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Epigallocatechin-3-gallate (EGCG) ameliorates hyperglycemia-induced embryonic vasculopathy and malformation by inhibition of Foxo3a activation

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Abstract

Objective—Maternal hyperglycemia increases the risk of congenital malformations. EGCG, a natural antioxidant purified from green tea, inhibits oxidative stress signaling. We propose that EGCG prevents hyperglycemia-induced malformation via inhibition of oxidative stress signaling. The objective of this study is to examine the effect of EGCG on hyperglycemia-induced adverse effects during embryonic development.

Study Design—E9 rat embryos were cultured under euglycemic (150 mg/dl glucose) and hyperglycemic (300 mg/dl glucose) conditions in the presence or absence of 1 or 10 μ M EGCG.

Results—Both 1 μ M and 10 μ M EGCG significantly ameliorated hyperglycemia-induced embryonic vasculopathy and malformations. Hyperglycemia inactivated Akt by reducing pAkt levels. EGCG reversed the inhibitory effect of hyperglycemia on Akt activation. EGCG also prevented hyperglycemia-reduced pFoxo3a levels.

Conclusion—EGCG prevents hyperglycemia-induced embryopathy through inhibition of Foxo3a activation. This may have been mediated via the activation of Akt. These findings offer the potential for a possible pharmacological prophylaxis for hyperglycemia-induced embryonic malformations

Keywords

EGCG; Foxo3a activation; Akt activation; hyperglycemia; malformations; vasculopathy

Introduction

Congenital malformations in children of diabetic women are significant clinical problems and the recent rise in diabetes¹ makes this pregnancy complication a continuing issue². Because glycemic control in diabetic women is difficult to control and maintain³, malformation rates of children of diabetic women are at least six times higher than those in non-diabetic women

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¹. Therefore, therapeutic interventions in addition to insulin are needed for diabetes-associated adverse pregnancy outcomes. Mechanistic studies are essential steps for the development of accessible, convenient and effective prevention strategies. Published data support the conclusion that congenital malformations during maternal hyperglycemia are the result of a disruption in the balance between intracellular reactive oxygen species (ROS) and endogenous antioxidant capacities^{4–7}. Thus, embryonic malformations under hyperglycemic conditions are the results of oxidative stress. Animal studies with dietary supplement of antioxidants such as multi-vitamins effectively prevent diabetic embryopathy⁸. However, human studies with conventional antioxidants do not result in any beneficial effects for human diseases associated with oxidative stress.

Prevention strategies for diabetic embryopathy must meet two important criteria: high effectiveness and lack of teratogenicity. EGCG, a polyphenolic component of green tea, has high antioxidant capacities⁹ and does not possess any teratogenic effects even at very high doses¹⁰. In addition to its antioxidant properties, EGCG is also a cell signaling modulator, suppressing pro-apoptotic signaling¹¹ and promoting pro-survival signaling in non-tumor cells¹². This is highly relevant to diabetic embryopathy. Hyperglycemia-induced oxidative stress activates pro-apoptotic signaling pathways¹³ and suppresses pro-survival pathways¹⁴ that lead to apoptosis in target embryonic cells. Oxidative stress-triggered apoptosis results in embryonic malformations. Since EGCG has both antioxidant and anti-oxidative stress signaling properties, it is reasoned that EGCG may be more effective in prevention of hyperglycemia-induced malformations than conventional antioxidants.

Our previous studies demonstrated a causative role of the pro-apoptotic JNK1/2 (c-Jun-N-terminal kinase) in the induction of diabetic embryopathy¹⁵. Forkhead transcription factors, Foxo1, Foxo3a and Foxo4, are downstream effectors of JNK1/2. Foxo factors are also oxidative stress mediators and apoptosis inducers. Foxo3a is enhanced whereas the activity of pro-survival signaling intermediate Akt (protein kinase B)¹⁶ is decreased. Akt and Foxo signaling are closely interrelated with the negative regulation of Foxo activities by Akt. Because EGCG is a potent cell signaling modulator that favors cell survival, we will evaluate the effect of EGCG in the present study on altered Foxo3a and Akt signals by hyperglycemia.

