Punicalagin, a polyphenol in pomegranate juice, downregulates p53 and attenuates hypoxia-induced apoptosis in cultured human placental syncytiotrophoblasts

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Chen B, Longtime MS, Nelson DM. Punicalagin, a polyphenol in pomegranate juice, downregulates p53 and attenuates hypoxia-induced apoptosis in cultured human placental syncytiotrophoblasts. Am J Physiol Endocrinol Metab 305: E1274–E1280, 2013. First published October 1, 2013; doi:10.1152/ajpendo.00218.2013.—Oxidative stress is associated with placental dysfunction and suboptimal pregnancy outcomes. Therapeutic interventions to limit placental injury from oxidative stress are lacking. Punicalagin is an ellagitannin and a potent antioxidant in pomegranate juice. We showed that both pomegranate juice and punicalagin decrease oxidative stress and apoptosis in cultured syncytiotrophoblasts. p53 is involved in the oxidative stress-induced apoptosis in trophoblasts. We now test the hypothesis that punicalagin limits trophoblast injury in vitro by regulating the levels of p53. We examined the expression of p53, mouse double minute 2 homolog, p21, hypoxia-inducible factor (HIF) α, and selected members of the B cell lymphoma 2 (BCL2) family of proteins in cultured syncytiotrophoblasts exposed to ≤1% oxygen in the absence or presence of punicalagin. We found that punicalagin attenuated hypoxia-induced apoptosis in syncytiotrophoblasts, as quantified by levels of cleaved poly-ADP ribose polymerase. This protective effect was in part mediated by reduced p53 activity shown by decreased expression of p21, lower HIF1α expression, and limited activity of caspases 9 and 3. There was no change in expression of proteins in the BCL2 family, which are also important in apoptosis. The data support a role for downregulation of p53 in the protection of human trophoblasts by punicalagin. Punicalagin, the fruit of Punica granatum, contains compounds that have not only antioxidant activity but also can affect expression of multiple genes (19, 23). Pomegranate juice contains high levels of polyphenols, including ellagitannins, gallotannins, and flavonoids. This juice improves cardiovascular function in animals and benefits endothelial cell function in vitro (6, 15) while also protecting neuronal cells from oxidative stress-induced injury in vivo (26). We recently showed that pomegranate juice reduces oxidative stress in human villous trophoblasts in vivo and in vitro, and pomegranate juice limits stimulus-induced cell death of human trophoblasts in explants and in primary cultures (12). As importantly, we revealed that punicalagin, a prominent ellagitannin in pomegranate juice, exerted protective effects similar to pomegranate juice for cell death and oxidative stress in primary human trophoblasts (12). The mechanisms by which pomegranate juice and punicalagin regulate the response of human trophoblasts to oxidative stress remain unclear. We have previously shown that p53 is involved in the oxidative stress-induced apoptosis in trophoblast (10). We herein test the hypothesis that punicalagin reduces apoptosis by modulation of the p53 pathway in primary cultures differentiated into syncytiotrophoblasts and exposed to hypoxia.

