Increased placental XIAP and caspase 3 is associated with increased placental apoptosis in a baboon model of maternal nutrient reduction

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OBJECTIVE: Our objective was to determine signaling molecules and apoptosis rate in the term placenta of a baboon model of maternal nutrient reduction (MNR).

STUDY DESIGN: Female baboons were fed ad libitum for controls (n = 7) or 70% of control baboon diet (MNR; n = 6) from 30-165 days of gestation with necropsy at 165 days of gestation. Placental tissues were collected and fixed for immunohistochemistry or snap frozen to measure extracellular signal-regulated kinases, protein kinase B, JUN NH2-terminal kinase, X-linked inhibitor of apoptosis protein, and caspase 3. Placental villous apoptosis was determined by terminal deoxynucleotidyl transferase-mediated 2′,3′-dideoxyuridine 5′-triphosphate nick-end labeling and cytokeratin 18 cleavage.

RESULTS: Compared with the control placentas, MNR placentas demonstrated reduced placental weight (P < .02), decreased phospho (p)-ERK (P < .04), increased placental villous apoptosis (P < .001), increased villous cytokeratin 18 cleavage, increased X-linked inhibitor of apoptosis protein (P < .007), and increased active caspase 3 (P < .02).

CONCLUSION: We conclude that placental apoptosis is increased in this baboon model of MNR at term and that the increase in X-linked inhibitor of apoptosis protein may be a protective mechanism against this apoptosis.

Key words: apoptosis, caspase 3, MNR, placenta, XIAP


Placental development is important for fetal growth and development. Abnormal placentation is associated with the development of complicated pregnancies in humans. Poor maternal nutrition is a common problem in the developing world, which can have many adverse effects on the fetus, including intrauterine growth restriction (IUGR). A higher degree of syncytiotrophoblast apoptosis is found in placentas from pregnancies that are complicated by fetal growth restriction and preeclampsia. Apoptosis, an active process of cellular destruction, serves an essential remodeling function in multicellular organisms and is a component of normal development and differentiation in most tissues, including the placenta. Abnormal regulation of apoptosis in different tissues has been implicated in the onset and progression of a broad range of diseases.

The X-linked inhibitor of apoptosis protein (XIAP) is the most potent member of a group of inhibitor of apoptosis proteins that regulate cell death. Inhibitor of apoptosis proteins possess a defining structural motif, called Baculovirus Inhibitor of Apoptosis Repeat (BIR), which is responsible for the inhibition of caspases 3, 7, and 9. Caspases are a family of cysteine proteases that play a central role in initiating and executing the apoptosis cascade. In trophoblast cells, caspases are known to cleave the cytokeratin 18 protein, which produces a neopeptide that is used as a marker of apoptosis. Controlling the activity of caspases is essential for the appropriate execution of cell death and the regulation of cell survival. XIAP is present in trophoblast throughout placental development, but expression is decreased significantly near delivery when apoptosis is maximal. A decrease in XIAP protein is associated with the presence of increased apoptosis during IUGR in pregnant sheep. This suggests a role for XIAP in the regulation of trophoblast apoptosis in normal and abnormal processes in pregnancy.

Signal transduction cascades serve to elicit a cellular response that is initiated at the cell surface. These cascades contain multiple components that involve a series of phosphorylation steps. The extracellular signal-regulated kinase 1/2 (ERK1/2) and the protein kinase B (AKT) pathways are found in many cell types and play various roles. These proteins mediate biologic responses like cel-
lular growth, proliferation, and cell survival.\textsuperscript{13} The c-Jun amino terminal kinase (JNK) is another signaling molecule that is activated to diverse stimuli that include DNA damage, ultraviolet radiation, heat shock, and others.\textsuperscript{14} There is a reduction of these signaling proteins in other models of IUGR.\textsuperscript{15}

