Placental TonEBP/NFAT5 Osmolyte Regulation in an Ovine Model of Intrauterine Growth Restriction

Author(s): Juan A. Arroyo, Pastora Garcia-Jones, Amanda Graham, Cecilia C. Teng, Frederick C. Battaglia, and Henry L. Galan
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Placental TonEBP/NFAT5 Osmolyte Regulation in an Ovine Model of Intrauterine Growth Restriction

Juan A. Arroyo, Pastora Garcia-Jones, Amanda Graham, Cecilia C. Teng, Frederick C. Battaglia, and Henry L. Galan

1Department of Obstetrics and Gynecology, University of Colorado Denver and Health Sciences Center, Aurora, Colorado
2Department of Neonatology, University of Colorado Denver and Health Sciences Center, Aurora, Colorado
3Department of Obstetrics and Gynecology, University of Kansas Medical Center, Kansas City, Kansas

ABSTRACT

TonEBP/NFAT5 (the tonicity-responsive enhancer binding protein/nuclear factor of activated T cells) modulates cellular response to osmotic changes by accumulating inositol and sorbitol inside the cells. Our objective was to assess placental osmolytes, TonEBP/NFAT5 RNA and protein expression, and signaling molecules across gestation between control and intrauterine growth restriction (IUGR) ovine pregnancies. Pregnant sheep were placed in hyperthermic conditions to induce IUGR. Placental tissues were collected at 55, 95, and 130 days gestational age (dGA) to measure inositol, sorbitol, TonEBP/NFAT5 (NFAT5), sodium-dependent myo-inositol transporter (SMIT; official symbol SLC5A3), aldose reductase (AR), and NADPH (official symbol DE-CR1). Placental weight was reduced in IUGR compared to controls at 95 and 130 dGA. Osmolyte concentrations were similar between control and IUGR placentas, but both groups demonstrated a significant decrease in inositol concentration and an increase in sorbitol concentration with advancing gestation. Cytosolic NFAT5 protein decreased significantly from 55 to 95 dGA in both groups, and nuclear NFAT5 protein increased only at 130 dGA in the IUGR group, but no differences were seen between groups for either cytosolic or nuclear NFAT5 protein concentrations. DE-CR1 concentrations were similar between groups and increased significantly with advancing gestational age. AR was lowest at 55dGA, and SLC5A3 increased with advancing gestational age. We conclude that both placental osmolytes and sorbitol (and their corresponding proteins SLC5A3 and AR) change with gestational age and are regulated, at least in part, by NFAT5 and DE-CR1 (NADPH). The inverse relationship between each osmolyte across gestation (e.g., inositol higher in early gestation and sorbitol higher in late gestation) may reflect nutritional needs that change across gestation.

hyperthermia, IUGR, osmoregulation, placenta, TonEBP/NFAT5

INTRODUCTION

Mammalian cells develop adaptive responses that enhance survival during various forms of cell stress. Osmolarity changes in mammalian cells result in an egress of intracellular water, which increases cellular ionic strength. Cells respond by accumulating organic osmolytes (polyols), such as sorbitol and inositol, thereby decreasing ionic strength by osmotic replacement [1]. Uncompensated osmotic stress leads to cell shrinkage, cell cycle delay apoptosis, DNA breakage, oxidative stress, and subsequently death [2–5]. The tonicity-responsive enhancer binding protein/nuclear factor of activated T cells (TonEBP/NFAT5) is a transcription factor that modulates cellular response to osmotic cell stress [6, 7]. Studies in mouse kidney cells have shown that an increase in osmolarity leads to activation of TonEBP/NFAT5 by phosphorylation and, subsequently, to nuclear translocation, transactivation, increased TonEBP/NFAT5 protein abundance, and mRNA stabilization [6–13]. Phosphorylation of this transcription factor is required for nuclear import and retention [9]. Activation of TonEBP/NFAT5 leads to increased expression of transmembrane proteins such as sodium-dependent myo-inositol transporter (SMIT/SLC5A3) and Na/Ci coupled osmolyte cotransporters as well as the induction of the aldose reductase enzyme (AR; responsible for sorbitol production), which regulate production and accumulation of inositol and sorbitol. Collectively, these regulate production and transport of organic osmolytes into cells to maintain normal osmolarity and cell volume [7, 14–16]. TonEBP/Nfat5 knockout mice, which undergo marked renal cell apoptosis, underscores the importance of the adaptive response to osmotic stress to cell survival [6]. In general, there is limited understanding of the significance of TonEBP/NFAT5 in other tissues besides the kidney. In particular, aside from a descriptive study, its role in the normal or pathologic placenta has not been previously investigated [17].