The well established whole-conceptus culture system is used to examine the effect of EGCG on hyperglycemia-induced adverse effects on embryonic development during period of organogenesis. The whole-conceptus culture system coupled with hyperglycemia faithfully mimics maternal diabetes-induced adverse pregnancy outcomes, avoids the maternal influences, and enables us to directly assess the protective effects of EGCG on conceptuses exposed to hyperglycemia. Embryonic vasculature is the first system to develop during embryonic development. Hyperglycemia induces embryonic vasculopathy¹⁷ resulting in either embryonic malformations or lethality. Using quantitative measurements, we have found that EGCG significantly reduces both hyperglycemia-induced embryonic vasculopathy and malformations, blocks hyperglycemia-induced Foxo3a activation, and reverses the inhibition of Akt by hyperglycemia. EGCG may be a possible therapeutic candidate for diabetic embryopathy.

Materials and Methods

Reagents

Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). EGCG was purchased from Sigma (St. Louis, MO) and dissolved in water.

Whole-conceptus culture

The procedures for animal use were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee. Sprague Dawley rats were paired overnight. Vaginal smears were examined the next morning, and the presence of spermatozoa in the examination indicated embryonic day (E) 0. Rat conceptuses at E9 were dissected out of the uteri in PBS (Invitrogen, La Jolla, CA). The parietal yolk sac was removed using a pair of fine forceps and the visceral yolk sac was left intact. Conceptuses (4/bottle) were cultured in 4 ml rat serum at 38°C in 30 rev/min rotation in the roller bottle system. For the initial 24 hr of culture, the bottles were gassed with 5% O₂/5% CO₂/90% N₂. For the following 12 hr, the bottles were gassed with 20% O₂/5% CO₂/75% N₂, and in the last 12 hr, 40% O₂/5% CO₂/75% N₂ were applied. Conceptuses were cultured under euglycemic (150 mg/dl glucose) and hyperglycemic (500 mg/dl glucose) conditions or in the presence and absence of 1 or 10 mM EGCG. After 48h culture, conceptuses were harvested for morphological examinations of yolk sac vasculature and embryonic malformations.

Morphologic Assessment of the yolk sac vasculature and Embryonic Malformations

Conceptuses were examined under a Leica MZ16F stereomicroscope (Bannockburn, IL) to assess yolk sac vasculature defect, then yolk sacs and embryos were separated and embryonic malformations were identified. Images of embryos with or without yolk sacs were captured by a DFC420 5 MPix digital camera with software (Leica, Bannockburn, IL) and processed with Adobe Photoshop CS2.

Yolk sac vasculatures were morphologically scored based on visible maldevelopment as previously described¹⁷. A morphological score of 4 indicated a fully development of the E11-like yolk sac vasculature with an arborizing interconnecting vascular network composed of arteries, veins, and capillaries exhibiting blood flow. A score of 3 represented only a minor defect of the yolk sac vasculature with less blood vessels than that of the yolk sac vasculature with score 4. A score of 2 indicated an arrest of the yolk sac vasculature development at the primary capillary plexus stage resulting in few yolk sac vessels. A score of 1 indicated a major defect of the yolk sac vasculature displaying an ecstatic vascular plexus with no signs of arborization and large, non-fused blood islands toward the ectoplacental cone. A score of 0 represented a completely devoid of the yolk sac vasculature with no visible or scattered blood islands. Normal embryos were classified as exhibiting correct body flexure, completely closed neural tube, and no evidence of other malformations. Malformed embryos were classified as showing evidence of failed closure of the neural tube or neural tube defect (NTD) and other malformations including cardiac abnormalities, incorrect body curvature, reverse tail flexion, and microcephaly.