Although there are many studies on the effects of controlled reductions in maternal nutrient intake on apoptosis in rodents and sheep, data are lacking in nonhuman primates.\textsuperscript{16–19} In the present study, we evaluated placental apoptosis and changes in key signaling molecules in the placentas of control pregnant baboons that were fed ad libitum and mothers that were exposed to nutrient reduction (MNR) and fed 70% of the feed consumed by the controls. Previous studies on the placentas showed that MNR decreased placental size near-term when villous volume and surface area, capillary surface area, and the villous isomorph coefficient were all decreased.\textsuperscript{1,20} Briefly, the subjects of this study were 13 female baboons aged 8–15 years that were maintained in group housing and bred as previously described in detail.\textsuperscript{1,20} Baboons were observed twice a day for well-being and 3 times a week for turgescence (sex skin swelling) and signs of vaginal bleeding to enable timing of pregnancy.\textsuperscript{1} Pregnancy was dated initially by observing the changes in the swelling of the sex skin and confirmed at 30 days of gestation by ultrasonography. Animals (Papio sp) were fed Purina Monkey Diet 5038 (Purina, St. Louis, MO). Diet was closely monitored in 7 animals that were fed ad libitum (control group) and their food intake was calculated weekly on a per kilogram basis. From confirmation of pregnancy, the 6 pregnant baboons in the MNR group received 70% of the average daily amount of feed eaten (on a weight-adjusted basis) by control animals at the same gestational age. Cesarane section deliveries were performed at 165.9 ± 1.1 days of gestation in control and at 162.1 ± 4.6 days of gestation in MNR animals. Placental sections were obtained from a central area of a cotyledon, fixed in formalin (10% buffered formalin), and embedded in paraffin for immunohistochemistry or snap frozen in liquid nitrogen for protein studies.

**Materials and Methods**

**Animal care**

All procedures were approved by the Southwest Foundation for Biomedical Research Institutional Animal Care and Use Committee and conducted in American Association for Accreditation of Laboratory Animal Care–approved facilities. Animal care was carried out as described by Schlabritz-Loutsevitch et al\textsuperscript{1,20} Briefly, the pregnant baboon presents several advantages for the study of effects of maternal nutrition on placental and fetal growth, compared with other nonhuman primates, including fetal size and similarities in placentation and placental structure with those of human pregnancy.\textsuperscript{1,16} We hypothesized that MNR would increase placental villous apoptosis and decrease abundance of XIAP at the end of gestation. Because this model is associated with a decrease in placental size, we further hypothesized an increase in cytokeratin 18 cleavage and active caspase 3 in placentas from MNR mothers. We also sought to determine changes in cell signaling proteins that are associated with cell proliferation (ERK), cell survival (AKT), and cell stress (JNK) in the placenta of controls and MNR animals.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)**

Placental sections were used for these experiments. TUNEL protocol was followed, as suggested by the manufacturer (Chemicon, Inc, Temecula, CA). Briefly, slides were dewaxed and postfixed with a solution of ethanol:acetic acid (2:1) for 5 minutes. The equilibration buffer was added to the slide for 10 seconds, followed by incubation with the terminal deoxynucleotidyl transferase enzyme for 1 hour at 37°C. After this, the antidigoxigenin conjugate was incubated on the slide for 30 minutes and 4',6-diamidino-2-phenylindole, dihydrochloride was used for nuclear staining. Slides were viewed with fluorescein excitation and emission filters. Analysis was performed in 2 placental slides per animal (3 controls; 3 MNR, selected randomly), and 20–30 fields were counted per slide with a mean count that was generated per slide for analysis purposes. The percentage apoptosis was calculated in the placental slides (12 slides total) as the number of TUNEL-positive cells divided by the total number of cells in 20–30 fields × 100.

**Western blot analysis**

Western blot was performed as previously described by Arroyo et al.\textsuperscript{12} Briefly, frozen placental tissues were homogenized, and protein tissue lysates (50 μg) were separated on 4–12% Bis-Tris gel sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were incubated with an antibody against mouse XIAP (at a dilution of 1:200; Transduction Laboratories, Lexington, KY) or antibodies against rabbit active (cleaved) caspase 3, caspase 3, phospho-AKT, total AKT, phospho-ERK or total ERK, and phospho-JNK or total JNK (all at 1:500; Cell Signaling Technology, Danvers, MA). A secondary anti-mouse or anti-rabbit immunoglobulin horse-radish peroxidase antibody (dilution 1:10,000; Upstate Cell Signaling Solutions, Lake Placid, NY) was incubated for 1 hour at room temperature. The membranes were incubated with enhanced chemiluminescence substrate (Amer-sham, Princeton, NJ) for 5 minutes, and the emission of light was detected with x-ray film. To determine loading consistencies, each membrane was stripped of antibodies and reprobed with an antibody against mouse beta-actin at a dilution of 1:4000 (MP Biomedicals, Solon, OH). The presence of these proteins was confirmed and quantified.

