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Altered mitochondrial apoptotic pathway in placentas from undernourished rat gestations

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Mitochondrial responses to apoptotic stimuli depend on the balance between anti- and pro-apoptotic members of the Bcl2 family of proteins (9). This family is characterized by four distinct Bcl2 homology domains (BH 1–4) (46), which are prime regulators of the mitochondrial apoptotic cascade. The apoptosis inhibitors (e.g., Bcl2 and Bcl-X₁) share four BH domains, whereas anti-apoptotic Mcl-1 contains only three domains (BH 1–3). Pro-apoptotic family members (e.g., Bak and Bad) contain BH 1–3 domains and promote cell death by heterodimerizing with anti-apoptotic proteins through the BH 3 domain. Members of the Bcl2 family of proteins are localized to the cytosol and the mitochondria. Following an apoptotic stimulus, pro-apoptotic Bak in the mitochondria and Bad, which translocates from the cytosol to mitochondria, induce apoptosis (34, 45, 50). This results in the release of cytochrome c to the cytosol, activation of initiator caspases such as caspase-9, with downstream cleavage of effector caspase-3 to yield cell death (10, 17). Conversely, a dominance of anti-apoptotic Bcl2 family of proteins prevents translocation of cytochrome c from the mitochondria and thus cell death (18, 19).

Peroxisome proliferator-activator receptor gamma (PPARγ) is a ligand-activated transcription factor that belongs to the nuclear hormone receptor family (35). In a basal state, PPARγ is bound to co-repressor proteins including nuclear receptor co-repressor and silencing mediator of retinoic acid and thyroid hormone receptor (20). Conversely, ligand binding induces PPARγ heterodimerization with retinoid X receptor-α, and its subsequent interaction with coactivators such as steroid receptor coactivators followed by binding to PPARγ response elements (PPREs) within target gene promoters (15) and activation of these genes (41). Notably, PPARγ can suppress apoptosis in many cell types (47, 48). PPARγ is also critical to placental development (3), but how this receptor influences placental apoptosis is unknown. Although the presence of a putative PPRE in the 3’ untranslated region of the anti-apoptotic Bcl-2 protein (8) may suggest a mechanism by maternal undernutrition; placenta; PPARγ; IUGR

ADEQUATE PLACENTATION PLAYS a central role in the establishment of optimal fetal growth and development (5, 33). The syncytiotrophoblast of the human placenta controls hormone production and maternal-to-fetal exchanges of nutrients. In rat placentas, the basal zone functions in hormone secretion and the labyrinth zone interfaces maternal-fetal exchange (31). These tissues undergo physiological cell death, called apoptosis, as part of normal placental remodeling and aging (11, 40) in both humans and rats. Apoptosis in combination with cell proliferation regulates cell turnover and facilitates removal of unwanted cells (2) without inflammation. Importantly, the pathway of cell death is excessively stimulated in placentas of pregnancies complicated by intrauterine growth restriction (IUGR) (7, 39). Apoptosis is mediated by caspases-dependent (Fas receptor, mitochondria, or reticulum endoplasmic) or caspases-independent (TNF-α) pathways following an apoptotic stimulus. The c-Jun NH2-terminal kinase (JNK) pathway is also associated with cell death via mitochondrial-dependent as well as mitochondria-independent pathways.

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which PPARγ may preserve mitochondrial integrity and prevents apoptosis by upregulating anti-apoptotic proteins.

Using a rat model of IUGR, we have shown that maternal undernutrition (MUN) during the second half of gestation results in reduced fetal and placental weights at term (7). Moreover, the IUGR placentas from the MUN pregnancies have increased apoptosis in part through activation of the Fas death receptor apoptotic pathway (7). Although the mitochondrial pathway is altered in IUGR pregnancies (23, 27), it is unknown whether this pathway is important in placentas from MUN gestations. Therefore, we test the hypothesis that the mitochondrial pathway to apoptosis contributes to the placental dysfunction of the pregnancies with MUN, using the MUN rat model of IUGR.

MATERIALS AND METHODS

Animals

Studies were approved by the Animal Research Committee of the Los Angeles BioMedical Research Institute at Harbor-UCLA (LABioMed), and were in accordance with the American Association for Accreditation of Laboratory Care (AALC), and National Institutes of Health Guidelines. Eight-week-old first-time-pregnant Sprague-Dawley rats (230–240 g body wt; Charles River Laboratories, Hollister, CA) were housed in a facility with constant temperature and humidity, a controlled 12:12-h light-dark cycle, and an ad libitum diet (AdLib) of standard laboratory chow (protein 23%, fat 4.5%, metabolizable energy 3030 kcal/kg; cat. no. 5001; Lab Diet, Brentwood, MO). At embryonic day 10 (E10) based upon day of expelled plug, dams were randomly allocated to a control diet (n = 6) in which they were continued on the AdLib diet or a 50% food-restricted (MUN) diet (n = 6) that was determined by quantifying normal intake of the rats that were fed AdLib at the equivalent stage of gestation. Respective diets were continued throughout the remainder of gestation. Notably, our rat chow contains the most essential nutrients and vitamins, and 50% global dietary restriction results in no abortion, no reabsorption, and a normal litter size. Furthermore, it parallels that of studies producing IUGR newborns following maternal protein restriction (30), uterine artery ligation (37), or other models. Although the nature and severity of the insults applied may vary greatly between the different models, the general finding is that either balanced undernutrition or restriction of specific nutrients promotes metabolic and physiological disturbance and also relative adiposity in adult life.

The 50% MUN diet was specifically started at E10 to avoid compounding effects of maternal nutrition on embryo implantation. Since most of placental development occurs during the second half of pregnancy (E10–E20), nutrient deficiency at this time in pregnancy will likely affect fetal growth by changing the complex interaction between the fetus and placenta. The long period of MUN (10 days) results in an irreversible slow rate of fetal growth (32). Overall our model is well placed for investigating the effect of maternal nutrient restriction on placental growth and development that are indicative of a healthy pregnancy.