MATERIALS AND METHODS

Isolation and culture of primary human trophoblasts. This study was approved by the Institutional Review Board of Washington University School of Medicine in St. Louis, MO. Primary human trophoblasts (PHTs) were isolated from placentas of 39 wk gestations after uncomplicated pregnancies and delivered by scheduled repeat cesarean section without labor under conduction anesthesia. The trophoblasts isolated from the Percoll band (11) at 1.050–1.060 were cultured for 4 h in DMEM (Life Technologies, Grand Island, NY) with 10% FBS and penicillin/streptomycin at 37°C in a 5% carbon dioxide-air atmosphere with 20% oxygen, to allow cell attachment. Some syncytial fragments are known to coalesce with cytotrophoblasts, and these fragments were eliminated by the approach reported by Guilbert and colleagues (16). The cultures at 4 h were washed three times with PBS, which eliminated visible villous fragments, since they have a lower affinity for adhesion to the culture plate compared with cytotrophoblasts (16). The cells were cultured an additional 24 h in the same medium and were then transferred to phenol red-free DMEM (Life Technologies), with 10% charcoal-stripped FBS (Sigma, St. Louis, MO), in the presence of glucose (7.5 mM; Sigma) with or without pomegranate juice (1% vol/vol; POM Wonderful), or punicalagin (16.9–67.6 μM; Chengdu Bio purity Phytochemicals) at 37°C in 20% oxygen for another 24 h. The cells were then cultured with glucose, pomegranate juice, or punicalagin, with exposure to <1% oxygen with 5% CO2, 10% H2, and 84% N2 for 24 h, with DMSO or p53 inhibitor Nutlin-3 (10 μM; Sigma). The <1% oxygen environment was supplied in an anaerobic glove box incubator.
(Thermo Electron, Marietta, OH) that allowed preaggregating of medium and handling of cultures without exposure to ambient oxygen.

Western blotting. PHTs were lysed in radioimmunoprecipitation buffer (1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS in PBS) containing a protease inhibitor cocktail and phosphatase inhibitors (Sigma). Twenty micrograms of proteins were separated in SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA) overnight at 150 mA at 4°C. The membrane was blocked in 5% nonfat dry milk in PBST (PBS with 0.05% Tween 20) for 1 h and then incubated overnight at 4°C or 4 h at room temperature with one of the following primary antibodies: rabbit monoclonal anti-cleaved poly-ADP ribose polymerase antibody (cl-PARP; Cell Signaling Technology, Danvers, MA), rabbit monoclonal anti-cleaved caspase 9 (Cell Signaling Technology), rabbit monoclonal anti-cleaved caspase 3 (Cell Signaling Technology), mouse monoclonal anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-mouse double minute 2 homolog (MDM2; Santa Cruz Biotechnology), rabbit polyclonal anti-hypoxia-inducible factor (HIF) 1α (Novus Biologicals, Littleton, CO), rabbit monoclonal anti-p21 (Cell Signaling Technology), rabbit polyclonal anti-MDMX (Abcam, Cambridge, England), rabbit monoclonal anti-BCLXL (BCL2; Oncogene, Cambridge, MA), goat polyclonal anti-BCLXs (Santa Cruz Biotechnology), mouse monoclonal anti-BAX (Santa Cruz Biotechnology), mouse monoclonal anti-BAD (Cell Signaling Technology), or goat polyclonal anti-actin (Santa Cruz Biotechnology), rabbit monoclonal anti-BCL2 (Cell Signaling Technology), rabbit monoclonal anti-actin (Santa Cruz Biotechnology) in 5% nonfat dry milk in PBST. The blot was then incubated at room temperature for 2 h and horseradish peroxidase-conjugated donkey anti-mouse, donkey anti-rabbit or donkey anti-goat IgG secondary antibodies (Santa Cruz Biotechnology), as appropriate. The blots were washed and processed for luminescence with SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific, Rockford, IL). Densitometry of bands on films was assessed with Epipacth-3 software (UVP BioImaging System, Upland, CA) and normalized to actin levels.

Gene expression analysis using quantitative RT-PCR. RNA was purified from PHTs by using TriReagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. The purified RNA was treated with deoxyribonuclease I (DNA-free; Ambion, Austin, TX). and reverse transcription was performed as described previously (11). Two milliliters of the product were used for qRT-PCR along with 300 nM of the appropriate forward and reverse primers and SYBRgreen PCR master mix (Applied Biosystems, Carlsbad, CA) in a total reaction volume of 15 μl. The primer pairs used are listed: p53: forward 5’-CCTATGGAACAATCTTCT-CCTGAAACA-3’ and reverse 5’-ACAGCATCAATATCCCAT-TGC-3’; MDM2: forward 5’-CCCTTGTTAGACCACCGGACAT-3’ and reverse 5’-GGCAGGCGGAAACAAATCTCC-3’; HIF1α: forward 5’-GGCGCGAACAGCAAGAAAGAG-3’ and reverse 5’-CCCTAT-CAGAGTGCGAAACTCACA-3’; p21: forward 5’-CTGTTCACTGG-TCTTGACCTCC-3’ and reverse 5’-GCTTGGTGGTGTGGTAA-GATA-ATCT-3’; and 18S: forward 5’-CAG GCC CGG TAC AGT GAA A-3’ and reverse 5’-AGA GGA GCC AGC GAC CAA-3’.

