Hypoxia downregulates p53 but induces apoptosis and enhances expression of BAD in cultures of human syncytiotrophoblasts

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Chen B, Longtine MS, Sadovsky Y, Nelson DM. Hypoxia downregulates p53 but induces apoptosis and enhances expression of BAD in cultures of human syncytiotrophoblasts. Am J Physiol Cell Physiol 299: C968–C976, 2010. First published September 1, 2010; doi:10.1152/ajpcell.00154.2010.—Hypoxia is commonly assigned a role in the placental dysfunction characteristic of preeclampsia and intrauterine growth restriction. We previously showed that hypoxia upregulates p53 and enhances apoptosis in primary cultures of human cytotrophoblasts. Here we tested the hypothesis that hypoxia also induces apoptosis in syncytiotrophoblasts by upregulation of p53. Primary cultures of human cytotrophoblasts that had differentiated into syncytiotrophoblasts by 52 h were exposed for ≤24 h to 20% or <1% oxygen in the presence or absence of staurosporine or the p53 modulators nutlin-3, pifithrin-α, and pifithrin-μ. Proteins were detected by Western blot analysis or immunofluorescence. Compared with 20% oxygen, exposure of syncytiotrophoblasts to <1% oxygen upregulated hypoxia-inducible factor (HIF)-1α and rapidly downregulated p53. Activity of p53 in hypoxic syncytiotrophoblasts was reduced by the higher expression of the negative p53 regulator MDMX and by the reduction of phosphorylation of p53 at Ser112, which reduces p53 activity. Conversely, staurosporine, a kinase inhibitor, and nutlin-3, a drug that enhances p53 expression, both raised p53 levels and increased the rate of apoptosis in syncytiotrophoblasts compared with vehicle controls. Immunofluorescence staining showed p53 immunolocalized to both cytoplasm and nuclei of nutlin-3-exposed syncytiotrophoblasts. The hypoxia-induced apoptosis in syncytiotrophoblasts correlated with enhanced expression of the proapoptotic BAD and a reduced level of antiapoptotic BAD phosphorylated on Ser112. We surmise that cell death induced by extreme hypoxia in syncytiotrophoblasts follows a non-p53-dependent pathway, unlike that of a nonhypoxic stimulus and unlike hypoxic cytotrophoblasts. We speculate that downregulation of p53 activity in response to hypoxia reduces or eliminates the apoptosis transduced by the p53 pathway in syncytiotrophoblasts, thereby limiting cell death and maintaining the integrity of this critical villous component.

placenta; trophoblast; injury

EARLY HUMAN PLACENTAL DEVELOPMENT normally occurs in a hypoxic environment, with an oxygen tension (PO2) < 20 mmHg (16). Perfusion of the intervillous space ensues at 10–12 wk of gestation, and the placental villi are then normally exposed to a PO2 of 40–80 mmHg from this time until delivery. Inadequate remodeling of the maternal spiral arterioles in early pregnancy results in retention of the muscle in the vessel walls, and these maladaptive changes expose villous trophoblasts to injury from underperfusion with hypoxia, hypoxia-reoxygenation, mechanical damage, or a combination of these stressors (1). Hypoxia and other stressors in the placenta predispose to placental dysfunction, which typically manifests as preeclampsia, intrauterine growth restriction (IUGR), or both (2, 38).

Syncytiotrophoblasts at the surface of placental villi creates the interface between the maternal and fetal circulations. Bathed in maternal blood in the intervillous space, this unique epithelium experiences fluctuating PO2 in the second half of pregnancy in vivo. Hypoxia, hypoxia-reoxygenation, or both induces the formation of reactive oxygen species that activate an apoptotic cascade in placental explants (5). We previously showed (25) that a low PO2 enhances apoptosis and markedly upregulates p53 expression in cytotrophoblasts in vitro. The increased expression of p53 in response to hypoxia in vitro correlates with enhanced p53 expression in villous cytotrophoblasts in placentas from pregnancies complicated by IUGR in vivo (26).

