OBSTETRICS

Curcumin ameliorates high glucose-induced neural tube defects by suppressing cellular stress and apoptosis

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OBJECTIVE: Curcumin is a naturally occurring polyphenol present in the roots of the *Curcuma longa* plant (turmeric), which possesses antioxidant, antitumorigenic, and antiinflammatory properties. Here, we test whether curcumin treatment reduces high glucose-induced neural tube defects (NTDs), and if this occurs via blocking cellular stress and caspase activation.

STUDY DESIGN: Embryonic day 8.5 mouse embryos were collected for use in whole-embryo culture under normal (100 mg/dL) or high (300 mg/dL) glucose conditions, with or without curcumin treatment. After 24 hours in culture, protein levels of oxidative stress makers, nitrosative stress makers, endoplasmic reticulum (ER) stress makers, cleaved caspase 3 and 8, and the level of lipid peroxides were determined in the embryos. After 36 hours in culture, embryos were examined for evidence of NTD formation.

RESULTS: Although 10 μmol/L of curcumin did not significantly reduce the rate of NTDs caused by high glucose, 20 μmol/L of curcumin significantly ameliorated high glucose-induced NTD formation. Curcumin suppressed oxidative stress in embryos cultured under high glucose conditions. Treatment reduced the levels of the lipid peroxidation marker, 4-hydroxynonenal, nitrotyrosine-modified protein, and lipid peroxides. Curcumin also blocked ER stress by inhibiting phosphorylated protein kinase RNA-like ER kinase, phosphorylated inositol-requiring protein-1α (p-IRE1α), phosphorylated eukaryotic initiation factor 2α (p-eIF2α), C/EBP-homologous protein, binding immunoglobulin protein, and x-box binding protein 1 messenger RNA splicing. Additionally, curcumin abolished caspase 3 and caspase 8 cleavage in embryos cultured under high glucose conditions.

CONCLUSION: Curcumin reduces high glucose-induced NTD formation by blocking cellular stress and caspase activation, suggesting that curcumin supplements could reduce the negative effects of diabetes on the embryo. Further investigation will be needed to determine if the experimental findings can translate into clinical settings.

Key words: caspase activation, curcumin, endoplasmic reticulum stress, high glucose, neural tube defects, nitrosative stress, oxidative stress

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Maternal diabetes increases the risk of congenital birth defects, including neural tube defects (NTDs).[1,2] Glycemic control by insulin treatment reduces the incidence of birth defects in both human beings and animal models.[3] However, glycemic control is difficult to achieve and maintain, and even transient exposure to high glucose can result in embryonic anomalies.[4] Offspring from diabetic women under modern preconception care still have a 2- to 5-fold higher incidence of birth defects, compared with offspring of mothers without diabetes.[5] Therefore, there is a great need for new therapeutics that inhibit the mechanisms underlying diabetic embryopathy.

Animal studies have shown that antioxidants, such as multivitamins, the tea polyphenol epigallocatechin gallate (EGCG), and the naturally occurring disaccharide trehalose, effectively ameliorate maternal diabetes-induced NTD formation.[6-8] However, human clinical trials have not shown similar results.[9,10] The beneficial effect of multivitamins in preventing birth defects in diabetic human pregnancies has not been clearly established.[8] EGCG use in patients with type 2 diabetes does not significantly affect the degree of hyperglycemia, insulin resistance, and other altered metabolic indices associated with type 2 diabetes.[11] A clinical trial on the effect of trehalose, an autophagy-inducing sugar, on cardiovascular diseases is ongoing. Therefore, it is unclear whether health benefits can actually be achieved by human consumption of trehalose.

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Because it is also uncertain if EGCG or trehalose can prevent diabetes-associated diseases, we need to identify new therapeutics that may work in human beings.

Curcumin is a phenolic compound present in the rhizomes of the turmeric spice plant that is used in traditional Indian medicine to treat a variety of diseases and conditions, including those of the skin, pulmonary, and gastrointestinal systems.[12] Curcumin is a potent antioxidant that has been shown to suppress diabetes-induced superoxide in vascular endothelial cells.[13] In addition to its antioxidant properties, curcumin appears to be able to modulate signal transduction and gene expression.[14] A previous study has demonstrated that curcumin blocks diabetes-induced inducible nitric oxide synthase (iNOS) expression in the adult heart.[15] Another study showed that curcumin improves diabetes-induced endothelial dysfunction by inhibiting protein kinase C activation.[13] Additionally, others have observed that curcumin abrogates endoplasmic reticulum (ER) stress, caspase activation, and apoptosis induced by either high glucose or hypoxia in noncancerous cells.[16] The antioxidant, anticellular organelle stress, signaling transduction, and gene expression-modulating effects of curcumin make it an ideal candidate therapeutic to prevent diabetic embryopathy.