Reactions were run in duplicate and analyzed using a Bio-Rad CFX96 Real-Time System. The PCR products of all primer pairs were run on agarose gels to verify a single band of the predicted size. All of the amplicons were sequenced to verify their authenticity. The amplification efficiency was determined using 10-fold dilutions of a trophoblast cDNA template on the same real-time PCR machine. The amplification efficiencies for p53, MDM2, HIF1α, p21, and 18S were 110, 103, 87, 95, and 97%, respectively. Amplifico melt curves were run on all reactions to ensure amplification of a single product with the appropriate melting temperature. Samples were normalized to parallel 18S reactions, and the fold increase relative to control was determined by the $2^{-ΔΔCt}$ method.

Immunofluorescence staining. Trophoblasts were fixed in −20°C methanol for 10 min, blocked in PBS/5% BSA for 1 h at room temperature, and then incubated for 2 h at room temperature with mouse anti-p21 antibody (Cell Signaling Technology). Control staining with preimmune mouse serum yielded no signal. After being washed with PBS, cells were incubated for 2 h at room temperature with Alexa-Fluor 546-donkey anti-mouse secondary antibody (Invitrogen) followed by 10 min incubation at room temperature with 0.1 μg/ml Hoechst (Pierce). Images were obtained at a final magnification of ×600 using a Nikon E800 epifluorescence microscope.

Statistical analysis. All experiments were repeated at least three times with PHTs from at least three different placentas. Statistical analysis for Figs. 1–4 is reported in the legends and included t-test, one-way ANOVA, or two-way ANOVA, with post hoc Bonferroni correction for the latter. Significance was deemed as $P < 0.05$.

RESULTS

Using an in vitro PHT system, illustrated in Fig. 1A, we first confirmed that pretreatment with punicalagin reduced hypoxia-induced apoptosis (Fig. 1B), as measured by the levels of cl-PARP as a nuclear marker of apoptosis. This protective effect was present even when punicalagin was added at the time of transfer to hypoxia, although to a lesser extent than with pretreatment (Fig. 1B). We thus chose to use 24 h of punicalagin pretreatment before exposure of trophoblasts to insults in our subsequent experiments.

We next examined the effect on hypoxia-induced apoptosis in syncytiotrophoblasts for a gradation of punicalagin concentrations selected because they bracket a concentration range of this polyphenol that can be present in vivo in humans (33, 34). We found an inverse relationship between the concentration of punicalagin and the level of apoptosis (Fig. 1C). We chose to use the middle concentration of punicalagin, 33.8 μM, for our subsequent experiments because this is equivalent to that present in culture medium with a 1:100 dilution of pomegranate juice, as we used previously (12).

The ability of punicalagin to reduce hypoxia-induced apoptosis in syncytiotrophoblasts suggested that punicalagin reduced caspase activation. Indeed, we found that punicalagin, and pomegranate juice, reduced the expression of active cleaved caspase-9 and of active cleaved caspase-3 in hypoxic syncytiotrophoblasts (Fig. 2A). We have previously shown that p53 is a major regulator of stress-induced apoptosis in trophoblasts (10). Thus, we hypothesized that the mechanism by which punicalagin reduces apoptosis in syncytiotrophoblasts involves a change in p53 activity. We thus examined p53 expression in syncytiotrophoblasts exposed to punicalagin compared with pomegranate juice in hypoxia for up to 24 h (Fig. 2B). We found both punicalagin and pomegranate juice significantly decreased p53 protein levels, and levels of cl-PARP, at up to 24 h exposure to hypoxia (Fig. 2, B and C). Notably, we also found that punicalagin significantly reduced p53 mRNA expression in syncytiotrophoblasts cultured in hypoxia for 24 h (Fig. 2D).