Tissue Collection

AdLib (n = 6) and MUN (n = 6) dams were killed at E20 using an overdose of 4% isoflurane. The uterus was delivered through a midline incision, and the gestational sacs were removed. All placentas were trimmed of membranes, and weights were recorded. Placentas from left proximal- and mid-horns were immediately fixed in 4% paraformaldehyde for 24 h prior to storage in 70% ethanol until histological processing. Placentas from right proximal- and mid-horn gestations were processed for protein extraction by gently removing the decidua from the basal zone and separating the placenta into basal and labyrinth components. The two zones were then individually weighed and flash-frozen in liquid nitrogen for protein extraction.

Terminal Deoxynucleotidyl Transferase dUTP-Mediated Nick-End Labeling Assay and Activated Caspase-3 Immunodetection

We used an ApopTag in situ apoptosis detection kit (Millipore, San Francisco, CA) as described (7). The terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay labels DNA strand breaks with digoxigenin nucleotides that are allowed to bind to an antidigoxigenin antibody conjugated with peroxidase. Placental cells were counterstained with methyl green (Sigma, St. Louis, MO). In the negative control, terminal deoxynucleotidyl transferase was replaced with PBS. The relative incidence of apoptotic cells in basal and labyrinth zones from AdLib and MUN placentas were analyzed and recorded using the Ariol automated scanning microscope and imaging analysis system (Applied Imaging, San Jose, CA) described below. The relative incidence of apoptotic cells was calculated as (%) = number of TUNEL stained nuclei in a zone/number of methyl green stained nuclei in the same zone ∗ 100. For each diet, six dams were used, and placental from proximal and mi-horn specimens were analyzed. The results are presented as an average of six proximal- or mid-horn placentas from six different dams for each diet.

To complement TUNEL assay results, we immunostained for cleaved caspase-3 as another marker for apoptosis. Five-micron sections of placental tissues were deparaffinized, rehydrated, subjected to antigen retrieval, and endogenous peroxidase activity was quenched in 3% hydrogen peroxide in PBS for 30 min. Nonspecific binding was blocked by incubating the sections in 5% normal goat serum. The sections were reacted with a nonimmune serum or with a rabbit polyclonal anticleaved caspase-3 (See Table 1 for dilution) overnight at 4°C, followed by three washes of 10 min in PBS and the addition of biotinylated anti-rabbit secondary antibody (Table 1) for 1 h. Further processing of the sections to detect activated caspase-3 was performed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Colorimetric detection was achieved using diaminobenzidine as chromogen and hydrogen peroxide as substrate for horseradish peroxidase (Vector Laboratories). The sections were counterstained with methyl green. Activated caspase-3 positive immunostaining in the entire basal or labyrinth zone from AdLib and MUN were scanned and analyzed using the Ariol system (Applied Imaging). The results are expressed as the percentage of activated caspase-3 positive immunostaining was calculated as activated caspase-3 positive stain in a zone (μm²)/total surface of that zone (μm²) ∗ 100. For each of the 12 dams, two sections from right proximal- and mid-horn placentas were used to calculate the percentage of immunostaining in each zone as described below.

TUNEL and Activated Caspase-3 Immunoreactivity Scoring

As mentioned previously, both TUNEL-positive nuclei and the degree of immunostaining for activated caspase-3 have been determined using Ariol software. First, all slides were blinded by a third party and scanned at ×20 objective magnification with three filters: red, green and blue (Ariol converts these three-channel images into color reconstructions). Secondly, the entire basal or labyrinth zone was drawn manually by using a marker crayon tool, and selected areas were highlighted by an editable colored outline, and then analyzed.
automatically with the Ariol software. Finally, the results were recorded as the number of positive and negative nuclei for TUNEL or as the number of positive and negative stain for activated caspase-3. Placental cells were classified as positive or negative based on predetermined thresholds that evaluate color and intensity of staining, as well as cell size, axis length, roundness, and compactness. Notably, methyl green staining of nuclei were clearly distinguished from brown diaminobenzidine reaction product. Ariol was trained to distinguish accurately positively stained cells from unstained cells by testing the set thresholds with a minimum of six tissue samples for each assay. Thresholds were adjusted iteratively after each test sample until the algorithm could correctly determine positive vs. negative staining.

Subcellular Fractionation

Cytosolic, mitochondrial, and nuclear fractions were isolated using the method of Hikim et al. (22). Tissues from frozen MUN and AdLib right proximal- and mid-horn placentas separated into basal and labyrinth zones were sonicated on ice in buffer A (0.25 M sucrose, 50 mM HEPES, 10 mM NaCl, 10 mM EDTA, and 2 mM dithiothreitol) supplemented with protease inhibitors (complete protease inhibitors; Roche, Indianapolis, IN). The crude homogenates were centrifuged at 1,000 g for 10 min at 4°C to isolate the nuclear fraction, whereas the resultant supernatant was centrifuged at 10,000 g for 15 min at 4°C to sediment the low speed fraction containing the mitochondria. These mitochondria were washed twice in buffer A and pelleted. The cytosolic and high-speed fractions were isolated after centrifugation of the supernatant fraction at 100,000 g for 60 min at 4°C. The resulting supernatant was the cytosolic fraction. The purity of the cytosolic, mitochondrial, nuclear fractions was assessed by Western blot analysis using antibodies to cytochrome c oxidase subunit IV (COXIV; Cell Signaling Technology, Danvers, MA) and LSD1 (Cell Signaling Technology). Protein concentration of the different fractions was determined by bicinchoninic acid solution (Thermo Scientific). All protein fractions were frozen at −80°C until use.