p53 regulates cell cycle arrest, senescence, apoptosis, autophagy, and metabolism (23, 24), and p53 mediates many of the responses to exogenous stimuli (45). p53 is rapidly degraded under basal conditions because MDM2 binds this protein and functions as an ubiquitin ligase that shuttles the p53 protein for degradation in the proteasome (18). MDMX, also called MDM4, is a complementary major negative regulator of p53, which limits coactivator binding to p53 and directs p53 for degradation by the proteasome independent of MDM2 (10, 13, 18, 46). This p53 degradation is blocked with extreme hypoxia in most cells, increasing the half-life of p53 from minutes to hours. The elevated level of p53 yields transcriptional and cytoplasmic effects that either kill cells or allow repair in response to injury. Posttranslational phosphorylation of p53 can further affect p53 activity by exposing the DNA binding domain to allow translocation of p53 to the nucleus or to alter p53 interaction with proteins from the Bcl-2 family in mitochondria. Notably, hypoxia-inducible factor (HIF)-1α is also constitutively synthesized and degraded by the proteasome under basal conditions. Hypoxia induces stabilization of HIF-1α, which can then interact directly with p53 or indirectly via MDM2 (4, 9, 36).

Collectively, the above findings highlight the complexity of the p53 response to cellular hypoxia. The regulation of p53 in syncytiotrophoblasts is poorly understood. To compare and contrast the p53 response of syncytiotrophoblasts exposed to hypoxia with the known response of the villous cytotrophoblast phenotype, we tested the hypothesis that enhanced p53 activity contributes to increased apoptosis in cultures of human syncytiotrophoblasts exposed to low PO2.
METHODS

Isolation and culture of primary trophoblasts. This study was approved by the Institutional Review Board of Washington University. Human placenta were obtained from uncomplicated pregnancies after elective cesarean section without labor at 39 wk of gestation by the manufacturer's instructions. Reverse

Primary human cytotrophoblasts were isolated from placenta by the trypsin-deoxyribonuclease-Dispase/Percoll method with modifications (21). Cultures were plated at 300,000 cells/cm² and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Sigma, St. Louis, MO) containing 10% fetal bovine serum (Sigma), 20 mmol/l HEPES (pH 7.4, Sigma), penicillin (100 U/ml), streptomycin (100 µg/ml), and fungicide (0.25 mg/ml), all from the Washington University tissue culture support center, at 37°C in a 5% carbon dioxide-air atmosphere with 20% oxygen. After 4 h to allow cell attachment, the cultures were washed thoroughly three times with PBS to eliminate nonattached cells and vili fragments, complete medium was replaced daily, and cultures were maintained for 52 h to allow abundant syncytiotrophoblasts to form in vitro, as previously described (31). We designated the phenotype at this stage as syncytiotrophoblast, after verifying morphological differentiation by immunostaining as described below. Fresh medium was provided at 52 h, and cultures were continued at 37°C in 20% oxygen as the control condition or maintained under <1% oxygen with 5% CO₂, 10% H₂, and 84% N₂. The <1% oxygen atmosphere was supplied in an anaerobic glove box incubator (Thermo Electron, Marietta, OH) that allowed preassing of medium and handling of cultures without exposure to ambient conditions.

Cultures were supplemented at the times and concentrations listed in Figs. 4 and 5 with staurosporine (Tocris, Ellisville, MO), nitulin-3 (Sigma), pifithrin-µ (Sigma), or pifithrin-α (Sigma). Cultures were harvested for proteins cytchemochemical staining at the times listed in Figs. 1, 3, 4, 5, and 7.