Intrauterine growth restriction (IUGR) complicates up to 5–8% of human pregnancies and is associated with increased fetal morbidity and mortality as well as serious sequelae for the newborn, child, and adult [18–20]. Placental insufficiency is the most common cause of IUGR with numerous placental abnormalities findings that include but are not limited to hypoxia, apoptosis, aberrant trophoblast invasion of spiral arteries, and increased placental resistance to blood flow [21–27]. In the placenta, trophoblast must thrive under conditions of both environmental stress, such as hypoxia, and intrinsic stress, such as volume changes associated with rapid cell division [28]. This is also true in IUGR cases with pre-eclampsia, where profound changes in volume shifts are well known.

A recent study demonstrates that proliferating and metabolically active murine thyroid and liver tissue microenvironments...
have high baseline osmolality when compared to serum, brain, and lung [29]. Similarly, the placenta is a metabolically active organ that undergoes phases of rapid cell proliferation. Thus, it is plausible that trophoblast also undergo osmotic cell stress during normal placentation and during pathologic obstetrical processes. Previous studies in our lab have demonstrated uptake of osmolytes into human and ovine umbilical circulation, high concentrations of sorbitol and inositol in placental tissue, and NFAT5 expression in the human and ovine placenta [17, 30, 31]. The objective of this study was twofold: 1) to determine the gestational age-related changes in TonEBP/NFAT5 and associated levels of enzymatic proteins responsible for osmolyte production across gestation in the placenta of normal ovine pregnancy and 2) to assess the effect of a pathologic pregnancy condition such as IUGR on these factors. We hypothesize that the placenta of IUGR pregnancies, compared to controls, will show an increase in osmolyte concentrations and in TonEBP/NFAT5 expression and a corresponding increase in production of AR and SLC5A3 enzymes.

MATERIALS AND METHODS

Animal Care and Tissue Preparation

We utilized a hyperthermia (HT)-induced ovine model of IUGR that has multiple features similar to the IUGR disease process seen in humans [31–34]. The University of Colorado at Denver Health Sciences Center Animal Care and Use Committee approved this study. Animal care was performed as previously described by Galan et al. [35]. A total of 28 sheep were studied. Fourteen sheep were placed in HT conditions beginning at 40 days of gestation (dGA; term 147 dGA) to induce IUGR, and 14 animals were placed in thermoneutral conditions as controls. The control and HT animals were distributed as follows: four animals per HT and control group representing early gestation (55 dGA), five animals per HT and control group representing midgestation (95 dGA), and five animals per HT and control group representing near term (130 dGA). At necropsy, the aqueous phase was transferred and RNA precipitated with 0.5 ml of 75% DE-PC-treated ethanol, dried at room temperature, and resuspended in 1 ml of TRI-REAGENT. After homogenization, samples were centrifuged, tissues supernatant were deproteinized and analyzed. A Dionex HPLC analyzer equipped with a CarboPac MA1 anion-exchange column was used for the separation the hexoses and polyols (Dionex, Sunnyvale, CA). The HPLC analysis was done as described by Arroyo et al. [17]. Briefly, ovine cotyledons were homogenized and sonicated in distilled water at 4°C. After centrifugation, tissues supernatant were deproteinized and analyzed. A Dionex HPLC analyzer equipped with a CarboPac MA1 anion-exchange column was used for the separation the hexoses and polyols (Dionex, Sunnyvale, CA). The analysis was run isocratically with 500 mM sodium hydroxide for 25 min, followed by a step change to 400 mM sodium hydroxide for 20 min at ambient temperature. The flow rate was 0.4 ml/h. The sodium hydroxide solution was prepared with deagionized water. All the peaks were quantified using a pulse anamperometric detector with a gold working electrode. The Dionex PeakNet software (Dionex) was used for instrument operation and data analysis. An internal xylitol standard was used to correct for instrument variances with the equation Concentration (μM) = (A/As)∗R∗D∗(X/NI).