SDS-PAGE and Western Blot Analysis

The effect of MUN on placental mitochondrial pathway protein expression levels was evaluated by Western blot analysis of the tissues’ lysates. Depending on the protein’s localization, cytosolic, mitochondrial, or nuclear fraction was used. For each sample, 50 μg of protein were separated on a 12% polyacrylamide gel (Invitrogen, Carlsbad, CA). The separated proteins were transferred electrophoretically to Immobilon-P membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were incubated in a blocking solution (5% powdered nonfat milk in Tris buffer saline [TBS] and 0.01% Tween-20 [TBS-T]) at room temperature for 1 h, and then in the same solution containing a primary antibody that reacts with one of the Bcl2 family members, cytochrome c, inducer caspase-9, or effector caspase-3 (see Table 1 for dilutions), at 4°C overnight. After washes in TBS-T, membranes were incubated with the corresponding horseradish peroxidase secondary antibody (Table 1) at room temperature for 1 h and visualized by the enhanced chemiluminescence signaling system (Den ville Scientific, Metuchen, NJ). Levels of protein expression normalized against their respective expression of β-actin for cytosolic fraction, anticytocrome oxidase subunit IV (COXIV) for mitochondrial fraction, or LSD1 for nuclear fraction were quantified densitometrically using Quantity One Software (version 4.6.7) (Bio-Rad).

Table 1. Antibodies used in immunohistochemistry, immunofluorescence, and Western blot analysis

<table>
<thead>
<tr>
<th>Primary and Secondary Antibodies</th>
<th>Commercial Source</th>
<th>Species</th>
<th>Clone</th>
<th>Working Concentration</th>
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<tr>
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<td>Rabbit</td>
<td>Poly</td>
<td>1:200&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Cell Signaling Technology</td>
<td>Rabbit</td>
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<tr>
<td>Biotinylated anti-rabbit IgG secondary antibody&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Vector Laboratories</td>
<td>Goat</td>
<td>Poly</td>
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<tr>
<td>Biotinylated anti-mouse IgG secondary antibody&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Invitrogen</td>
<td>Goat</td>
<td>Mono</td>
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<tr>
<td>Alexa Fluor 647&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Poly</td>
<td>1:300&lt;sup&gt;b&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup>PPAR<sub>α</sub>, peroxisome proliferator-activator receptor-γ; <sup>b</sup>COXIV, cytochrome c oxidase subunit IV; <sup>c</sup>Immunohistochemistry; <sup>d</sup>Immunofluorescence; <sup>e</sup>Western blot analysis.
Immunohistochemical Localization of Anti- and Pro-Apoptotic Bcl2 and Nuclear PPARγ Proteins

Following deparaffinization and rehydration, 5-μm proximal- and mid-horn placenta sections were reacted with a specific antibody (see Table 1 for dilutions) overnight at 4°C, followed by three washes of 10 min in TBS-T and the addition of biotinylated anti-rabbit or anti-mouse secondary antibody (Vector Laboratories) for 1 h. After colorimetric detection, as described earlier, the sections were counterstained with methyl green (Sigma), dehy-

Fig. 1. Placental basal (A) and labyrinth (B) weights from mid- and proximal-horn positions from maternal undernutrition (MUN) and ad libitum-fed (AdLib) pregnant rats. C: % TUNEL-positive nuclei in basal and labyrinth zones. Representative activated caspase-3 positive immunostaining in basal zone (C and D) and labyrinth (F and G) zones from MUN and AdLib proximal-horn placentas. Negative controls (E and H) with no immunostaining. I: quantitative analysis of activated caspase-3 in basal and labyrinth zones from both mid- and proximal-horn placentas. *Significant difference between zone weights, % TUNEL-positive nuclei, or activated caspase-3 protein immunostaining from MUN (black bars) vs. AdLib (white bars). aSignificant difference between proximal- and mid-horn placental positions. Red arrows (C–D, F–G) indicate brown positive cell stains. Scale bars = 50 μm.
drated, and then mounted with Permount (Sigma). For each of the six, two-sections from left proximal-horn placentas were used. To control for specificity of the immunogen reactions, adjacent control sections were subjected to the same immunoperoxidase method, except that primary antibodies were replaced with a matching concentration nonimmune serum at the same dilutions as the specific primary antibody. The percentage of positive immunostaining was calculated as positive stain in a zone (μm²)/total surface of that zone (μm²) × 100. Positive immunostaining in the entire basal or labyrinth zone from AdLib and MUN were scanned and analyzed using the Ariol system (Applied Imaging). For each of the 12 dams, two sections from left proximal-horn placentas were used for immunostaining scoring.

**Double Immunofluorescence Staining of Mitochondrial Bcl2 Family of Proteins**

Five-micron transverse deparaffinized and rehydrated placental sections were subjected to antigen retrieval using citrate buffer and then blocked for 30 min with 5% BSA in TBS-T. The sections were double stained with a mixture of anti-MnSOD antibody and one of the anti- or pro-apoptotic antibodies at dilutions indicated in Table 1 overnight at 4°C after which the sections were washed three times with TBS-T and incubated with a mixture of Alexa Fluor 546 anti-rabbit IgG (Invitrogen, Carlsbad, CA) and Alexa Fluor 647 goat anti-mouse IgG (Invitrogen) for 1 h. After several washes with TBS-T, the slides were mounted with Vectashield mounting medium (Vector Laboratories). Sections of each tissue were incubated with a