**M30 cytoideath immunohistochemistry**

Immunohistochemistry was performed on paraffin-embedded placental sections. Slides were dewaxed with 100% xylene. Slides were washed in phosphate-buffered saline solution (PBS), and sections were blocked for 1 hour with 10%...
normal goat serum/PBS. Slides were then incubated for 1 hour with a mouse monoclonal primary antibody against M30 cytodeath (the neopeptide produced from cytokeratin 18 cleavage; dilution 1:200; Roche Diagnostics Corporation, Indianapolis, IN) or a mouse immunoglobulin G1 (dilution of 1:500) for negative control. Sections were washed in 1 × PBS. Sections were then incubated for 45 minutes with a biotin-labeled anti-mouse secondary antibody. Slides were washed in 1 × PBS, incubated in streptavidin-biotin-horseradish peroxidase solution, and developed with diaminobenzidine Vectastain ABC DAB kit (Vector Laboratories, Inc, Burlingame, CA). Diaminobenzidine (brown) was used to stain for the M30 positive cells in serial placental sections. Hematoxylin was used for nuclear counterstaining. Slides were mounted with Permount mounting media (Fisher Scientific, Pittsburgh, PA).

**Statistical analysis**

Comparisons were made between control and MNR groups with a rank sum test (Mann-Whitney U test) for the following elements: fetal and placental weights and XIAP, caspase 3, ERK, and AKT Western blot analysis. For TUNEL assay, a comparison was made of mean number of apoptotic cell counts per slide between both groups (n = 5); slides for each group were checked for normality and Student t test was performed. Data are shown as mean ± SE; a probability value of < .05 was considered significant for the statistical comparisons.

**RESULTS**

MNR baboons showed a significant decrease in placental weight (177 g ± 8 g vs 145 g ± 7 g; P < .02) and a mean fetal weight decrease of 7% (744 g ± 32 g vs 669 g ± 33 g; P = .1) at 165 days of gestation (Figure 1). In our study, there were no pregnancy miscarriages or fetal deaths, fetal growth restriction, or fetal abnormalities.

**Placental apoptosis**

For the TUNEL studies, approximately 265 microscopic fields were available for analysis (MNR group, 120 fields; control group, 145 fields). Figure 2 shows the difference in apoptosis that was achieved by TUNEL staining in the placenta of a control ad libitum fed mother and a placenta of mother in the nutrient reduction group. It can be seen that the percentage of apoptosis increased by approximately 100% in the presence of MNR (Figure 2). Immunofluorescence was performed to identify trophoblast cells (Figure 3). Figure 3 also shows the presence of M30 in negative control, treated, and control animals. Figure 3 shows immunofluorescence staining for
pan-cytokeratin to identify the trophoblast cells. Immunohistochemistry for M30 showed an increased cytokeratin 18 cleavage in the trophoblast of placentas from mothers in the MNR group, compared with controls (Figure 3).

**Placental XIAP and caspase 3 proteins**

XIAP protein was significantly increased at 165 days of gestation (1.5-fold) in the placentas of mothers in the MNR group (Figure 4). Similarly, active caspase 3 protein (5.8-fold) was also increased in the placentas of mothers in the MNR group (Figure 5).

**ERK, AKT, and JNK**

PERK protein increased 10.0-fold ($P < .04$); neither pAKT nor pJNK show differences in the placentas of the 2 groups (Figure 6).

**COMMENT**

The baboon model of MNR is characterized by a decrease in villous number and volume and a decrease in overall placental size near term. We found that the smaller placentas of the MNR baboon group were characterized by an increase in apoptosis (TUNEL studies), which was further validated by a qualitative increase in M30 from the immunohistochemistry studies. To further elucidate the underlying mechanisms of apoptosis, XIAP and caspase 3 protein levels were determined in the placentas of MNR and control animals. XIAP protein was significantly increased in MNR-treated animals, compared with controls. Similarly, active caspase 3 was also increased in the placenta of MNR animals, which suggests that the increase in XIAP protein could be a result of the increased apoptosis that was found at this point.