Western immunoblotting. Western immunoblotting was performed according to published protocols on total protein extracts of cultured trophoblasts (3). Briefly, the proteins from cultured syncytiotrophoblasts were extracted and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) overnight at 4°C and 300 mA. The blot was blocked 1 h with 5% nonfat dry milk in 1× PBS with 0.05% Tween 20 (PBST) and then incubated for 2 h at room temperature or overnight at 4°C with the following primary antibodies: mouse monoclonal p53 antibody (DO-1, 0.1 µg/ml, Calbiochem, Gibbstown, NJ), mouse monoclonal MDM2 antibody (0.2 µg/ml, Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal M30 antibody that detects the apoptotic cleavage products of cytokeratin 18 intermediate filaments (1:2,000, as recommended by the manufacturer, Roche, Indianapolis, IN), mouse monoclonal anti-p21 antibody (0.2 µg/ml, Santa Cruz), mouse monoclonal anti-MDMX antibody (1 µg/ml, Sigma), rabbit monoclonal anti-BAD antibody (0.2 µg/ml, Cell Signaling, Danvers, MA), rabbit monoclonal anti-BAD-Po4Ser155 antibody (0.2 µg/ml, Cell Signaling), mouse monoclonal anti-p53-Po4Ser156 antibody (0.1 µg/ml, Calbiochem), rabbit polyclonal anti-HIF-1α antibody (10 µg/ml, Novus Biologicals, Littleton, CO), rabbit polyclonal anti-lamin A/C antibody (0.2 µg/ml, Cell Signaling), or goat polyclonal anti-actin (0.2 µg /ml, Santa Cruz) in 5% nonfat dry milk in PBST. The blot was incubated for 2 h with horseradish peroxidase-conjugated sheep anti-mouse, donkey anti-mouse, goat anti-rabbit, or donkey anti-GoG IgG secondary antibody (0.02–0.1 µg/ml, Santa Cruz) at room temperature, washed, and processed for luminescence with the Amersham Pharmacia ECL kit (Amersham Pharmacia Biotech, Arlington Heights, IL). To determine semiquantitative levels of proteins, densitometry of films was assessed with Epichemi-3 software (UVP BioImaging System, Upland, CA) and normalized to actin levels.

Quantitative real-time PCR. RNA was purified from cultured syncytiotrophoblasts with TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. Reverse transcription and quantitative real-time PCR were performed as previously described (3) with primers listed in Table 1.

Immunofluorescence staining and microscopy. The number of nuclei in mononucleated cells (cytotrophoblasts) versus multinucleated syncytia was determined by immunofluorescence staining as previously described (31) with the following modifications. Primary human trophoblasts from three different placenta were plated in 20% oxygen, and at 52 h in culture cells were fixed with 2% paraformaldehyde for 10 min and stained for E-cadherin, which is a marker for plasma membranes (7), with a rabbit monoclonal antibody (1:300, as recommended by the manufacturer, Abcam, Cambridge, MA) and for nuclei with Draq 5 (5 µM, Biostatus, Shepshed, UK). The absence of E-cadherin in regions with multiple nuclei is indicative of syncytiotrophoblast formation (7). Specimens were mounted with Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA) and observed with a confocal microscope (Nikon, ECLIPSE E800, Melville, NY) equipped with a C1 confocal laser scanning head and three lasers with emissions of 488 nm, 546 nm, and 633 nm and ×60 oil-immersion lens. After image optimization, 24 Z-stack images were acquired for each wavelength with a Z-step size of 0.5. A maximum projection image was then generated with Nikon EZC1 software. For each placenta, 10 fields were selected randomly, digital images were captured as above, and the number of nuclei in cytotrophoblasts and syncytiotrophoblasts was determined and expressed as a percentage of total nuclei. For the data in Fig. 6, the syncytiotrophoblasts were exposed to nutlin-3 and DMSO vehicle control, fixed with 2% paraformaldehyde for 2 min, permeabilized with cold 80% methanol at −20°C for 5 min, blocked with 2% bovine serum albumin in PBS, and incubated overnight at 4°C with mouse monoclonal p53 antibody (DO-1, 1 µg/ml, Calbiochem) and rabbit monoclonal antibody to E-cadherin (1:300, as recommended by the manufacturer, Abcam) or without primary antibody. The cells were washed with cold PBS and incubated for 2 h at room temperature with Alexa-conjugated goat anti-mouse (Alexa 555, 10 µg/ml, Invitrogen, Carlsbad, CA) to detect p53, goat anti-rabbit (Alexa 488, 10 µg/ml, Invitrogen) to detect surface membrane E-cadherin, and Draq 5 to identify nuclei. A maximum projection Z-stack image was then generated with the Nikon EZC1 software as described above, and multiple images were analyzed for p53 immunostaining of cytoplasm and nuclei in both villous phenotypes.