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**Fig. 2. Effects of MUN on the level of anti-apoptotic Bcl2 and Bcl-XL protein expression from mid- and proximal-horn placentas as determined by Western blot analysis, and densitometric analysis. Representative immunoblots of Bcl2 (A and C) and Bcl-XL (E and G) protein expression in basal and labyrinth zones. Densitometric analysis of cytosolic and mitochondrial expression of Bcl2 (B and D), and Bcl-XL (F and H) proteins in basal and labyrinth zones. *Significant difference between zones’ anti-apoptotic protein expression levels from MUN (black bars) vs. AdLib (white bars).**
Table 2. Expression of BclII family of protein in cytosolic and mitochondrial fractions from maternal undernutrition proximal-horn and mid-horn placentas at embryonic day 20 using Western blot analysis

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Cytosol</th>
<th>Mitochondria</th>
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<tbody>
<tr>
<td></td>
<td>Proximal-Horn</td>
<td>Mid-Horn</td>
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<tr>
<td>BclII</td>
<td>&lt;sup&gt;↑&lt;/sup&gt;</td>
<td>&lt;sup&gt;↓&lt;/sup&gt;</td>
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<tr>
<td>Bax</td>
<td>&lt;sup&gt;↑&lt;/sup&gt;</td>
<td>&lt;sup&gt;↑&lt;/sup&gt;</td>
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<tr>
<td>Bak</td>
<td>&lt;sup&gt;↑&lt;/sup&gt;</td>
<td>&lt;sup&gt;↑&lt;/sup&gt;</td>
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<tr>
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<td>&lt;sup&gt;↑&lt;/sup&gt;</td>
<td>&lt;sup&gt;↑&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Blank box indicates no protein determination. Arrows: <sup>↑</sup> decrease in protein expression, <sup>↓</sup> increase in protein expression, and <sup>→</sup> unchanged protein expression. Two arrows in the same box indicate that basal and labyrinth zones were differentially modulated.

matching concentration of nonimmune serum at the same dilution as the first antibody.

Quantified Confocal Immunofluorescence

All slides were blinded by a third party, and digital images of the Bcl2 family members (red) and mitochondrial MnSOD (green) staining were captured at four randomly selected fields using a Leica TCS SP2 confocal laser-scanning microscope (Leica Microsystems, San Francisco, CA) at ×40 objective magnification utilizing the 578-nm (anti- and pro-apoptotic proteins), and 650-nm (MnSOD protein) laser excitation at 75% of maximal power, and emission filters at 603-nm and 668-nm, respectively. The spectral overlap region between fluorophore emission spectra was excluded from acquired images. Colocalization between each of the Bcl2 family proteins of cells and mitochondrial MnSOD signals in either basal or labyrinth zones from proximal-horn placentas was measured, after background correction, using Colocalizer Express software (Tokyo, Japan). The contribution of the red channel (M<sub>red</sub>) in the colocalized areas of the image to the overall amount of colocalized fluorescence was defined as: M<sub>red</sub> = ΣR<sub>colored</sub>/ΣR<sub>total</sub> (R represents red pixels) (49). M<sub>red</sub> is the sum of the intensities of red pixels that have a green component by the total sum of red intensities.

Statistical Analysis

Placental weights and protein expression using Western blot analysis and immunostaining were analyzed using NCSS97 software. Placental weights, level of apoptosis, and Bcl2 family of anti- and pro-apoptotic protein expression by Western blot analysis were compared with a three-way ANNOVA (with horn positions, zones, and diet, as causes of variation). When interaction occurs between these factors, subsequent analyses were carried out using a one-way ANOVA test. Immunohistochemistry and immunofluorescence levels were compared using a two-way ANOVA (with diet and positions as causes of variation) followed by Tukey-Kramer post hoc test. Since differences between zones and placental positions were evident, data are presented separately for basal and labyrinth zones, and mid- and proximal-horn placentas. Data are presented as the means ± SE and were considered significant at P < 0.05.

RESULTS

Placental Zone Weights

MUN basal and labyrinth zones from either mid-horn or proximal-horn positions weighed significantly less than AdLib zone- and position-matched placentas (Fig. 1A; P < 0.05). Among MUN placentas, the basal and the labyrinth zones from mid-horn placentas weighed significantly less than zone-matched tissues from proximal-horn sites (Fig. 1A; P < 0.05), while uterine position had no impact on the weights of the two placental zones in AdLib gestations.

Apoptotic Index and Activated Caspase-3 Expression Increase in MUN Placentas

As placental cell death influences placental development (21, 38), we used TUNEL assay to assess placental apoptosis, and predicted that both MUN mid- and proximal-horn placentas would have higher levels of cell death in basal and labyrinth zones than the respective AdLib zones. Thus, in the MUN mid- and proximal-horn placentas, the apoptotic indices were significantly increased in basal and labyrinth zones compared with their respective AdLib zones (Fig. 1B; P < 0.04). However, when comparing horns, MUN and AdLib basal and labyrinth zones from mid-horn placentas had comparable incidence of apoptosis than zone-matched from proximal-horn placentas.

Consistent with results from the TUNEL assay indicating enhanced apoptosis in MUN placentas, either MUN placental basal (Fig. 1, C and H) or labyrinth (Fig. 1, F and H) zones from mid- and proximal-horns had significantly higher levels of activated caspase-3 immunostaining than zone-matched and position-matched placentas from AdLib controls (Fig. 1, D, G, and H; P < 0.05). When comparing positions, the MUN placenta showed significantly higher activated caspase-3 immunostaining in either basal or labyrinth zones from the mid-horn uterine position compared with proximal-horn placentas (Fig. 1I). In AdLib placentas, activated caspase-3 immunostaining was also significantly higher in the labyrinth zone (Fig. 1I) of the mid-horn than proximal-horn placentas.
(I) Cytosolic anti-apoptotic proteins and scoring

Bcl2  Bcl-X<sub>L</sub>  Negative control

MUN  MUN  MUN

M

Basal zone

AdLib  AdLib  AdLib

B  F  J

C  G  K

D  H  L

Labyrinth zone

(II) Mitochondrial anti-apoptotic protein expression

Bcl2  Bcl-X<sub>L</sub>  Negative control

MUN  MUN  MUN

M'

Basal zone

AdLib  AdLib  AdLib

A'  E'  I'

B'  F'  J'

C'  G'  K'

D'  H'  L'

Labyrinth zone
although activated caspase-3 expression in the basal zone was not different between the two uterine positions (Fig. 1H). There was no detectable staining for activated caspase-3 in specimens treated with nonimmune serum without the primary antibody (Fig. 1, E and H).