Cell death or apoptosis is present normally during cell growth and in the development of tissues, including the placenta. Increased trophoblast apoptosis is present in clinical obstetric disease, such as preeclampsia and IUGR. Trophoblasts are epithelial cells that facilitate the exchange of nutrients and wastes between maternal and fetal compartments. Proper functioning of these cells is critical for placental development that leads to a successful pregnancy. Results from the current study, including the TUNEL assay and cleavage of cytokeratin 18, indicate that apoptosis is present in the placenta in this baboon model of MNR and that it is greater than in the matched controls. This may contribute to the decreased placental weight that was observed in our model. Interestingly, the decrease in weight that was seen in the placenta was not seen in the fetus between groups near term. Although unexpected, this finding has been described in other animal models of MNR. Ozaki et al showed that there was no significant decrease in fetal weight by day 20 of gestation, but growth restriction was present at birth in the rat dietary restriction model. Collectively, these results suggest that decreased placental weight and apoptosis do not affect fetal size near term in this baboon model of MNR, although it remains to be seen whether fetal organ systems are impacted by these placental changes.

In addition, experiments were performed to determine signaling molecules that were associated with the aforementioned findings during MNR. More specifically, the protein activation (phos
phorylation) of ERK, AKT, and JNK was studied. There was a significant decrease in ERK activation in the MNR placenta compared with controls. In contrast, no significant differences were observed in the activation of placental AKT or JNK proteins during MNR in the baboon. The association of decreased ERK with the decrease in placental size and increase in apoptosis is not entirely surprising, because ERK is associated commonly with cell proliferation. Although the aforementioned findings seem logical and consistent with published literature in apoptosis, some findings were not interpreted as easily. We did not observe any changes in the activation of AKT or JNK proteins. This was unexpected because these proteins usually are involved in apoptosis and cell stress. At the same time, we observed an increase in the anti-apoptotic protein XIAP and an increase in caspase 3 activation. Given that XIAP normally inhibits caspase activation and subsequent apoptosis, this increase may represent a compensatory mechanism for the trophoblast for survival in the placenta of these MNR animals. We speculate that the XIAP increase could be involved in protecting the trophoblast from stress, resulting in the lack of activation of AKT or JNK that leads to improved trophoblast survival and function during the MNR in this baboon model. The fact that there is an increase in activation of caspase 3 and M30 staining suggests that other mechanisms that are independent of XIAP could be a factor that induces this activation and leads to the apoptosis that we see in the MNR placenta. Although there is no direct clinical application of the findings in this study, it remains relevant to normal and abnormal human placental nutrient transport for several reasons. The mechanisms that underlie the effect of diminished maternal nutrition on fetoplacental development and its role in fetal origins of adult disease have received considerable interest over the last few decades, which includes worldwide interest.26,27 The Barker28 hypothesis is based on end organ changes in function and structure during fetal life that subsequently lead to disease in adults. Several studies have shown that moderate degrees of nutrient restriction can impact organ structure and function, which includes the kidney, heart, liver, and placenta.29,30 The finding of increased placental apoptosis in this baboon model of MNR at term is a finding that has been described in the human IUGR placenta.31 Why the finding of an increase in XIAP, which is known to be protective against apoptosis, did not prevent apoptosis, compared with controls, is not known. It may be that, without XIAP, apoptosis is not controlled, which is a question that may be answered only with knock-out models.

Finally, we note that both groups of baboons were of mixed age and parity. The extent to which these factors affect the changes that we report remains to be determined. We have shown recently that placental efficiency that is calculated as fetal weight divided by placental weight is higher in mature adult multiparous baboons than in primiparous baboons of the same weight (unpublished observations). The degree to which that
may be due to a lesser degree of apoptosis in multiparity remains to be evaluated.

In summary, apoptosis is increased in the baboon placenta, which leads to a reduction in placental weight in this MNR baboon model. In addition, XIAP protein expression is increased in the placenta near-term in this model of MNR. We speculate that a possible mechanism of protection for the increased apoptosis that was observed in the placenta of treated animals was caused by an increase in XIAP expression in the placenta of MNR animals, compared with controls. Also, we speculate that, although XIAP was increased, this increase is not sufficient to prevent the decrease in placental weight in this model of MNR. To our knowledge, this is the first report to show an increase in XIAP protein that is associated with an increase in placental apoptosis during MNR in animal or human studies. Further studies are needed to determine the role of XIAP and trophoblast apoptosis earlier in gestation in this baboon model of MNR.

REFERENCES