Western Blotting

Western Blotting was performed as described by Yang, *et al.*¹⁵. Briefly, after 48h culture, embryos were collected and were sonicated in 80 µl ice-cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 2 mM Na orthovanadate, 1 mM PMSF and 1% Triton 100) containing a protease inhibitor cocktail (Sigma, St. Louis, MO). Equal amounts of protein (50 µg) were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane (Schleicher & Schuell). Membranes were incubated with primary antibodies against phospho-threonine 32-Foxo3a (Cell Signaling, Beverly, MA) or phospho-threonine 473-Akt (Cell Signaling), followed by peroxidase-conjugated secondary antibodies. Membranes were exposed to goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibodies. To ensure that equivalent amounts of protein were loaded among samples; membranes were stripped and probed with a mouse antibody against β-actin (Abcam, Cambridge, MA). Signals were detected using an Amersham ECL Advance Detection Kit (GE Healthcare, Piscataway, NJ) and chemiluminescence emitted from the bands was directly

captured using a UVP Bioimage EC3 system (UVP, Upland, CA). Densitometric analysis of chemiluminescence signals were performed by VisionWorks LS software (UVP, Upland, CA). Images of representative immunoblots were arranged using Adobe Photoshop and Microsoft PowerPoint software. All experiments were repeated three times with the use of independently prepared tissue lysates.

Statistics

Data are presented as means $\pm$ SE (standard error). One way ANOVA was performed using SigmaStat 3.5 software. In ANOVA analysis, Tukey-test was used to estimate the significance of the results. Statistical significance was accepted at $p < 0.05$. Significant difference between groups in malformation incidences was analyzed by *Chi-square* test.

Results

EGCG significantly ameliorates hyperglycemia-induced embryonic vasculopathy

Defective embryonic vasculatures results in either embryonic lethality or congenital malformations. A morphological score system has been developed to quantitatively assess the defects of yolk sac vasculatures. Conceptuses cultured under hyperglycemic conditions had lower morphological scores than those cultured under euglycemic conditions (Figure 1B). EGCG at concentrations 1 μ M and 10 μ M did not have any adverse effects on embryonic vasculature development because vasculature morphological scores in EGCG groups did not differ from those in the euglycemic control group (Figure 1B). EGCG treatment at 1 μ M and 10 μ M significantly reversed the reduction of vasculature morphological scores by hyperglycemia (Figure 1B). Conceptuses cultured under euglycemia or under hyperglycemia with EGCG had fully developed vasculature networks after 48 h culture (Figure 1A). In contrast, conceptuses cultured under hyperglycemia alone were devoid of vessels (Figure 1A) or has variety of defects ranging from arrest at the blood island stage to the presence of few blood vessels.

EGCG effectively reduces hyperglycemia-induced embryonic malformations

Hyperglycemia-induced oxidative stress and resultant apoptosis are responsible for hyperglycemia-induced malformations. EGCG is an antioxidant and is able to inhibit pro-apoptotic signals. To determine if EGCG reduces hyperglycemia-induced malformations, conceptuses cultured under hyperglycemia (300 mg/dl glucose) were treated with 1 or 10 μ M EGCG. As previously reported¹⁸, hyperglycemia resulted in a 50% embryonic malformation rate (Table 1). Conceptuses cultured under euglycemia (150 mg/dl) had a low embryonic malformation rate (7.14%) (Table 1). No embryonic malformations were observed in conceptuses cultured under euglycemia plus 1 or 10 μ M EGCG (Table 1), suggesting that not only does EGCG have no teratogenic effects, but it also supports normal embryonic development. With 1 or 10 μ M EGCG treatment, hyperglycemia-induced malformations were completely prevented (Table 1). After 48 h culture, E9 embryos in the euglycemia group, and the hyperglycemia plus 1 μ M or 10 μ M EGCG groups, developed to normal E11-like structures with well-closed neural tube and correct body curvature (Figure 2A, C and D). In contrast, embryos in the hyperglycemia group exhibited neural tube defects with failed neural tube closure and incorrect body curvature (Figure 2B). These results provide strong *in vitro* evidence in support of EGCG as a possible therapeutic candidate for hyperglycemia-induced embryonic anomalies.