These findings support the premise that MUN is a stimulus for increased apoptosis in placentas. Furthermore, the apoptotic indices suggested that the effect of MUN was exacerbated in the mid-horn placentas.

Expression of Anti- and Pro-Apoptotic Bcl-2 Family Proteins in Cytosolic and Mitochondrial Placental Fractions

The role of the mitochondrial pathway in the increased placental apoptosis is unknown. We determined whether the Bcl-2 family of anti- and pro-apoptotic markers of apoptosis were accompanied by activation of the mitochondrial pathway in placental basal and labyrinth zones in using Western blot analysis, immunoperoxidase staining, and double immunofluorescence analyses.

Expression of anti-apoptotic Bcl2 family of proteins. Using Western blot analysis, we evaluated the expression of anti-apoptotic regulators Bcl2, Bcl-XL, and Mcl-1 in the cytosolic and mitochondrial compartments of basal and labyrinth zones from proximal and mid-horn placentas. For each of these proteins, we detected a single immunoreactive band of the expected molecular weight in both cytosolic and mitochondrial protein lysate fractions (Fig. 2 A, C, E, and G). When analyzed by densitometry, the MUN mid-horn basal zone showed a significantly lower anti-apoptotic Bcl2 expression in the mitochondrial fraction (Fig. 2D; Table 2), but not in the cytosolic fraction (Fig. 2B; Table 2) compared with the zone-matched AdLib controls. In the MUN proximal-horn basal zone, cytosolic and mitochondrial Bcl2 expression were significantly downregulated (Fig. 2, B and D; Table 2) compared with zone-matched AdLib controls. Conversely, in MUN mid- and proximal-horn labyrinth zone, cytosolic and mitochondrial Bcl2 expression were significantly downregulated (Fig. 2, B and D; Table 2) compared with zone-matched AdLib controls. Additionally, Bcl-XL protein was significantly lower in cytosolic (Fig. 2F; Table 2) and mitochondrial fractions (Fig. 2H; Table 2) from both placental zones and uterine positions in the MUN placenta compared with the zone- and position-matched AdLib controls. In contrast, cytosolic and mitochondrial Mcl-1 protein expression was not affected by MUN, regardless of zone or position (data not shown).

To confirm results of the Western blot analysis for anti-apoptotic proteins, we used immunoperoxidase staining and immunofluorescence analyses focusing on the proximal-horn placentas, as both mid-horn and proximal-horn placentas showed similar trends in protein expression by Western blot analysis. Thus, immunostaining of cytosolic Bcl2 in the MUN placenta was unchanged in basal zone (Fig. 3, A and M), but significantly decreased in the labyrinth zone (Fig. 3, C and M) compared with zone-matched AdLib controls (Fig. 3, B, D, and M). Furthermore, Bcl-XL immunostaining was significantly lower in either basal (Fig. 3, E and N; P < 0.05) or labyrinth (Fig. 3, G, and N; P < 0.05) zones of MUN placenta compared with the matched AdLib controls (Fig. 3, F, H, and N). Conversely, Mcl-1 immunostaining was prominent in both MUN and AdLib placentas (data not shown). No immunostaining was observed for any anti-apoptotic proteins in the negative controls (Fig. 3, I–L).

Using double immunofluorescence labeling with MnSOD as a mitochondrial marker, we next examined whether the expression of the Bcl2 family anti-apoptotic proteins studied shifted from cytosol to mitochondria. The MnSOD protein stained as green puncta colocalization with anti-apoptotic proteins (Fig. 3, A′–H′), confirming the presence of the Bcl2 family proteins in mitochondria. Mitochondrial Bcl-2 immunofluorescence staining was comparable in both MUN and AdLib basal zones (Fig. 3, A′–B′, M′; P = 0.56). Conversely, Bcl-2 expression was significantly lower in MUN labyrinth zone (Fig. 3, C′ and M′; P < 0.05) compared with zone-matched AdLib controls (Fig. 3, D′ and M′). Moreover, Bcl-Xl immunofluorescence staining was significantly lower in basal (Fig. 3, E′ and N′; P < 0.01) and labyrinth zones (Fig. 3, G′ and N′; P < 0.03) of MUN placentas compared with zone-matched AdLib placentas (Fig. 3, F′, H′, and M′). Strong Mcl-1 immunoreactivity was detected in MUN and AdLib basal and labyrinth zones (data not shown). No immunofluorescence staining was observed for any of the anti-apoptotic proteins in the negative controls (Fig. 3, I′–L′).

Collectively, these results provide evidence that the increased placental apoptosis is accompanied by downregulation of the Bcl2 and Bcl-Xl proteins but unchanged Mcl-1 expression.

Expression of pro-apoptotic Bcl2 family of proteins. The balance between pro- and anti-apoptotic factors, and the formation of heterodimers act as checkpoints in the progression of pro-apoptosis signals in the mitochondrial pathway (25). We evaluated the expression of pro-apoptotic Bcl-2 family members Bak and Bak proteins that function as an obligate gateway for the activation of apoptosis.