Cytosolic, nuclear, and total protein extracts. Syncytiotrophoblasts cultured for 24 h in either 20% oxygen or <1% oxygen had cytosolic and nuclear proteins extracted with a Nuclear Extract Kit (Active Motif, Carlsbad, CA). In brief, cells were washed twice with ice-cold PBS with protease and phosphatase inhibitors, resuspended in hypotonic buffer, and lysed with detergent. The lysate was centrifuged to separate cytoplasmic and nuclear fractions, the nuclear pellet was resuspended in complete lysis buffer and recentrifuged, and the supernatant containing extracted proteins was transferred to prechilled

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<th>Transcript</th>
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<tr>
<td>p53</td>
<td>F</td>
<td>CCAAACTGGAAATCTTCTCCAAACAAACAA</td>
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<tr>
<td>MDM2</td>
<td>R</td>
<td>CCCTCGTTGAGAACAAAGCGAT</td>
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<td>18S</td>
<td>F</td>
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F, forward primer; R, reverse primer.
microcentrifuge tubes and stored at −80°C until being used for immunoblotting. Total protein extracts were prepared as described previously (3).

Statistical analyses. Student’s t-test was performed for all statistical analyses with KaleidaGraph software, version 4.1.0 for Macintosh (Synergy Software, Reading PA). A \( P < 0.05 \) was determined as significant.

RESULTS

Confirmation of the syncytiotrophoblast paradigm. We focused on placental syncytiotrophoblasts, and to again verify that our cultured primary human trophoblasts efficiently fused to form syncytiotrophoblasts, we conducted immunofluorescence staining for E-cadherin, which stains plasma membranes among trophoblast cells, and for Draq 5 staining of nuclear DNA in three primary cultures. The absence of E-cadherin in regions with multiple nuclei is indicative of a syncytium (7). In our conditions, we found that 85–94% of the cell nuclei among three primary cultures were within syncytiotrophoblasts by 52 h in culture, with the remainder in cytotrophoblasts (Fig. 1A). We refer below to cultures at 52 h and beyond as syncytiotrophoblasts.

Effect of oxygen on apoptosis in human syncytiotrophoblasts. Syncytiotrophoblasts exposed to <1% oxygen showed a 2- to 10-fold increase in caspase-mediated cytokeratin 18 intermediate filament cleavage products, indicative of apoptosis (Fig. 1B, \( P < 0.05 \)). HIF-1α plays an essential role in cellular responses to hypoxia (49), and we verified that the cultures responded to hypoxia by upregulation of HIF-1α. As expected, <1% oxygen markedly increased the protein level of HIF-1α in nuclear extracts compared with 20% oxygen (Fig. 1C, \( P < 0.05 \)). The expression of lamin A/C, an apoptotic substrate for caspases in nuclei, was decreased in syncytiotrophoblasts exposed to <1% oxygen (Fig. 1C, \( P < 0.05 \)), indicating that hypoxia-induced apoptosis occurred. Collectively, these data show that <1% oxygen upregulates HIF-1α expression while increasing the level of apoptosis in cultured syncytiotrophoblasts.

Oxygen effects on expression of genes involved in p53 regulation. Multiple genes are regulated by p53 including mdm2, and MDM proteins modulate p53 expression and function in trophoblasts. We first examined p53 and mdm2 expression by real-time PCR and found that mRNA expression in syncytiotrophoblasts cultured in <1% oxygen was not different from expression in 20% oxygen (Fig. 2A and B). We previously reported (3) that ndrg1 was upregulated in primary cultures of human cytotrophoblasts exposed to <1% oxygen and that NDRG1 negatively regulated p53 protein to reduce hypoxic injury to cytotrophoblasts. We found that ndrg1 mRNA levels were also higher in syncytiotrophoblasts exposed to <1% oxygen (\( P < 0.01 \)) compared with 20% oxygen (Fig. 2C), showing that the ndrg1 response to hypoxia was not phenotype dependent.
We next asked whether the reduction of p53 expression in hypoxic syncytiotrophoblasts reflected diminished p53 activity, despite the decrease in the expression level of the negative regulator, MDM2. MDMX, also called MDM4, is another negative regulator of p53 whose function does not overlap with MDM2 (27). We found that MDMX protein levels were increased within 1 h of exposure of syncytiotrophoblasts to 1% oxygen, and MDMX remained elevated for the entire 24 h (Fig. 3C, P < 0.05). Collectively, these data show that the response of p53 to hypoxia in primary cultures of villous trophoblasts is phenotype dependent and that hypoxic syncytiotrophoblasts have decreased p53 activity, perhaps due to enhanced levels of the negative regulator MDMX, compared with cells cultured in standard conditions of 20% oxygen.