RNA Extraction

Total RNA was extracted from the collected tissues using the TRI-REAGENT method. Protocol was followed as suggested by the manufacturer (Sigma, St. Louis, MO). Briefly, 100 mg of each tissue were homogenized in 1 ml of TRI-REAGENT. After homogenization, samples were centrifuged, supernatants were transferred, and chloroform was extracted. After centrifugation, the aqueous phase was transferred and RNA precipitated with 0.5 ml of cold isopropanol followed by centrifugation. The RNA pellet was washed in 75% DE-PC-treated ethanol, dried at room temperature, and resuspended in DE-PC treated water. The extracted RNA was then further purified with a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). To quantify the RNA, sample absorbance was measured at 260 and 280 nm using a GE Healthcare Ultraspec 4300 Pro-UV-VIS spectrophotometer (GE Healthcare, Piscataway, NJ). For RNA quality, samples were run on a gel, and 260/280 ratios were calculated. Only samples with a ratio above 1.9 were used for these experiments.

The cDNA was produced through reverse transcription using the First-Strand cDNA Synthesis protocol from the SuperScript III kit by Invitrogen (Invitrogen, Carlsbad, CA). Protocol was followed as suggested by the manufacturer. Briefly, 5 μg of total RNA were mixed with 50 μmol/L of oligo (dT) primers, 10 mmol/L dNTP mix, and DE-PC-treated water. Next, the samples were incubated at 65°C for 5 min and then placed on ice for at least 1 min. Then, 0.2 μL of each Oligo dT (25 μmol/L, MgCl2, 0.1 mmol/L DTT, RNase out, and SuperScript III RT) were added to each sample and then incubated at 50°C for 5 min. Reactions were terminated by incubation at 70°C for 15 min. RNase H (1 μL) was added to each sample and then incubated at 37°C for 20 min. The samples were stored at −20°C until further used.

RT-PCR and Sequencing of TonEBP/NFAT5

Using the cDNA (produced above), RT-PCR was performed (MJ Research PTC-200 Thermal Gradient Cycler; Bio-Rad, Hercules, CA). Tissue-specific cDNA was used with NFAT5 forward (5′-TCT TGG GTT ACA GGG CTC TTC AGT-3′) and reverse (5′-GGT TTC TGG TTG GTG AGG CAT-3′) primers at an annealing temperature of 60.3°C during the RT-PCR procedure. The TonEBP/NFAT5 PCR product was purified using a QiAquick PCR Purification kit and sequenced for authenticity.

Real-Time PCR

Quantitative real-time PCR was used to quantify mRNA concentrations in our samples. Ten nanograms of each sample cDNA were subjected to real-time PCR using human NFAT5 forward/reverse primers (same as above) and compared to a standard curve generated by known quantities of NFAT5 cDNA to determine starting quantity. To normalize our NFAT5 data, sample cDNA were subjected to real-time PCR using primers (forward 5′-TCA ACC AGG TGG AGA TCA ACG 3′ and reverse 5′-GTT TCC AGC GGC TTT GTG ATG 3′) for ribosomal protein S15 and a standard curve of known quantities of S15 cDNA. The amplification efficiencies were 95–105% for NFAT5 and S15. The threshold setting was kept at 0.02 during the analysis of all RT-PCR data to allow for comparisons across gestation.