Next, we determined if the reduction of p53 levels by punicalagin in hypoxic syncytiotrophoblasts affects p53 activity by assaying expression of p21 and MDM2, two targets of p53 activity. Punicalagin reduced expression of p21 protein (Fig. 3A, left and middle) and mRNA (Fig. 3A, right). Similarly, punicalagin reduced the expression of MDM2 protein (Fig. 3B, left and middle) and mRNA (Fig. 3B, right). HIF1α is a transcription factor induced by hypoxia that can interact with MDM2, resulting in altered p53 activity and apoptosis (13, 20, 28, 35, 39). Notably, we found that punicalagin...
significantly reduced expression of HIF1α protein and mRNA in hypoxic syncytiotrophoblasts (Fig. 3C).

Another key set of proteins regulating apoptosis in human trophoblasts is the members of the BCL2 family (10, 17, 18). To determine if multiple apoptotic pathways are affected by punicalagin, we examined its effect on BCL2 family members. We found that punicalagin did not affect expression of BCL2, BCL2XL, BAK, BAX, or BAD proteins in hypoxic syncytiotrophoblasts (Fig. 3D), suggesting changes in expression of these BCL2 family proteins are not involved in the protective effects of punicalagin, highlighting the relative specificity of the effect on the p53 pathway.

To study if punicalagin had a posttranscriptional effect on the expression of p53 in syncytiotrophoblasts under hypoxia,
we next assayed cells treated with punicalagin in the presence or absence of Nutlin-3 (Fig. 4A), a specific inhibitor of the p53 and MDM2 interaction. As expected, Nutlin-3 significantly increased p53 levels and trophoblast apoptosis, as measured by cl-PARP (Fig. 4, B and C), consistent with our previous results (10). Punicalagin significantly decreased the Nutlin-3-induced elevation of p53 levels (Fig. 4, B and C) and, concomitantly, decreased the Nutlin-3 induction of apoptosis (Fig. 4, B and C).

As expected, the Nutlin-3 induction of p53 increased expression of p21 (Fig. 4, B–D), a downstream target of p53, and punicalagin reduced this Nutlin-3-mediated increase of p21 levels (Fig. 4, B–D). Expression of MDM2 (another downstream target of p53) was modestly increased by Nutlin-3, and this increase was prevented by punicalagin (Fig. 4, B and C). Finally, expression of MDMX, another important regulator of p53, was not affected by punicalagin or Nutlin-3 treatment (Fig. 4, B and C).

**DISCUSSION**

The data show that punicalagin limits hypoxia-induced apoptosis in cultures of syncytiotrophoblasts as measured by cl-PARP, active caspase 9, and active caspase 3. This protective effect associates with decreased expression of p53 and HIF1α. Moreover, punicalagin decreased p21 and MDM2 levels, reflecting the decreased p53 activity. Importantly, punicalagin uniquely decreased Nutlin-3-induced expression of p53 and the downstream targets for p53, including p21 and MDM2, but not MDMX. Notably, the effect on the p53 pathway is relatively selective, since punicalagin had no effect on expression of the members of the BCL2 family or MDMX. We conclude that punicalagin attenuates hypoxia-induced trophoblast death through modulation of p53 activity.