p53 is upregulated with a nonhypoxic stimulus in syncytiotrophoblasts. The marked reduction in p53 activity in hypoxia raised the question of whether or not this was a response of syncytiotrophoblasts exposed to any apoptotic stimulus. We addressed this question by exposure of syncytiotrophoblasts to staurosporine, a protein kinase inhibitor that increases apoptotic death through the mitochondrial pathway. Addition of staurosporine resulted in an increase in p53 and MDM2 proteins with peak expression at 24 h, when apoptosis was also highest, compared with vehicle control cultures (Fig. 4, P < 0.05). Thus, unlike hypoxia that downregulates the level of p53 in syncytiotrophoblasts, a nonapoptotic stimulus upregulates p53 in this phenotype.

Syncytiotrophoblast responses to agents altering p53 stability. Although there are many phosphorylation sites on p53 that have different effects on p53 activity (12), phosphorylation of the Ser392 site stabilizes the p53 tetramer to activate transcription of target genes (20, 35). We tested the hypothesis that hypoxia downregulates phosphorylation of p53-Ser392 to limit p53 activity in syncytiotrophoblasts. We found higher than time 0 baseline levels for phosphorylated p53-Ser392 in syncytiotrophoblasts exposed to 20% oxygen after 24 h (Fig. 5A, P < 0.05), while phosphorylation of p53-Ser392 in hypoxic syncytiotrophoblasts in <1% oxygen rapidly diminished from time 0 baseline levels to be barely detectable beyond 2 h of culture in hypoxia. These data complement the p53 expression data detailed above and indicate that limitation of p53 activity is a common theme in hypoxic syncytiotrophoblasts.

Nutlin-3 increases p53 levels in cells by inhibiting the interaction of p53 and MDM2 to limit p53 degradation (43). We tested the effect of nutlin-3 on the p53 response to different P02 in cultured syncytiotrophoblasts. Syncytiotrophoblasts cultured in 20% oxygen showed the expected increase in p53 levels after both 4-h and 24-h exposure to nutlin-3 (Fig. 5B), and the increase in p53 correlated with enhanced transcriptional activity, as measured by the gene expression for p21 and mdm2, two targets of p53 transcriptional activity. Moreover, nutlin-3 induced a higher level of apoptosis in syncytiotrophoblasts under 20% oxygen than under <1% oxygen (Fig. 5C). These results indicate that overexpression of p53 can induce cell death in syncytiotrophoblasts.

Pifithrin-μ can inhibit the binding of cytoplasmic p53 to the antiapoptotic proteins BclXL and Bcl-2 in mitochondria (42), and pifithrin-α is reported to be a relatively selective inhibitor of p53-mediated transcription in the nucleus. Neither pifithrin-μ nor pifithrin-α altered p53 expression in syncytiotrophoblasts, with levels undetectable and similar to

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**Fig. 2. RT-quantitative (q)PCR analysis of p53-regulated genes in syncytiotrophoblasts cultured in oxygen concentration of 20% or <1% (n = 6). A: p53. B: mdm2. C: ndrg1. *P < 0.01. D: puma. E: noxa. *P < 0.05.**

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*puma* and *noxa* are BH3-only members of the Bcl-2 family that facilitate apoptosis and that are transcriptional targets of p53 (17, 30, 44, 47). We postulated that the enhanced apoptosis in syncytiotrophoblasts exposed to hypoxia resulted from downstream transcriptional effects of p53 on these proapoptotic genes. Interestingly, mRNA levels for noxa were significantly (P < 0.05) downregulated under conditions of <1% oxygen, compared with 20% oxygen, while levels of puma were not different in the two oxygen concentrations (Figs. 2, D and E). These data indicate that mRNA expression for p53 and mdm2 is not affected by hypoxia, that ndrg1 is increased by hypoxia independent of villous trophoblast phenotype, and that p53-regulated gene expression for the BH3 proapoptotic mediators noxa and puma could not account for the proapoptotic effect of low oxygen levels on syncytiotrophoblasts.