Western Blot Analysis

Western blotting was performed as previously shown by Arroyo et al. [36]. Briefly, Cotyledons tissues were homogenized in lysis buffer. Protein tissue lysates (50 μg) were separated on 4–12% Bis-Tris gel SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with a antibody against mouse NFAT5 (catalog no. H00010725M01 at a dilution of 1:500; Affinity Bioreagents, Golden, CO), mouse SLC5A3 (SMT; catalog no. NC9739718 at a dilution of 1:200; Fisher Scientific, St. Louis, MO), or antibodies against rabbit AR (catalog no. SC-2313 at a dilution of 1:500; Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were then incubated with an secondary horseradish peroxidase (HRP)-conjugated antibody for 1 h at room temperature. The membranes were then incubated with ECL substrate, and the emission of light was detected using x-ray film. To determine loading consistencies, each membrane was stripped of antibodies and reprobed with an antibody against mouse beta-actin. Presence of these proteins was confirmed and quantified.

Nuclear Extraction and Western Blot

Nuclear proteins were extracted from thawed placental tissue using the NE-PER nuclear protein extraction kit (Pierce, Rockford, IL). Briefly, 100 mg of placental tissues were weighed and placed in a microcentrifuge tube. Five hundred microliters of cytoplasmic extraction reagent I (Cotyledon, Sunnyvale, CA). The analysis was run isocratically with 500 mM sodium hydroxide for 25 min, followed by a step change to 400 mM sodium hydroxide for 20 min at ambient temperature. The function of the extraction was tested by running Western blots of both the cytoplasmic and the nuclear extracts with an anti-lamin (nuclear protein) antibody. Western blotting was then performed as shown earlier in the Western blot analysis section.
TABLE 1. Fetal and placental weights (g) in control and HT (IUGR) animals.

<table>
<thead>
<tr>
<th>dGA</th>
<th>Control</th>
<th>IUGR</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>Fetal</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>55</td>
<td>32 ± 2.6</td>
<td>29 ± 2.3</td>
<td>0.22</td>
</tr>
<tr>
<td>95</td>
<td>715 ± 11</td>
<td>682 ± 205</td>
<td>0.79</td>
</tr>
<tr>
<td>130</td>
<td>2914 ± 201</td>
<td>1718 ± 433</td>
<td>&lt;0.008</td>
</tr>
<tr>
<td>Placental</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>181 ± 20</td>
<td>135 ± 3</td>
<td>≤0.053</td>
</tr>
<tr>
<td>95</td>
<td>440 ± 50</td>
<td>186 ± 18</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>130</td>
<td>349 ± 21</td>
<td>169 ± 43</td>
<td>≤0.004</td>
</tr>
</tbody>
</table>

**DECR1 Determination**

DECR1 concentrations were determined using the Fluorescent NADP/DECR1 (NADPH) detection kit by Cell Technology (Mountain View, CA). Protocol was followed as shown by the manufacturer. Briefly, 100 mg of cotyledon tissues were homogenized in DE-CR1 extraction buffer on ice. Homogenates were heated up at 60°C followed by the addition of reaction buffer. Samples were vortexed and stored at −80°C until needed. Samples and DE-CR1 calibration curve standards were incubated for 10 min in reaction buffer, followed by the addition of detection buffer and incubation for 60 min at room temperature. Plates were read in a fluorescence plate reader using an excitation of 530 nm and an emission of 590 nm.