Polyphenols in pomegranate juice exert anti-tumor effects through inhibition of the NF-κB pathway, effects on cellular proliferation and HIF1α-mediated angiogenesis, and by induction of apoptosis (1–4). Punicalagin protects human trophoblasts from hypoxia-induced cell death, which is opposite to the induction of apoptosis observed in cancer cells. This finding underscores the cell type-specific nature of the responses to punicalagin. Moreover, punicalagin reduces HIF1α mRNA and protein expression in trophoblasts. Importantly, our findings suggest that punicalagin has biological activities greater than simply being an antioxidant, since it modulates the steady-state mRNA levels of p53, p21, and MDM2 in human trophoblasts. Whether these responses involve alterations in transcription rates, RNA stability, or both remains a topic for further investigation.
Fig. 4. The effect of punicalagin on the expression of cl-PARP, p53, p21, MDM2, and MDMX in syncytiotrophoblasts exposed to Nutlin-3 (10 μM) compared with DMSO under hypoxia. A: diagram of experimental approach. B: Western blots of cl-PARP, p53, p21, MDM2, and MDMX. C: summary graphs of densitometry of Western blots of cl-PARP, p53, p21, MDM2, and MDMX. In A–C, n = 6. *P < 0.05, two-way ANOVA. D: immunofluorescence staining of p21 in syncytiotrophoblasts under hypoxia, exposed to glucose control or punicalagin in the presence or absence of Nutlin-3 or DMSO solvent.
The p53 pathway is regulated by multiple mechanisms, including changes in protein stability mediated by MDM2 and MDMX, changes in transcription, and a multitude of regulated phosphorylations (21, 22, 25). MDM2 is a ubiquitin ligase that modifies p53 so that the protein is degraded by the proteasome. MDM2 expression levels are transcriptionally regulated by p53, similar to transcriptional regulation of p21. MDMX, also called MDM4, is another major negative regulator of p53, but, unlike MDM2, MDMX expression is not regulated by p53. MDMX interacts with p53 to inhibit p53 transactivation activity, instead of contributing to p53 degradation induced by MDM2. Recent evidence suggests that MDM2 and MDMX form a heterodimeric protein complex with p53 on the promoters of specific p53 target genes to execute distinct functions (21). Our previous data indicate that hypoxia enhances MDMX expression to inhibit p53 activity (10). Notably, we found that punicalagin did not alter protein levels of MDMX in hypoxic syncytiotrophoblasts, likely because hypoxia maximizes the expression of MDMX. We postulate that the ratio of MDM2/MDMX heterodimers is at least partly responsible for the changes in p53 activity in the syncytiotrophoblasts exposed to punicalagin, although further evidence is needed to verify this. Collectively, our data indicate that punicalagin works by at least two mechanisms to yield decreased p53 activity in hypoxic syncytiotrophoblasts. First, punicalagin yields decreased expression of p53 mRNA, which partially determines p53 levels. Second, because punicalagin specifically decreases Nutlin-3-induced p53 levels, it is likely punicalagin also has posttranscriptional effects on p53 levels in syncytiotrophoblasts.

The BCL2 family of proteins is a critical regulator of cell death. For example, interaction of p53 with Bak causes oligomerization of Bak and release of cytochrome c from mitochondria (24). Surprisingly, none of the BCL2 family members we examined showed changes in expression in hypoxic syncytiotrophoblasts exposed to punicalagin. These findings suggest that punicalagin inhibition of apoptosis in syncytiotrophoblasts is relatively specific for the p53 and not just an overall antiapoptotic cell response to punicalagin.

Collectively, our results show punicalagin reduces trophoblast death by reducing p53 transcription, protein expression, and activity. Pomegranate juice in general, and the polyphenol punicalagin in particular, is worthy of further study as a therapeutic approach to limit trophoblast injury resulting from oxidative stress.

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GRANTS

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DISCLOSURES

All the coauthors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Author contributions: B.C. and D.M.N. conception and design of research; B.C. performed experiments; B.C. analyzed data; B.C., M.S.L., and D.M.N. edited and revised manuscript; B.C., M.S.L., and D.M.N. approved final version of manuscript.

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