*p53, MDM2, and MDMX expression changes in hypoxic syncytiotrophoblasts.* As noted in the introduction, p53 activity is regulated by MDM2 and MDMX. Thus we next examined expression of all three proteins as a surrogate measure for p53 activity in syncytiotrophoblasts exposed to <1% and 20% oxygen for up to 24 h. We found that p53 and MDM2 levels were rapidly diminished by 2 h of exposure to <1% oxygen, and they remained low for 24 h (Fig. 3A, P < 0.05). This rapid, marked downregulation of p53 and MDM2 proteins in syncytiotrophoblasts exposed to <1% oxygen was distinctly different from the robust upregulation of p53 we reported for cultured cytotrophoblasts exposed to <1% oxygen (25) and confirmed here with the same primary cultures allowed to form the two villous phenotypes (Fig. 3B, P < 0.05).
Fig. 3. p53, MDM2, and MDMX expression in syncytiotrophoblasts cultured in 20% and <1% oxygen. Time zero was 52 h from the initiation of primary culture. In this and subsequent figures, all densitometry values are normalized to actin. A, left: representative Western blot of p53 and MDM2 expression in syncytiotrophoblasts exposed to 20% or <1% oxygen for up to 24 h. Center and right: summary graphs of densitometry of p53 and MDM2 (n = 4). B: p53 and MDM2 expression in syncytiotrophoblasts compared with cytotrophoblasts. Top left: representative Western blots of p53 and MDM2 in syncytiotrophoblasts. Top center and right: summary graphs of densitometry of Western blots (n = 3). Bottom left: representative Western blots of p53 and MDM2 in cytotrophoblasts. Bottom center and right: summary graphs of densitometry of Western blots (n = 3). C: MDMX expression in syncytiotrophoblasts cultured up to 24 h in 20% or <1% oxygen. Left: representative Western blot of MDMX. Right: summary graphs of densitometry of Western blots (n = 4). *P < 0.05.

Fig. 4. Effect of staurosporine on the expression of p53, MDM2, and cyt 18 in syncytiotrophoblasts. Expression of p53, MDM2, and cyt 18 in syncytiotrophoblasts exposed to 0.3 μM staurosporine for up to 24 h is shown. Left: representative Western blots of p53, MDM2, and cyt 18. Right: summary graphs of densitometry of Western blots (n = 4). *P < 0.05. Ctrl, control; Stau, staurosporine.

p53 immunolocalizes to both cytoplasm and nucleus of syncytiotrophoblasts. We previously found (14) that in cultures of syncytiotrophoblasts p53 coimmunoprecipitated with Bak, a proapoptotic member of the Bcl-2 family of proteins that is...
localized in mitochondria. This result, combined with the above results with nutlin-3 and the two pifithrins, raised the possibility that p53 functions in both the cytoplasm and the nucleus of syncytiotrophoblasts. To test this hypothesis, we used nutlin-3 to enhance expression of p53 in syncytiotrophoblasts and confocal immunofluorescence microscopy to visualize the subcellular immunolocalization of p53 reactivity. Compared with the absence of staining in nutlin-3-treated control cultures with no primary antibody, we detected specific, but low-level, fluorescence for p53 in the nuclei and cytoplasm of syncytiotrophoblasts and residual cytotrophoblasts in vehicle control cultures (Fig. 6). Moreover, syncytiotrophoblasts exposed to nutlin-3 expressed intense specific immunofluorescence staining for p53 in most nuclei as well as specific p53 immunofluorescence in punctate structures within the cytoplasm. These data indicate that when p53 is overexpressed a higher level of apoptosis results, and p53 appears in both the nuclei and the cytoplasm of syncytiotrophoblasts, suggesting a role for p53 in effecting apoptotic death in both subcellular compartments.

Expression of BAD correlates with apoptosis in hypoxic syncytiotrophoblasts. As hypoxia reduced levels of p53 in syncytiotrophoblasts yet increased apoptosis, we surmised that the apoptosis involved a non-p53-dependent mechanism. BAD is a proapoptotic member of the Bcl-2 family that is sequestered in the cytoplasm of cytotrophoblasts by 14-3-3 proteins when phosphorylated at Ser112 (15). Increased levels of BAD, or reduced phosphorylation of BAD-Ser112, activate the mitochondrial pathway of apoptosis (48). We tested the hypothesis that increased expression of BAD, decreased phosphorylation of BAD-Ser112, or both was associated with enhanced apoptosis of hypoxic syncytiotrophoblasts. Indeed, we found a marked increase in the expression of BAD and a decrease in the relative level of phosphorylated BAD-Ser112 in syncytiotrophoblasts exposed to hypoxia compared with standard conditions (Fig. 7, \(P < 0.05\)). These results suggest that BAD contributes to hypoxia-induced apoptosis in syncytiotrophoblasts, unlike the undetectable role for BAD we previously observed in cytotrophoblasts (15).