EGCG blocks hyperglycemia-induced Foxo3a activation

The mechanisms underlying the preventive effect of EGCG on hyperglycemia-induced malformations may be due to its antioxidant property. However, EGCG appears more effective

than conventional antioxidants. We reason that EGCG may block the pro-apoptotic key intermediates whose activation is triggered by hyperglycemia and mediates the teratogenic effect of hyperglycemia. Foxo3a is a key intermediate in hyperglycemia-induced apoptosis. Upon stress stimuli, Foxo3a is dephosphorylated into its active state, resulting in an increase of its transcription activity leading to apoptotic gene expression. Under euglycemic conditions, embryonic Foxo3a was highly phosphorylated (Figure 3). Upon hyperglycemia, pFoxo3a levels were reduced (Figure 3). Densitometric analysis revealed that the reduction of pFoxo3a levels were significant when compared to the levels of pFoxo3a in the euglycemic group (Figure 3). At euglycemic conditions, EGCG did not affect the levels of pFoxo3a (Figure 3). EGCG treatments at concentration of 1 or 10 μ M significantly blocked hyperglycemia-induced pFoxo3a (Figure 3). These results demonstrate for the first time that EGCG can prevent hyperglycemia-induced Foxo3a activation.

EGCG prevents hyperglycemia-inhibited Akt activation

Akt is a key intermediate in the phosphatidylinositol-3-kinase (PI3K) pathway. Akt phosphorylates Foxo3a at threonine 32 and thus inactivates Foxo3a¹⁹. Because Foxo3a is dephosphorylated upon hyperglycemic exposures, it is postulated that Akt activity may be correspondingly reduced. When Akt is activated, it is phosphorylated at threonine 473. Levels of pAkt were determined in embryos cultured under euglycemic or hyperglycemic conditions with or without EGCG. Hyperglycemia significantly reduced pAkt levels when compared to that of the euglycemic group (Figure 4). Under euglycemic conditions, addition of EGCG did not alter the levels of pAkt (Figure 4). Both 1 μ M and 10 μ M EGCG treatments reversed hyperglycemia-induced pAkt reduction (Figure 4). The findings strongly suggest that EGCG prevents the adverse effects of hyperglycemia on embryonic development during the period of organogenesis by inhibition of Foxo3a activity.

Comment

The role of polyphenols in the treatment of metabolic disorders and their associated complications has recently been of great interest²⁰. The findings in the present studies may set an example for future development of EGCG as a therapeutic intervention to a broad spectrum of diabetic complications. EGCG effectively reduces hyperglycemia-induced embryonic vasculopathy and embryonic malformations. Importantly, EGCG suppresses hyperglycemia-induced Foxo3a activation and reverses hyperglycemia-inactivated Akt. Since alteration of Foxo proteins and Akt activity are involved in many disease processes, EGCG, as a signaling modulator in favor of cell survival, may have great promise in treatment of diseases related to oxidative stress and apoptosis.

EGCG is a major green tea component and thus is suitable for an accessible and convenient dietary supplement. Although green tea consumption is generally not associated with adverse outcomes in birth, it is important to evaluate the safety of EGCG since purified EGCG may be consumed at much greater levels in prevention of human diseases than drinking green tea. EGCG has non-genotoxicity based on data from both *in vivo* and *in vitro* assay systems^{10, 21, 22}. The safety of EGCG exposure during organogenesis and embryonic development has been evaluated¹⁰. In a main teratogenicity study, feeding pregnant rats diets supplemented at 1400, 4200 or 14,000 ppm during organogenesis is non-toxic to both dams and fetuses. A two generation study in rats fed 1200, 3600 or 12,000 ppm EGCG preparation shows no adverse effects on reproduction or fertility¹⁰. Although the EGCG concentrations used in the present studies are much less than the lowest dose, 1200 ppm, in these reported animal studies^{10, 21, 22}, these EGCG doses effectively reduce hyperglycemia-induced adverse effects on embryonic development. Together, these findings demonstrate that EGCG is effective in prevention of embryopathy induced by hyperglycemia and is safe for both mothers and the developing

conceptuses. EGCG may be a possible therapeutic intervention that targets both hyperglycemia-induced oxidative stress and key molecular intermediates mediating the induction of diabetic embryopathy.