**Immunofluorescence**

Immunofluorescence (IF) was performed for localization of NFAT5 in the sheep placenta. Serial sections were incubated overnight with a mouse antibody against NFAT5 (catalog no. H00010725M01 at a dilution of 1:250; Affinity Bioreagents), a mouse Pan cytokeratin antibody (catalog no. c-1801 at a dilution of 1:100; Sigma), or an immunoglobulin G1-negative control (Jackson Laboratories, West Grove, PA). Anti-mouse fluorescein or Texas red-conjugated secondary antibody was incubated for 1 h; 40,6-diamidino-2-phenylindole dihydrochloride was used for nuclear counterstaining. Slides were viewed on an Olympus BX51 microscope system and digitally captured with a Pixera 600CL camera fluorescence microscope with the appropriate excitation and emission filter (fluorescein or rhodamine filters).

**Statistical Analysis**

A comparison of the following endpoints was made between and within each control and IUGR pregnancies: fetal and placental weights, inositol and sorbitol concentration, TonEBP/NFAT5 mRNA, nuclear and cytosolic protein concentrations, DE-CR1 (also known as NADPH) levels, and AR and SLC5A3 proteins. All data were assessed for normality, and differences were determined using Mann-Whitney test, with P < 0.05 considered significant. A Kruskal-Wallis test was used to compare differences within groups using Dunn post hoc test with P < 0.05 considered significant.

**RESULTS**

Table 1 shows the fetal and placental weights for control and IUGR groups at each gestational age study time point. Compared to controls, the IUGR group showed a 1.8-fold reduction in fetal weight at 130 dGA (P < 0.008); however, no differences were seen at 55 and 95 dGA. Significantly reduced placental weights were noted in the IUGR group at 95 dGA (P < 0.003) and 130 dGA (P < 0.004), and a trend was seen for a decrease at 55 dGA (P < 0.053).

**Placental Inositol and Sorbitol Concentration**

We first investigated inositol and sorbitol levels in placental tissues across gestation in the control and IUGR animals. Kruskal-Wallis analysis showed a significant decrease in inositol (P < 0.002) and a significant increased in sorbitol concentrations in the cotyledons (P < 0.01) across gestation within each control and IUGR groups (Fig. 1). There were no differences in osmolyte concentrations between control and IUGR groups at any given time across gestation.

**NFAT5 mRNA**

Given that osmolar changes induce accumulation of inositol and sorbitol and that NFAT5 is associated with cell osmotic changes, we first investigated NFAT5 mRNA expression in the placenta of control and IUGR animals. Compared to controls, placental NFAT5 mRNA in the IUGR group was not altered between 95 and 130 dGA. However, at 55 dGA, there was a significant increase (3.4-fold; P < 0.002) in the IUGR group of NFAT5 mRNA compared to controls (Fig. 2). There was a significant increase in control NFAT5 mRNA (P < 0.003) between 55 and 130 dGA in the control tissues (Fig. 2). In contrast, NFAT5 mRNA was decreased in the IUGR group between the 55 and 95 dGA time points (P < 0.002; Fig. 2) but increased between 95 and 130 dGA time points (P < 0.02; Fig. 2).

**TonEBP/NFAT5 Protein**

We next investigated the cytosolic and nuclear expression of NFAT5 in the cotyledon of these animals. A characteristic Western blot for these studies is depicted in Figure 3A. There were no significant differences in the Western blot analysis of cytosolic or nuclear TonEBP/NFAT5 protein of controls vs. IUGR groups (Fig. 3B). Mann-Whitney test showed a significant decrease in cotyledony NAFT5 cytosolic protein
between 55 and 95 dGA time points for both control (\(P < 0.008\)) and IUGR (\(P < 0.03\)) groups (Fig. 3B). In contrast, there was a significant increase between 55 and 130 dGA (\(P < 0.03\)) and 95 and 130 dGA (\(P < 0.02\)) only in the IUGR group in the nuclear expression of the NFAT5 protein (Fig. 3B).