**DISCUSSION**

The data show that hypoxia increases expression of HIF-1α, induces apoptosis, but reduces p53 expression and activity in cultures of human syncytiotrophoblasts compared with standard culture conditions of 20% oxygen. This contrasts with the
rapid induction of p53 in hypoxic cytotrophoblasts (3, 25) to indicate that there is a phenotype-dependent response of p53 in villous trophoblasts exposed to hypoxia. p53 activity is reduced by enhanced MDMX expression as a negative regulator for p53 and by the limited phosphorylation of p53 at Ser392, a site important for p53 transcriptional activity. Conversely, staurosporine as a nonhypoxic stimulus and nutlin as a drug that causes overexpression of p53 each enhance p53 activity to induce apoptosis in syncytiotrophoblasts. p53 immunolocalizes to both the cytoplasm and the nucleus under the influence of nutlin-3, implicating transcriptional and nontranscriptional functions for p53 in syncytiotrophoblasts. The data indicate that hypoxia-induced apoptosis is not primarily the result of p53 transcriptional activity but instead correlates with the proapoptotic expression of BAD and the proapoptotic isoform of BAD with limited phosphorylation at Ser112. We speculate that downregulation of p53 activity in response to hypoxia reduces or eliminates the apoptosis transduced by the p53 pathway in syncytiotrophoblasts and thereby limits injury and maintains the integrity of this critical villous component.

Hypoxia in the fetal circulation of pregnancies with severe IUGR in the second and third trimesters of pregnancy may be severe, with Po2 levels of 10–20 mmHg (39). This contrasts with the normal Po2 of average-growth babies, with a Po2 in the 40–80 mmHg range (32). The low Po2 in IUGR are likely to reveal the extremes of responses of the villous trophoblast to hypoxia.

We chose to study the effects of standard culture conditions of 20% oxygen and 1% oxygen with theoretical Po2 values of 150 mmHg and <15 mmHg, respectively. Notably, because of cellular oxygen consumption and low diffusion of oxygen through the medium, the measured pericellular Po2 is between 78 and 110 mmHg in a variety of cell types when cultured in a 20% oxygen environment (29, 33).

**Fig. 6.** Immunofluorescence of p53 in nutlin-3-exposed syncytiotrophoblasts. Top: no primary antibody (Ab) control in nutlin-3-exposed syncytiotrophoblasts. Middle: p53 in vehicle (DMSO)-exposed syncytiotrophoblasts with both primary and secondary antibodies to detect p53. Bottom: p53 in nutlin-3-exposed syncytiotrophoblasts with both primary and secondary antibodies to detect p53. Immunoreactive p53 is stained red. Surface membranes are marked by E-cadherin staining (green), and DNA in nuclei is stained with Draq 5 (blue).

**Fig. 7.** Expression of BAD and BAD phosphorylated at Ser112 in syncytiotrophoblasts cultured in 20% or 1% oxygen for 24 h. A: representative Western blot of BAD and summary graph of densitometry of Western blots (n = 3). *P < 0.05. B: representative Western blot of BAD phosphorylated at Ser112 and summary graph of densitometry of Western blots (n = 3). *P < 0.05.
p53 is expressed in both cytotrophoblast nuclei and cytoplasm of the invasive trophoblasts of the first-trimester placenta (6) and in nuclei of the villous cytotrophoblasts at term (11). Syncytiotrophoblast in normal term villi prominently expresses MDM2 but only a low level of p53 (11). Importantly, apoptosis is higher and p53 is upregulated in villi from placentas with IUGR (26). Notably, syncytiotrophoblasts in culture are more resistant to apoptotic stimuli compared with primary cytotrophoblasts (8, 19). Our data on reduced p53 expression in hypoxic syncytiotrophoblasts offer one pathway for this greater resistance to cell death. What remains unknown is whether the downregulation of p53 activity affects trophoblast resistance directly by limiting p53 transduction of apoptosis or indirectly by p53 effects on metabolism. For example, inhibition of p53 limits oxidative phosphorylation, and this p53 response may indirectly guide metabolism toward glycolysis, which may protect the trophoblast layer in the presence of low Po2 (45).