We, and others, have demonstrated that oxidative stress is a central causal event in diabetic embryopathy.[3,17-22] Oxidative stress-induced kinase signaling triggers ER stress in the developing embryo, leading to NTD formation.[19] iNOS expression and its associated nitrosative stress are induced by maternal diabetes, and deletion of the *iNos* gene alleviates NTD formation in diabetic pregnancies.[23] Maternal diabetes-induced specific protein kinase C isoform activation is a key component of the causal events in NTD formation.[18,24] It is possible that curcumin can target all critical events that lead to diabetic embryopathy. Thus, we propose that curcumin ameliorates high glucose-induced NTD formation by suppressing oxidative stress and ER stress.

In the present study, we assessed the effect of curcumin on NTD formation in murine embryo culture under high glucose conditions, and revealed its impact on high glucose-induced cellular stress and apoptosis in the developing embryo.

Materials and Methods

Animals and whole-embryo culture

Wild-type C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). The procedures for animal use were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee. The procedure of whole-embryo culture has been previously described.[6,25] C57BL/6J mice were paired overnight. The next morning was designated embryonic day (E)0.5 if a vaginal plug was present. Mouse embryos at E8.5 were dissected out of the uteri in phosphate-buffered saline (Invitrogen, La Jolla, CA). The parietal yolk sac was removed using a pair of fine forceps, and the visceral yolk sac was left intact. Embryos (4 per bottle) were cultured in 25% Tyrode salt solution and 75% rat serum freshly prepared from male rats. The embryos were cultured at 37°C in 30 rpm rotation in the roller bottle system. The culture bottles were gassed 5% O_2/5% CO_2/90% N_2 for the first 24 hours and 20% O_2/5% CO_2/75% N_2 for the last 12 hours.

Embryos were cultured for 24 or 36 hours with 100 mg/dL glucose, a value close to the blood glucose level of nondiabetic mice, or 300 mg/dL glucose, which is equivalent to the blood glucose level of diabetic mice, in the presence or absence of curcumin (Sigma-Aldrich, St. Louis, MO). We started our whole-embryo culture experiments using 0, 10, and 20 μmol/L curcumin. At the end of 24 hours, embryos were dissected from the yolk sac for biochemical and molecular analyses. At the end of 36 hours, embryos were dissected from the yolk sac and examined under a Leica MZ16F stereomicroscope (Leica Microsystems, Bannockburn, IL) to identify embryonic malformations.

Images of the embryos were captured by a DFC420 5 MPix digital camera (Leica Microsystems). Normal embryos were classified as possessing a completely closed neural tube and no evidence of other malformations. Malformed embryos were classified as showing evidence of failed neural tube closure or of an NTD. NTDs were verified by histological sections.

Lipid hydroperoxide quantification

The degree of lipid peroxidation was quantitatively assessed by the lipid hydroperoxide (LPO) assay, as previously described,[26] and using the Calbiochem LPO assay kit (Millipore, Bedford, MA), following the manufacturer's instructions. Briefly, embryos cultured for 24 hours under normal and high glucose conditions were homogenized in HPLC-grade water. The LPO of the embryonic tissue were extracted by deoxygenated chloroform, and then measured by the absorbance of 500 nm after reaction with chromogen. The results were expressed as μmol/L LPO per μg protein. Protein concentrations were determined by the BioRad DC protein assay kit (BioRad, Hercules, CA).

Immunoblotting

Immunoblotting was performed as described by Yang et al[17] and Li et al.[20] To extract protein, a protease inhibitor cocktail (Sigma-Aldrich) in lysis buffer (Cell Signaling Technology, Beverly, MA) was used. Equal amounts of protein and the Precision Plus Protein Standards (BioRad) were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore). Membranes were incubated in 5% nonfat milk for 45 minutes, and then were incubated for 18 hours at 4°C with the following primary antibodies at dilutions of 1:1000 in 5% nonfat milk: phosphorylated protein kinase RNA-like ER kinase (PERK); PERK; p-eIF2α; eIF2α; C/EBP-homologous protein; binding immunoglobulin protein; IRE1α nitrotyrosine (Cell Signaling Technology); p-IRE1α (Abcam, Cambridge, MA); 4-hydroxynonenal (HNE) (Millipore); caspase 8 (mouse specific) (Alexis Biochemicals, San Diego, CA); and caspase 3 (Millipore). Membranes were exposed to HRP-conjugated goat antirabbit or goat antimouse (Jackson ImmunoResearch Laboratories, West Grove, PA) or goat antirat (Chemicon, Temecula, CA)