Using Western blot analysis, we detected a single immunoreactive band of the expected molecular weight in both cytosolic and mitochondrial protein fractions for each of Bax and Bak proteins (Fig. 4, A, C, and E). When analyzed by densitometry, our results show that in contrast to the anti-apoptotic protein Bcl-XI, pro-apoptotic Bax was significantly upregulated in cytosolic (Fig. 4B, Table 2) and mitochondrial (Fig. 4D, Table 2) fractions of the MUN placental basal and labyrinth zones from mid- and proximal-horn uterine positions compared with zone, and positions matched AdLib controls. Moreover, pro-apoptotic protein Bak, which resides mainly in the mitochondria (43), was significantly induced by MUN in the basal zone from mid-horn and proximal-horn (Fig. 4F, Table 2) placentas, while in the labyrinth zone, Bak was unchanged in the mid-horn but significantly increased in proximal-horn (Fig. 4F, Table 2) placentas compared with matched AdLib controls. Notably, when comparing positions, the MUN placentas showed significantly increased expression of mitochondrial Bak (Fig. 4D) in basal zone and Bak (Fig. 4F) in labyrinth zone from mid-horn placentas compared with proximal-horn placentas (Fig. 4, D and F).

Immunoperoxidase staining analysis of pro-apoptotic Bax and Bak substantiates the Western blot analysis results. Indeed, Bax immunostaining was significantly higher in basal (Fig. 5, A and M) and labyrinth (Fig. 5, C and M) placental zones from the MUN group compared with the matched AdLib specimens (Fig. 5, B, D, and M; P < 0.05). As expected, Bak immunostaining was similar in both MUN and AdLib placental basal and labyrinth zones (Fig. 5, E–H, and N; P < 0.05), as this
protein is mainly localized in the mitochondria. No detectable staining was observed for any of the pro-apoptotic proteins in the negative controls (Fig. 5, I–L).

During apoptosis, the pro-apoptotic Bcl-2 family member Bax translocates from the cytosol to mitochondria, where it oligomerizes and permeabilizes the mitochondrial outer membrane to promote apoptosis (13). Consistent with this finding, our results showed that mitochondrial Bax immunofluorescence staining was significantly stronger in the basal (Fig. 5, A' and M'; P < 0.002) and labyrinth zones (Fig. 5, C' and M'; P < 0.001) of MUN placentas compared with zone-matched AdLib controls (Fig. 5, B', D', and M'). Similarly, Bak immunostaining was significantly higher in the basal (Fig. 5, E' and N'; P < 0.01) and the labyrinth (Fig. 5, G' and N'; P < 0.04) zones of MUN placentas compared with zone-matched AdLib placentas (Fig. 5, F', H', and N'). Again, no detectable staining was observed for Bax or Bak proteins in the negative controls (Fig. 5, I'–L').
(I) Cytosolic pro-apoptotic proteins immunostaining and scoring

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(II) Mitochondrial pro-apoptotic protein immunofluorescence

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Overall, the immunostaining results complemented the Western blot analysis data, indicated that MUN placentas expressed higher levels of the pro-apoptotic proteins Bax within the cytosol and Bax and Bak in the mitochondria, and suggested that Bax and Bak were critical for apoptosis induction.

Pro-Apoptotic Cytochrome c Release and Caspases Activation

Since expression profiles for the anti- and the pro-apoptotic Bcl2 family of proteins clearly indicated activation of the mitochondrial apoptotic pathway, we next determined whether this Bcl2 family of proteins expression upregulation was accompanied by cytochrome c release from the mitochondria into the cytosol of MUN placental cells and activation of cytosolic caspase-9 and -3 using Western blot and immunoperoxidase staining analyses.

Using Western blot analysis, we demonstrated that in either MUN or AdLib specimens, immunoreactive band corresponding to cytochrome c was detected in the cytosolic fraction from both basal and labyrinth zones of mid- and proximal-horn placentas (Fig. 6A), whereas expression of cleaved caspase-9 and -3 revealed two polypeptides of the expected molecular weights (Fig. 6, C and E). Densitometric analysis demonstrated that the release of cytochrome c into the cytosolic fraction was significantly higher in the MUN basal and labyrinth zones from mid- and proximal-horn placentas than in zone-matched AdLib placentas (Fig. 6B; P < 0.05) (Table 2). Likewise, levels of activated caspase-9 and -3 proteins triggered by cytochrome c release were significantly higher in MUN from either basal or labyrinth zones compared with zone-matched AdLib placentas (Fig. 6, D and F; P < 0.05) (Table 2).

Immunoperoxidase staining also demonstrated that the release of cytochrome c into the cytosol was significantly higher in either placental zones of the MUN group (Fig. 7, A, C, and M) compared with zone-matched AdLib placentas (Fig. 7, B, D, and M). Notably, cytochrome c is a highly water-soluble protein; therefore, the diffuse intercellular staining observed may reflect its rapid cytoplasmic dispersion once released from the mitochondria. Activated caspase-9 immunostaining was significantly higher in basal (Fig. 7, E and N) and labyrinth (Fig. 6, G and N) zones of MUN placentas compared with AdLib placentas (Fig. 7, B, H, and N). Again, no detectable staining was observed for cytochrome c or activated-caspase-9 in the negative controls (Fig. 7, I–L).

The Western blot analysis and immunoperoxidase staining data demonstrated that the Bcl2 family protein expression profiles correlated with the release of cytochrome c from mitochondria and activation of caspases, consistent with activation of the mitochondrial apoptotic pathway.

Nuclear PPARγ Protein Downregulation in the MUN Placentas

Since PPARγ prevents apoptosis in many tissues (48), we determine whether the downregulation of this nuclear receptor accompanies the increased apoptosis in MUN placentas using Western blot analysis and immunoperoxidase staining analyses.