The present study identifies EGCG as a safe and effective treatment to prevent hyperglycemia-induced adverse embryonic development. This is an important finding in the field of diabetic embryopathy because the use of specific pharmacological inhibitors that target the signaling pathways mediating the teratogenicity of hyperglycemia may be limited by their teratogenic effects. This is the case for JNK1/2 inhibitors. Our previous pharmacological and genetic evidences¹⁵ strongly suggest that JNK1/2 activation mediates the deleterious effect of hyperglycemia on embryonic development. JNK inhibitors may be useful in prevention of diabetic embryopathy. However, pharmacological JNK inhibitors exert teratogenic effects on pre-implantation embryos²³. Conventional antioxidants may also be limited in treatment of diabetic embryopathy because of their teratogenic effects. For example, the doses of vitamin E required to block the adverse effects of maternal hyperglycemia are much higher than the recommended daily doses, and very close to the doses that can cause pregnancy loss, particularly in diabetic pregnancy. Since vitamin E is lipid-soluble and can be stored in maternal deposits for long periods of time, prophylactic use of vitamin E could increase, rather than decrease, risk for embryonic malformations. Because of the uncertainty of effectiveness and potential adverse effects of pharmacological inhibitors to cell signaling intermediates and of conventional antioxidants in human pregnancies, strategic interventions in prevention of diabetic embryopathy are agents that have antioxidant property with proven effectiveness and diminished teratogenic effects. Most importantly, they should also target key signaling intermediates mediating the adverse effects of hyperglycemia. EGCG is a potential candidate that meets the above criteria in effective prevention of diabetic embryopathy.

Our study uses a well-established whole-conceptuses culture system^{7, 15, 24}. The advantage of this system is that it avoids maternal influences while directly assessing the effectiveness of EGCG. Recently, it has been demonstrated that EGCG can prevent diabetes²⁵, therefore, EGCG may affect maternal diabetes status if used for *in vivo* animal studies. The present findings will open new direction for studies on the mechanisms underlying the beneficial effects of EGCG in ameliorating human diseases.

Our unpublished studies using *foxo3a*^{-/-} mice demonstrate that Foxo3a plays a causative role in the induction of embryonic malformations by diabetes. EGCG inhibits Foxo3a activation thereby reducing hyperglycemia-induced embryonic vasculopathy and malformations. This is the first study to show that EGCG inactivates Foxo3a by blockade of its phosphorylation at Thr32, a phosphorylation site of Akt. In consistent with this finding, hyperglycemia-reduced Akt activity is also blocked by EGCG. Thus, EGCG exerts beneficial effects via modulation of the Akt-Foxo3a pathway in hyperglycemia-induced adverse embryonic outcomes.

In summary, we provide strong direct evidence that EGCG effectively prevents the adverse effect of hyperglycemia on embryonic development. EGCG corrects the aberrant Akt-Foxo3a pathway triggered by hyperglycemia. The findings provide important insights for the development of EGCG as a therapeutic intervention in diabetes-associated adverse pregnancy outcomes.