**TonEBP/NFAT5 Localization**

In order to determine that NFAT5 protein is localized to the trophoblast cells, IF was performed. An antibody to pan-cytokeratin was used to identify the trophoblast cells within the cotyledon of the placenta (Fig. 4; red fluorescence). Using a serial section of placental tissue, TonEBP/NFAT5 protein IF (green fluorescence) localized to the trophoblast cells in the cotyledon of the sheep (Fig. 4).

**DECR1, AR, and SLC5A3 Expression**

We further investigated the proteins associated with the production of inositol and sorbitol osmolytes that induced by the action of NFAT5. The expression of DE-CR1 (used in the production of osmolytes) was first determined in the cotyledons of the control and IUGR groups. There were no differences in DE-CR1 concentrations between the control and IUGR groups at any gestational time point studied (Fig. 5). In contrast, Kruskal-Wallis test group analyses showed a significant increase in DE-CR1 levels across gestation in the control samples (\(P < 0.03\); Fig. 5). Similarly, a trend for a significant increased in DE-CR1 levels was observed in the IUGR cotyledons (\(P < 0.08\); Fig. 5).

We next determined the expression of placental AR and SLC5A3 across gestation. A characteristic Western blot for these studies is depicted in Figure 6A. The cotyledonal AR enzyme (involved in sorbitol production) was significantly increased only in the near-term IUGR group (1.5-fold, \(P < 0.02\)) when compared to controls (Fig. 6B). Analysis within groups showed a significant increase in AR expression between 55 and 95 dGA in control samples (\(P < 0.03\)), while a significant increase was seen between 55 and 130 dGA (\(P < 0.03\)) in the IUGR samples (Fig. 6B).

SLC5A3, the inositol transporter, was then assessed in cotyledon tissues of control and IUGR animals. SLC5A3 levels in cotyledon tissues were significantly decreased only in the IUGR group at 95 dGA (2.5-fold, \(P < 0.02\)) as compared to controls (Fig. 6C). Within groups, Mann-Whitney testing

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**FIG. 2.** Expression of cotyledonal NFAT5 mRNA across gestation during IUGR. NFAT5 mRNA was increased between IUGR (HT) and control groups at 55 dGA (\(P < 0.002\)). A significant increase in NFAT5 mRNA (\(P < 0.003\)) was observed between 55 and 130 dGA within the control placentas. There was a decreased in NFAT5 mRNA in the IUGR group between the 55- and 95-dGA time points (\(P < 0.002\)). Data presented as the mean ± SEM.

**FIG. 3.** Cytosolic and nuclear TonEBP/NFAT5 protein content in cotyledons from control and IUGR (HT) groups. A) A characteristic Western blot of cytosolic and nuclear NFAT5 lysates at 130 dGA. B) Cytosolic TonEBP/NFAT5 protein was decreased between 55 and 95 dGA (\(P < 0.03\)) in both control and IUGR groups. Nuclear TonEBP/NFAT5 protein was increased between 55 and 130 dGA and 95 and 130 dGA (\(P < 0.03\)) in the IUGR group. Data presented as the mean ± SEM.
and 130 dGA in both the control (showed a significant decrease in SLC5A3 protein between 55 with NFAT5 and pan-cytokeratin antibodies (FIG. 4. NFAT5 immunofluorescence of cotyledon tissue. Serial sections cotyledons (Image 46x509 to 297x738) osmolytes between groups, the finding of a progressive unexpected given that IUGR is a pathologic condition like IUGR. Although there were no differences in placental growth is reduced and placental apoptosis increased. This suggests that osmolarity is similar even in a pathologic condition like IUGR. Although there were no differences in osmolytes between groups, the finding of a progressive decrease in inositol and a progressive increase in sorbitol in both groups is interesting and suggests different roles for these osmolytes at different times of gestation. Preliminary data from tissue osmolarity studies in our laboratory (data not shown) suggest that there is a progressive increase in placental osmolarity with advancing gestation in normal ovine pregnancy, and sorbitol may be the osmolyte responsible. The higher level of sorbitol may be associated with the release of sorbitol to both maternal and fetal circulations observed in these animals near term [31]. Interestingly, DE-CR1 levels showed a similar pattern as the one observed for sorbitol, suggesting that perhaps DE-CR1 is used for the production of sorbitol and less so for inositol.