Using Western blot analysis, we detected an immunoreactive band corresponding to PPARγ in Western blots of nuclear protein extracts from MUN and AdLib basal and labyrinth zones from proximal- and mid-horn placentas (Fig. 8A). The level of PPARγ was significantly lower in MUN basal and labyrinth zones from mid- and proximal-horn placentas compared with zone-matched AdLib placentas (Fig. 8B; P < 0.05). Notably, AdLib placentas further exhibited a significantly higher PPARγ expression in labyrinth zones of mid-horn placentas compared with proximal-horn placentas (Fig. 8B; P < 0.05), a result not found in MUN placentas.

Immunoperoxidase staining was performed to localize PPARγ and identify placental expression patterns. PPARγ immunostaining was significantly lower in the basal (Fig. 8, C and I) and labyrinth (Fig. 8, F and I) zones from MUN proximal-horn placentas compared with zone-matched position-matched specimens from the AdLib placentas (Fig. 8, D, G, and I). Notably, PPARγ was prominently identified as a black deposit in the nucleus of the AdLib placentas (Fig. 8, D and G), likely due to the formation of excess peroxidase reaction product from high PPARγ expression. There was also weak immunoreactivity in the cytoplasm of AdLib placentas, while no staining was observed in negative controls in which anti-PPARγ was replaced by nonimmune serum (Fig. 8, E and H).

These data complement the findings from Bcl2 protein expression analysis and indicate that PPARγ expression inversely correlates with apoptosis and the expression profiles of pro-apoptotic Bcl2 family proteins.

DISCUSSION

Placentas isolated from MUN rats exhibited clear signs of enhanced apoptotic cell death relative to AdLib controls as detected by two well-established markers of apoptosis, TUNEL staining and immunostaining for activated caspase-3. Moreover, Western blot analysis and immunohistochemical staining revealed that the Bcl2 family proteins with multiple BH 1–4 domains had a pro-apoptotic expression profile in both the basal and labyrinth zones of placentas isolated from the mid- and proximal-horn positions of the rat uterus during MUN pregnancies. Specifically, there was a reduction in the expression of the anti-apoptotic Bcl2 and Bcl-xL proteins and en-
hanced expression of the pro-apoptotic proteins Bax and Bak. Conversely, expression of Mcl-1, a multi-domain Bcl-2 family protein, was unaffected by MUN treatment. The subcellular expression profiles of pro-apoptotic Bax and Bak in cells of the MUN placentas showed enhanced immunolocalization with the mitochondrial marker manganese superoxide dismutase (24). These data indicate that MUN enhanced pro-apoptotic regulators, leading to mitochondrial release of cytochrome c into the cytoplasm. Cytosolic cytochrome c then activated caspase-9 and effector caspase-3, leading to mitochondria-dependent apoptotic cell death. These new findings complement our previous study showing that Fas ligand-receptor interactions contribute to placental dysfunction in MUN (7). Collectively, these data indicate that placental dysfunction in MUN pregnancies derives from multiple signaling pathways induced by limiting maternal nutrient supply. The inverse relationship between PPARγ expression and placental apoptosis in MUN pregnancies suggests that this nuclear receptor, a known regulator of placental development and function (3), may be a prime determinant of placental apoptosis.

The significant reduction in placental zone weights may be at least partially caused by enhanced apoptosis in the MUN placentas. This is supported by microscopic examination showing enhanced activated caspase-3 in basal and labyrinth zones.
As apoptosis is critical for placental development, we have recently demonstrated activation of Fas receptor-dependent apoptosis in MUN placentas. Our studies suggest that the mitochondrial pathway acts in parallel with the Fas pathway to induce placental dysfunction under conditions of limited nutrient availability. The degree of placental dysfunction and aberrant effects on fetal development could depend on which part of the placenta is most seriously affected. The basal zone is the site of hormone production, while the labyrinth is the site of feto-maternal exchange. The degree of fetal dysfunction may be exacerbated by the uterine position, whether mid- or proximal-horn, as the uterus has nonuniform vascularity. The basal zone from the mid-horn placentas showed decreased levels of both mitochondrial anti-apoptotic proteins Bcl2 and Bcl-XL in MUN placentas relative to zone- and position-matched AdLib placentas, whereas the basal zone from proximal-horn MUN placentas exhibited comparable Bcl2 expression but lower Bcl-XL expression. Since Bcl-XL provides stronger protection against apoptosis than Bcl2 (16), the decrease in Bcl-XL in the proximal horn may lead to more pronounced changes in the balance among Bcl2 family proteins and enhanced apoptosis. Unlike the basal zone, the labyrinth zone from both mid- and proximal-horns exhibited decreased expression of both Bcl2 and Bcl-XL proteins in MUN placentas relative to matched AdLib placentas, indicating that the MUN-induced apoptosis may be due to an inappropriate decrease in anti-apoptotic proteins. Contrary to expectation, however, both basal and labyrinth zones showed comparable levels of anti-apoptotic Mcl-1 in MUN and AdLib placentas irrespective of position. This striking difference between pro-survival Mcl-1 and Bcl2/Bcl-XL may be due to distinct interacting proteins. The Mcl-1 protein lacks the BH 4 domain and so interacts with a distinct set of proteins to modulate apoptosis (14). Unlike Bcl2 and Bcl-XL proteins, pro-apoptotic Bax and Bak protein expression was increased in all regions except for the mid-horn labyrinth zone, suggesting that other pro-death proteins were activated in this horn. In conjunction with pro-apoptotic Bax and Bak, MUN-induced placental apoptosis could also be triggered directly by BH 3-only proteins. Our recent results demonstrating upregulation of the tBid caspase-activated form of a BH 3-only apoptotic protein in the placental labyrinth zone supports this premise (7).