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Figure 1. EGCG ameliorates hyperglycemia-induced embryonic vasculopathy

EGCG ameliorates hyperglycemia-induced embryonic vasculopathy. Representative images of yolk sac vasculatures in E9 conceptuses after 48 h culture were shown in **A**. **a**, **b**, **c** showed yolk sac vasculatures with morphological score 4 in the euglycemia (150 mg/dl glucose) group, the euglycemia + 1 μ M EGCG, and the euglycemia + 10 μ M EGCG, respectively. The yolk sac vasculature from the hyperglycemia (300 mg/dl glucose) group in **d** had a morphological score 0. **e** and **f** showed yolk sac vasculatures with morphological score 4 in the hyperglycemia + 1 μ M EGCG group and the hyperglycemia + 10 μ M EGCG group, respectively. **B**, morphological scores of yolk sac vasculatures in each group were expressed as mean $\pm$ standard errors. **Eu**: Euglycemia (150 mg/dl glucose) and **Hy**: hyperglycemia (300 mg/dl). * denotes significant difference ($P < 0.05$) when compared to other groups. Scale bar=1 mm.

Figure 2. EGCG ameliorates hyperglycemia-induced embryonic malformation

Representative images of normally developed E9 embryos after 48h culture from the euglycemia group (150 mg/dl glucose) (**A**) or the hyperglycemia (300 mg/dl glucose) + 1 μ M EGCG group (**C**) or the hyperglycemia + 10 μ M EGCG group (**D**), and malformed embryos (**B**) manifested with neural tube defect (exencephaly) and incorrect body curvature from the hyperglycemia group. White arrow denotes an open neural tube. Scale bar=1 mm.

Figure 3. EGCG blocks Foxo3a dephosphorylation induced by hyperglycemia

EGCG blocks Foxo3a dephosphorylation induced by hyperglycemia. Levels of pFoxo3a at Thr32 were detected by Western blotting in E9 embryos after 48h culture from the euglycemia (150 mg/dl glucose) group (**Eu**), the hyperglycemia (300 mg/dl glucose) group (**Hy**), the euglycemia + 1 μM EGCG group (**Eu + 1 μM EGCG**), the euglycemia + 10 μM EGCG group (**Eu + 10 μM EGCG**), the hyperglycemia + 1 μM EGCG group (**Hy + 1 μM EGCG**), and the hyperglycemia + 10 μM EGCG group (**Hy + 10 μM EGCG**). β-actin was served as an equal protein loading control. Experiments were repeated for three times. Representative images were shown in **A**. Densitometrical analysis of pFoxo3a against β-actin was shown in **B**. Data were expressed as mean ± standard errors with n = 3. * denotes significant difference ($P < 0.05$) when compared to other groups.

Figure 4. EGCG reverses hyperglycemia-reduced Akt activity

EGCG reverses hyperglycemia-reduced Akt activity. Levels of pAkt at Thr473 were detected by Western blotting in E9 embryos after 48h culture from the euglycemia (150 mg/dl glucose) group (**Eu**), the hyperglycemia (300 mg/dl glucose) group (**Hy**), the euglycemia + 1 μ M EGCG group (**Eu + 1 μ M EGCG**), the hyperglycemia + 1 μ M EGCG group (**Hy + 1 μ M EGCG**), and the hyperglycemia + 10 μ M EGCG group (**Hy + 10 μ M EGCG**). β -actin was served as an equal protein loading control. Experiments were repeated for three times. Representative images were shown in **A**. Densitometrical analysis of pAkt against β -actin was shown in **B**. Data were expressed as mean $\pm$ standard errors with $n = 3$. * denotes significant difference ($P < 0.05$) when compared to other groups.

Table 1

Effect of EGCG treatments on hyperglycemia-induced embryonic malformations

Experimental groups	Total number of Embryos	Total number of malformed Embryos	Malformation rates (%)
Euglycemia (150 mg/dl)	14	1	7.14
Hyperglycemia (300 mg/dl)	14	7 *	50 *
Euglycemia + 1 μ M EGCG	12	0	0
Euglycemia + 10 μ M EGCG	12	0	0
Hyperglycemia + 1 μ M EGCG	11	0	0
Hyperglycemia + 10 μ M EGCG	10	0	0

The data were resulted from three independent experiments. The asterisk (*) indicate significant differences compared to all other groups by Chi-square test ($P < 0.05$). Malformation rate= malformed embryos/total number of embryos.