
<td>CSD3</td>
<td>CSD3 molecule, delta</td>
<td>ATCTCCGGCCATCCTGAGTCTG</td>
<td>GGCATGTCGCTCTGTAG</td>
</tr>
<tr>
<td>CSD3G</td>
<td>CSD3G molecule, gamma</td>
<td>ATTCTCTGAGGAGGAGGATT</td>
<td>TGCTAGGACCTGCTGATTCA</td>
</tr>
<tr>
<td>CSD5</td>
<td>CSD5 molecule</td>
<td>CAGTCCCTGCTCTCTGTAG</td>
<td>TTCTGGCCTGCTGATT</td>
</tr>
<tr>
<td>StAR</td>
<td>steroidogenic acute regulatory protein</td>
<td>GCTGTCGCTGGCTTGTGA</td>
<td>TGCTAGGACCTGCTGATTCA</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>11β hydroxylase</td>
<td>AACGCGGCGAGGCTGACTTAT</td>
<td>TGCGCGGCGAGGCTGATTCA</td>
</tr>
<tr>
<td>CASP8</td>
<td>caspase 8</td>
<td>CCAGTGCGGCGAGGCTGACTCAT</td>
<td>TCCTGGCCTGCTGATTCA</td>
</tr>
</tbody>
</table>

1 The online version of this article contains supplemental material.
hypoxic compared with normoxic fetuses. The volcano plot, shown in Fig. 1, left, illustrates that there is a larger cohort of genes downregulated by hypoxia compared with the cohort of genes upregulated by hypoxia. Network analysis revealed that 485 of the upregulated genes could be organized into a single network (not shown). GO analysis of the network of upregulated genes revealed a network of related GO terms (Fig. 2), with highest order statistically significant ontology terms including response to oxygen stimulus, RNA splicing, antiapoptosis, vascular smooth muscle proliferation, vasculature development, positive regulation of Notch receptor target. Network analysis revealed that 826 of the downregulated genes could also be organized into a single network (not shown). GO analysis of the downregulated gene network revealed a network of related GO terms (Fig. 3), including: antigen receptor-mediated immunity, macromolecular complex assembly, S-phase, translation elongation, RNA splicing, protein transport, posttranscriptional regulation, negative regulation of protein metabolism, cellular metabolism.

Analysis of both up- and downregulated genes in the KEGG genome database revealed that 100 of the DR genes are significantly associated with metabolic pathways, with reduced gene expression in the energy metabolic pathways, including tricarboxylic acid cycle and oxidative phosphorylation. Fatty acid and sphingolipid biosynthetic pathways are upregulated. Nucleotide metabolic pathways were downregulated. Glycan biosynthetic and metabolic pathways were downregulated. Other pathways with large numbers of DR genes include pathways in cancer (48 genes), HTLV-1 infection (41 genes), PI3K-Akt signaling (36 genes), Herpes simplex infection (31 genes), Epstein-Barr virus infection (28 genes), T cell receptor signaling (26 genes), leukocyte transepithelial migration (21 genes), Wnt signaling (18 genes), and NF-κB signaling (18 genes). With regard to neural and endocrine signaling, the glutamatergic synapse (12 genes, upregulated NMDA mGluR1/2/7/8, glutamate transporter, HOMER1 and decreased mGluR4 signaling), TGF-β (10 genes, increased TNF-α, BMP, and decreased SMAD), chemokine signaling (21 genes), T cell receptor signaling (26 genes), and VEGF pathway (10 genes).

The identification of glucocorticoid synthesis in the GO analysis suggested that several DR genes might be glucocorticoid sensitive. Analysis for overlap with known glucocorticoid receptor-controlled genes revealed only five DR genes, with GADD45B, PTGS2, RGS2, and SRGN upregulated and ANGPTL4 downregulated.
Ventilatory hypoxia increased six and decreased 13 genes known to be controlled by HIF1α. We compared our lists of up- and downregulated genes to a list of HIF1α-controlled genes (Fig. 4), as reported by Benita and coworkers (1). Analysis of all genes upregulated or downregulated ($P < 0.05$) by hypoxia in the current study revealed that these genes are significantly related to apoptosis and prostaglandin biosynthesis.