Comparison of placental positions showed greater immunostaining for activated caspase-3 in mid-horn placentas than in proximal-horn placentas, possibly due to differences in maternal blood supply (6). Moreover, there was significantly lower Bax and Bak protein expression in MUN mid-horn placentas compared with proximal-horn placentas, suggesting that other Bcl2 family proteins with BH 1–4 domains were activated, possibly to compensate for some of the apoptotic events.
concomitant with mitochondrial dysfunction. Overall, our results demonstrate zone-specific and position-specific activation of the mitochondrial apoptotic pathway in MUN placentas following limited maternal nutrient supply, resulting in impaired placental growth and most likely in placental dysfunction.

Remarkably, during the 10 days of feed restriction, maternal weight loss (60 g) was markedly higher than placental and fetal...
weight reduction (90 mg and 200 mg, respectively) (7), suggesting that the dams have lost significant fat during the MUN period through fat mobilization. The maternal fat loss would more likely result in elevated maternal circulating fatty acids that could possibly activate placental JNK mitochondrial-dependent pathway in the MUN placentas. Although, additional experiments are required to differentiate between the contribution of mitochondrial pathway alone or in concert with JNK-dependent pathway to placental weight and function alterations.

The decreased weight of the basal and labyrinth zones in MUN rats may limit the placental capacity for hormone production (29) and nutrient transfer (21) with subsequent impact on fetal growth and physiological functions. The MUN-induced placental apoptosis in both zones may result in a defective placental barrier enhancing exchange across the maternal/fetal interface (26), which has been shown to impact fetal growth and development. In addition, a permeable interface may explain the finding of increased maternal levels of α-fetoprotein (42) in pregnancies with growth-restricted fetuses. Notably, fetal growth restriction in response to MUN has been attributed primarily to reduced fetal nutrient availability (26). These results indicate that impaired placental growth, and probably function, may also play a role in the reduced fetal growth that predisposes to adult metabolic disorders.

The impact of nutrient restriction in human pregnancies was demonstrated in a study from Saudi Arabia where babies in the second or third trimester of gestation during Ramadan (day-fasting time) had a mean placental weight below that of matched newborns not in utero during this period (1). Similarly, in our rat model, nutrient restriction during the second half of pregnancy (E10–E20) resulted in fetal intrauterine growth restriction and lower birth weight (12). This model of an IUGR programming adult-related obesity is similar to that seen in today’s contemporary society, which is characterized by reduced activity coupled with increased food intake. In spite of these similarities, caution is warranted when extrapolating animal models to human pathophysiology because the timing and effects of nutritional insults commonly experienced during human pregnancy range across a continuum, and are rarely as
severe as nutrient deprivation in the MUN model (50%). Furthermore, the human in utero experience is confounded by multiple genetic and environmental variables (36), whereas the rats in our study were inbred, fed a homogeneous diet, reared in a homogenous environment, and exposed to a severe dietary insult only during the second half of gestation. Additionally, placental position is a factor in only a minority of human births. Despite these limitations, our findings are similar to those in human placentas from IUGR pregnancies that show increased placental apoptosis and reduced placental weight compared with placentas from control pregnancies (28).

As PPARγ prevents apoptosis in many cell death models by preserving the mitochondrial transmembrane potential (44), it may be possible that this nuclear receptor might regulate MUN-mediated placental cell death. Consistent with this, nuclear PPARγ was significantly lower in the MUN placentas, suggesting that MUN-induced apoptosis may be due to PPARγ downregulation. Besides the presence of PPRE in Bcl2 (8), a search of both Bcl-XL and Mcl-1 promoters also revealed several potential PPRE sites in Bcl-XL but not in Mcl-1. Thus, upregulation of PPARγ could potentially result in increased expression of anti-apoptotic Bcl2 and Bcl-XL, which subsequently prevent formation of the mitochondrial permeability transition pore and cytoplasmic release of cytochrome c (Fig. 9A).

Conversely, downregulation of PPARγ may decrease Bcl2 and Bcl-XL expression, shifting the balance toward pro-apoptotic species that promote cytochrome c release (Fig. 9B). These observations point to a potentially important novel role for PPARγ in placental development and homeostasis through the control of cell turnover. Indeed, downregulation of PPARγ may represent a mechanism for PPARγ-mediated increased apoptosis in the MUN placentas. Further studies are required to dissect the mechanism through which this process occurs and the exact role of PPARγ in MUN placentas. We speculate that PPARγ might regulate placental weight and function through control of apoptosis, and work is underway to verify this hypothesis. As we continue to address specific signaling pathways that lead to placental apoptosis, we will further elucidate how PPARγ may downregulate both Fas receptor-dependent and mitochondrial apoptotic pathways.

In summary, suboptimal maternal nutrition results in reduced placental weight and increased placental apoptosis through the mitochondrial apoptotic pathway, possibly in conjunction with the Fas pathway (7) (Fig. 9). The subsequent reduction in newborn weight associates with a higher prevalence of metabolic syndromes later in life (4). The marked effect of MUN on mid-horn placentas indicates that this may be the most unfavorable position, which may result in the most severe fetal damage. Clarifying the role of PPARγ and the Bcl2 family of proteins in the placentas of MUN rats is of utmost importance as this may provide insights into novel therapeutic strategies to address placental dysfunction and birth defects. Currently, PPARγ agonists are in use for the treatment of diabetes, and small molecule inhibitors of the Bcl2 proteins are already being developed for use in cancer. We may, therefore, already have some of the pharmacological tools for clinical intervention in utero.

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

AUTHOR CONTRIBUTIONS
Author contributions: L.B., M.D., D.M.N., and M.G.R. conception and design of research; L.B. performed experiments; L.B. analyzed data; L.B. and D.M.N. interpreted results of experiments; L.B. prepared figures; L.B. drafted manuscript; L.B. and D.M.N. edited and revised manuscript; L.B., M.D., D.M.N., and M.G.R. approved final version of manuscript.

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MUN IN PREGNANCY STIMULATES PLACENTAL APOPTOSIS