We postulated that the enhanced apoptosis in syncytiotrophoblasts exposed to hypoxia was the result of transcriptional effects by p53 to enhance expression of proapoptotic genes, such as puma and noxa. However, as the mRNA levels of both these genes were unchanged or slightly reduced, respectively, increased transcription of these genes could not account for the increased apoptosis in hypoxic syncytiotrophoblasts. Real-time PCR indicated that p53 and mdm2 mRNA levels were unchanged by hypoxia, a finding that is not surprising since regulated protein degradation is most crucial in determining the levels of these proteins in cells. As we found in hypoxic cytotrophoblasts (3), Ndrg1 mRNA was increased in hypoxic syncytiotrophoblasts, likely reflecting a protective function for NDRG, as we proposed previously (3). We also found that HIF-1α was upregulated in the syncytiotrophoblasts under hypoxic conditions, as expected. HIF-1α expression inversely correlates with p53 status (37), possibly due to competition for p300 (34).

Posttranslational modifications alter the stability of p53, the MDM2-p53 interaction, or both, resulting in altered p53 activity. MDMX is a key negative regulator of p53 (28). The increased expression of MDMX in hypoxic syncytiotrophoblasts limits p53 activity, compared with syncytiotrophoblasts exposed to 20% oxygen. Phosphorylation of p53 at Ser392 decreases the transcriptional activity of p53, and this phosphorylation of p53 at Ser392 increases the transcriptional activity of p53, and this phosphorylation is reduced in hypoxic syncytiotrophoblasts compared with cultures in 20% oxygen. Collectively, the data show that a combination of reduced p53 expression and activity limits the effects of p53 in apoptosis in hypoxic syncytiotrophoblasts.

Our data also show that the p53 pathway can be activated in syncytiotrophoblasts. Nutilin-3 is a nongenotoxic agent that elevates p53 expression by direct inhibition of p53 interaction with MDM2. Nutilin-3 enhances p53 expression in trophoblasts, and this overexpression allowed us to localize p53 to nuclei and to punctate cytoplasmic structures, consistent with mitochondria. The reagents pifithrin-α and pifithrin-μ are proposed to be antiapoptotic agents that selectively inhibit p53 activity. Our data indicate that they are not selective p53 inhibitors in syncytiotrophoblasts, and other cultured cells have varied responses to these agents (41, 42, 50). This may result from inadequate p53 inhibition or from the non-p53 actions recently reported in cultures treated with pifithrins (22, 40). We conclude that pifithrins are not adequate inhibitors for p53 in cultured trophoblasts when used at nontoxic levels.

Although Bcl-2 family members are important regulators of apoptosis, the function of the Bcl-2 family in placental physiological and pathological conditions is still poorly understood. Bax, Bak, and Mcl-1S levels are increased in preeclampsia placental tissues or placenta explants in hypoxia-reoxygenation conditions, while Bcl-2 levels are unchanged (38).

Importantly, we here identify that the proapoptotic protein BAD is increased in hypoxic syncytiotrophoblasts. This contrasts with the lack of change in expression of BAD in hypoxic cytotrophoblasts (15), again underscoring the phenotype-dependent response of villous trophoblasts to hypoxia. Enhanced apoptosis in hypoxic cytotrophoblasts correlates with reduced phosphorylation of BAD-Ser112. Interestingly, the prosurvival effects of epidermal growth factor are mediated in part by enhanced phosphorylation of BAD-Ser112 in hypoxic cytotrophoblasts (15). The minimal phosphorylation of the BAD Ser112 site in hypoxic syncytiotrophoblasts further enhances the death-inducing effects of overexpression of this BH3-only Bcl-2 family member.

In conclusion, we have shown that enhanced MDMX expression level and the reduced phosphorylation of the Ser392 residue on p53 combine to limit p53 activity in hypoxic syncytiotrophoblasts, despite enhanced apoptosis. We correlate enhanced expression of BAD as a central proapoptotic mediator in the higher than control cell death in syncytiotrophoblasts exposed to low oxygen. We speculate that rapid down-regulation of p53 activity in syncytiotrophoblasts is one survival response to protect the integrity of the villous syncytiotrophoblast layer exposed to low Po2 during pregnancy.

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DISCLOSURES

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REFERENCES

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