The exact role for these osmolytes and regulatory proteins (e.g., NFAT 5) remains uncertain, but there is some evidence to highlight the importance in embryonic and fetal life. Although the fetus is dependent on placental, not kidney, function for solute and waste filtration, Nfat5 knockout mice exhibit dramatically reduced embryonic viability [6]. The polyol pathway, once considered vestigial, is now understood to provide an alternative pathway to the production of lactate during ATP formation in low-oxygen environments, such as the developing embryo, thus avoiding excessive metabolic acidosis [28, 38]. During early development, polyols are thought to draw water and solutes across the human placenta and help to expand the gestational sac [1, 38]. Hod et al. [39, 40] have shown that changes in glycogen and polyol concentrations in cultured rat embryos were associated with increased congenital anomalies. Groenen et al. [41] have recently shown an association between low maternal myoinositol concentrations and increased incidence of spina bifida in humans. Multiple studies in TonEBP/NFAT5 or polyol regulation point toward an important role for this molecule in the development of T-cell immune response during embryogenesis, integrin-induced cellular migration, and cell proliferation [29, 31, 38, 42, 43]. These data suggest an important role for osmolytes and TonEBP/NFAT5 during placental and fetal development.

The TonEBP/NFAT5 transcription factor regulates the accumulation of some organic osmolytes, such as inositol and sorbitol [12–15, 44–46]. Accumulation of osmolytes is also regulated by several molecules, such as AR, SLC5A3, and others [15, 47]. NFAT5 is a transcription factor known to stimulate genes responsible for the production of these enzymes and transporters [17]. Activation of this transcription factor involves three processes: nuclear translocation, upregulation of transcriptional activity, and increased NFAT5 synthesis at the RNA and protein levels [14, 48]. TonEBP/
NFAT5 is the only known gene regulator of the mammalian adaptive response to osmotic stress. Relatively little is known about the role of TonEBP/NFAT5 and osmotic stress regulation in mammals in other tissues besides the kidney. Its role in the placenta has been limited to its identification in sheep and human placentas [17].

Mechanistically, several signaling molecules, such as extracellular signal-regulated kinase, FYN, cAMP-dependent protein kinase, and p38 mitogen-activated kinase signaling, are required for in vivo hypertonic activation of TonEBP/NFAT5 in various cell types [10, 44, 49]. A recent study demonstrates that phosphorylation by casein kinase 1 (CK1) mediates nucleocytoplasmic trafficking of NFAT5 in response to both hypertonicity (nuclear translocation) and hypotonicity (nuclear exclusion) in HeLa cells [50].