We performed quantitative PCR analysis on a subset of genes that were significantly DR or, if not on the array, were predicted to be DR by our network analysis (Fig. 5). These are genes that are related to hypothalamic releasing hormone synthesis, immune response, and glucocorticoid synthesis.

**DISCUSSION**

A remarkable characteristic of the genomic response of the fetal hypothalamus to a 30 min period of maternal ventilatory hypoxia is the preponderance of downregulation of gene expression relative to upregulation. KEGG analysis of the cohorts of up- and downregulated genes reveals that a major aspect of the response to hypoxia is a decrease in glycolysis and oxidative phosphorylation, a result that corresponds directly with the known physiological response to hypoxia in the late-gestation fetus (20). Jones et al. (21) reported that the fetus responds to hypoxia with a decrease in glucose consumption. Rurak et al. (35) reported that the fetus defends oxygen consumption ($\dot{V}O_2$) early in a continuous bout of hypoxia but that sustained hypoxia, especially when accompanied by acidemia, reduces $\dot{V}O_2$. In the fetal hindlimb circulation, the fetus maintains oxygen consumption until arterial oxygen content decreases to less than half (1.5 mM) of the normoxic level (3.2 mM) (9). Interestingly, the reduction in oxygen consumption might be related to the ambient oxygen tension, as fetal skeletal muscle cells in vitro reduce oxygen consumption when exposed to an...
hypoxic gas mixture (10). Nevertheless, not all investigators
have been able to measure reduced fetal oxygen consumption
during hypoxia (6, 7), suggesting that measured decreases in
oxygen consumption might be triggered by more than oxygen
delivery alone. It is likely, as stated by Richardson and Bock-
ing (33), that the fetus generally defends oxygen delivery and
uptake until the arterial oxygen content is reduced by more
than half, at which time the oxygen consumption decreases.
Fetal hypoxia also decreases tyrosine (27) and leucine (26)
uptake by the fetus, indicating that protein biosynthesis from
amino acids is also impaired by hypoxia. Of interest is that
none of the members of the SLC6 family of transporters,
thought to be amino acid transporters (11), were DR in the
present study. On the other hand, this might be due to the
specific timing of tissue collection in the present study.

Recently, Murray (28) has posited that the fetus and placenta
respond to hypoxia with a coordinated response, one that
cannot be accounted for simply by the supply of and demand
for oxygen. The genomic response to transient fetal hypoxia
reported in the present study support this argument. Reduction
of fetal PO2 from 19 to 9 would be expected to reduce the
oxygen content of the arterial blood within the range of oxygen
supply in which oxygen consumption is homeostatically main-
tained in the fetus (7). Nevertheless, the genomic signature of
the response to this transient hypoxia is a downregulation of
genes that encode enzymes involved in glycolysis and oxida-
tive phosphorylation, suggesting that the fetus mounts a mo-
lecular response to the hypoxia that underlies the eventual
decrease in oxygen consumption. In other words, the molecular
response comes first. What actually triggers the molecular
response is an interesting topic of speculation but is most likely
the result of changes in neurotransmission after stimulation of
the arterial chemoreceptors (4, 5).

GO analysis revealed a statistically significant probability
that RNA transcription, elongation, and splicing are altered by
hypoxia. While this makes sense in light of the expectation that
the hypoxic stress will elicit a vigorous genomic response, it is
also interesting in light of reports that RNA splicing can be
altered by hypoxia and that this response in the brain might
endow neurons with protection against hypoxic damage by
alternatively splicing the erythropoietin receptor (12). In hu-
man umbilical cord endothelial cells, hypoxia has been shown
to stimulate alternative splicing of mRNA’s encoding extracel-
lar matrix proteins (37).

Fig. 4. Venn diagram illustrating the overlap between populations of upregu-
lated (left, red, “Up”) and downregulated (right, yellow, “Down”) genes with
known HIF-1α responsive genes (bottom, green, “HIF1A”).