We observed a significant decrease in NFAT5 mRNA at early gestation during HT (55 dGA). This was not sustained at the other gestational time points studied. TonEBP/NFAT5 results at 55dGA supports posttranscriptional regulation, as the increase in mRNA seen at 55dGA was not matched with an increase in protein. Early gestation is normally characterized by rapid growth by cell division as opposed to growth by cell hypertrophy, which is found in later stages. Exposure to hyperthermia diminishes this growth phase. The increase in NFAT5 mRNA in the IUGR group, noted only in early gestation, suggests a possible TonEBP/NFAT5-mediated compensatory mechanism to placental (and possibly fetal) growth restriction caused by exposure to HT. The early gestation mRNA results also suggest a higher level of osmotic stress in the early HT-exposed placenta, leading to increased NFAT5 mRNA stabilization.
When cytosolic NFAT5 protein levels were determined, we did not see any differences at any gestational point between control and treated cotyledons. This suggests that in the placenta, TonEBP/NFAT5 may be posttranscriptionally regulated. We did observe that this protein was significantly decreased between 55 and 95 days gestation in both controls and HT placentas. Although we did not see an association between cytosolic TonEBP/NFAT5 and the osmolyte production, we did observe a similar pattern between the nuclear TonEBP/NFAT5 protein expression and placental sorbitol. This suggests that nuclear expression of this protein may be a better indicator of osmolarity changes within the placenta, as it mimics the osmolyte levels found within these tissues. While nuclear protein levels were affected, we did not assess the phosphorylation form of this protein in this study. Thus, true TonEBP/NFAT5 activity may not be reflected in our protein expression results despite targeting activation via nuclear translocation by measuring TonEBP/NFAT5 levels after nuclear extraction. Interestingly, AR protein was found to be increased at 130 days gestation in the placenta of our IUGR model. In this case, we found a significant decrease in this transporter as we compared control and treated animals. Further studies are needed to determine the significance of this finding, but it may suggest the importance of inositol at this gestational stage, where the placenta is already compromised in this model of IUGR. In control animals, a trend for a decrease in SLC5A3 expression was found as gestation progressed. This SLC5A3 pattern of expression matches the progressively decreased inositol levels in the placenta, suggesting that direct transport by SLC5A3 is one of the factors directly involved in inositol regulation across gestation in the sheep placenta. We observed an opposite trend for expression of this transporter and the TonEBP/NFAT5 protein levels for both controls and HT samples, suggesting that perhaps the nuclear levels of TonEBP/NFAT5 are more directly correlated with the production of placental sorbitol than inositol during gestation in this model of IUGR.

We speculate that a mechanism distinct from TonEBP/NFAT5 could be involved in the regulation of polyol expression in the ovine IUGR placenta and that other factors, such as activity of a yet unidentified gene regulator or de novo placental production of osmolytes, may lead to the high levels of inositol found in the fetal circulation and placenta during early gestation [17, 30]. A recent study in gene expression profiling of rat brain cells exposed to hypertonicity proposed new transcription factors that may mediate osmoadaptation [52]. In this study, the authors noted rapid induction of activating transcription factor 3 (ATF3), APOLD1 (also known as Verge [vascular early response gene]), and Krüppel-like factor 4 (KLF4) transcription factors and delayed increased expression of the SLC38A2 (also known as SNAT2 [sodium-dependent neutral amino acid transporter 2]) amino acid transporter. Whereas NFAT5 mRNA labeling in neurons remained unchanged, TonEBP/NFAT5 protein labeling rapidly increased, suggesting that the rapid tonicity-induced activation of ATF3, APOLD1, and KLF4 may regulate genes involved in osmoadaptation.

In this model of IUGR, nuclear TonEBP/NFAT5 seems to be involved in the sorbitol regulation. The present study suggests a role for NFAT5 in maintaining osmotic homeostasis in the placenta during normal and IUGR pregnancies. Further studies are needed to understand the role of this transcription factor in the production of inositol early in gestation during pregnancy in this model of IUGR. We chose to study osmolytes, the tonicity-responsive enhancer binding protein (NFAT5), and related proteins for two reasons. The first is that the placenta is actively proliferating and metabolically active, and such cells are associated with high baseline osmolarity compared to cells that are not as active [29]. The second is that IUGR is a common condition affecting pregnancy that impacts placental growth with enhanced apoptosis and likely associated with osmolarity changes compared to normal pregnancies. While ovine and human placental structure and placentation are different vastly, prior studies in our laboratory have shown that both the human and the sheep placenta express NFAT5 mRNA and protein [17]. Although not previously investigated, it would be interesting to assess the osmolarity factors in other conditions in pregnancy, such as diabetes with large glucose shifts and pre-eclampsia with a hemoconcentrated maternal state, where large swings in osmolarity may occur.

ACKNOWLEDGMENT

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