Fig. 5. Differentially regulated genes as measured by real-time PCR. All changes are
statistically significant (*P < 0.05) with the exception of AVP (P = 0.11). Data are
represented as fold change relative to control.
The appearance of immune responses in the GO analysis suggests a role for cells within the hematopoietic cell lineage in the response of the fetal brain to hypoxia. Supporting this analysis are increasing gene expression of CD3G, CD5, CD8A, CD247 and decreasing gene expression of CD1B, CD3D, CD3E, CD34, CD36, CD93, CD99, CD163L1. The pattern of change of the various CD markers suggests lymphocyte infiltration of the fetal brain after the period of hypoxia, similar to that observed in the adult animal after hypoxia/ischemia and stroke (38). It is also possible that the increase in Notch signaling is related to determination of T cell fate for precursor cells in the fetal hypothalamus (22). Functions related to immune cell function and cytokine signaling include alteration in the NF-κB cascade from the KEGG analysis. In adult animals, stroke promotes the local production of cytokines in the affected region of the brain. These cytokines can be involved in the relative opening of the blood-brain barrier, a step that promotes and allows lymphocyte infiltration into the brain tissue (15). On the other hand, the blood-brain barrier is not complete in the fetal sheep (39), and it is possible that leukocytes can traverse between blood and brain parenchyma as a feature of normal fetal physiology. The present results suggest activity of the innate and adaptive immune systems within the fetal brain and that the activity of the adaptive immune system is altered after fetal hypoxia.

The number of DR genes known to be glucocorticoid sensitive was relatively small. The number of glucocorticoid-regulated genes might have been limited by the upregulation of HSD11B2 and downregulation of HSD11B1, perhaps increasing local conversion of cortisol to cortisone, limiting the effect of the increased plasma glucocorticoid concentrations on cells expressing the GR in the fetal hypothalamus. It is also possible that we might have observed more glucocorticoid-regulated genes if we had investigated a later time point (allowing more time for genomic actions of transient increases in plasma cortisol concentrations 30–60 min earlier). With regard to HIF1A as a possible mediator of the cellular response to hypoxia, we detected changes in expression of several HIF1A-responsive genes. However, genes that are transcriptionally activated by HIF1A were downregulated in the present study (1). We therefore suspect that, at this early time point, the association with HIF1A-controlled genes is more correlation than cause and effect. The involvement of HIF1A might have been limited by the relatively mild and short-lived bout of hypoxia and the fact that the majority of the genomic response to the stress is likely to be mediated by changes in neurotransmission.

It is important to note that the design of this study focuses on the acute (30–60 min) response to transient hypoxia. The genes that respond within this relatively short time frame illustrate the response of the fetal hypothalamus at this specific time, and it is unlikely that the cohort of differentially regulated genes at later times would be the same. For example, the transcriptomic signature of reduced metabolism found in the present study matches the results of physiology experiments in other studies, but we would not expect this response to persist for days or perhaps even hours. For this reason, it is important to interpret these results within the context of time and to remember that these results cannot be generalized to later time points or to different periods of hypoxia (e.g., chronic hypoxia lasting hours or days).

To summarize the results of this gene array study, we have used the Agilent 15k ovine array, previously annotated in our laboratory (31), to identify genes in the ovine fetal hypothalamus that are significantly up- and downregulated 1 h after the onset of a 30 min period of maternal ventilatory hypoxia. There were major alterations in the transcriptome in response to the hypoxia. GO and pathway analysis revealed alterations in glucose metabolism and oxidative phosphorylation that reflect the well-known reduction in cerebral metabolism during hypoxia. GO also suggested an infiltration of T lymphocytes into and an acceleration of development of lymphocytes within the hypothalamus. The possible involvement of immune cells in the fetal response to a transient and nonlife threatening period of hypoxia is a novel result that could have broad implications for a better understanding of neuroimmune responses to relatively common stresses during fetal life. We conclude that the application of genomics technology and systems biology analysis to the fetal sheep model reveals potential mechanisms of stress responsiveness and stress survival that were heretofore unappreciated or unknown. In particular, these results emphasize that the cellular response to hypoxia features reduced metabolism, the involvement of the fetal immune system, and the importance of glucocorticoid signaling.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES