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Protein Kinase C β 2 inhibition reduces hyperglycemia-induced neural tube defects through suppression of a caspase8-triggered apoptotic pathway

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Abstract

Background—Neural tube defects (NTDs) in infants of diabetic mothers are associated with increased protein kinase C β 2 (PKC β 2) activity and programmed cell death (apoptosis) in the neuroepithelium during early embryogenesis. Apoptosis in diabetic embryopathy is triggered by caspase8, an initiator caspase which activates a cascade of molecular events involving members of the caspase and Bcl-2 families, such as caspase3 and Bid, as well as Cytochrome C. Whether PKC β 2 regulates caspase8-induced apoptosis remains to be addressed.

Methods—Mouse embryos at embryonic (E) day 8.5 (E8.5) were cultured in a high concentration of glucose (22 mM) and treated with PKC β 2 inhibitor (50 nM) for 48 hours. The embryonic development and NTDs were examined. Apoptosis was assessed using TUNEL assay. Changes in activation of apoptotic factors were assessed using Western blot assay.

Results—Inhibition of PKC β 2 significantly reduced NTD rate to the similar level as in euglycemic control (8.3 mM). Activation of caspase8, as indicated with a cleaved form, was significantly lower than that in the hyperglycemic group ($p < 0.05$), and similar to that in euglycemic control. Similar results were also observed for Cytochrome C, caspase3, and Bid ($p < 0.05$).

Conclusion—PKC β 2 mediates the effect of maternal hyperglycemia on embryonic NTDs in diabetic embryopathy by influencing a caspase8-regulated apoptotic pathway.

Keywords

caspase8; diabetic embryopathy; neural tube defect; caspase; hyperglycemia; protein kinase C

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Introduction

Diabetes mellitus in pregnancy is largely associated with variety of birth defects in infants, including malformations in the central nervous system (1,2). However, the precise mechanism of neural tube defect (NTD) has not been fully elucidated, although increased oxidative stress and apoptosis is considered (3,4).

Apoptosis is a major factor in the development of NTDs in diabetic embryopathy (5,6). It is a complex process regulated by a number of factors in which caspases play the key roles. Caspases are cysteine proteases and divided into initiator and effector caspases (7). The effector caspases (e.g., caspase3, 6 and 7) are directly responsible for the execution of apoptosis. However, the initiator caspases including caspase8 and caspase9 are carrying apoptotic signals by triggering a molecular cascade to activate effector caspases. Caspase8 triggers a series of molecular events to activate caspase9 which, in turn, activates effector caspases. Activated caspase8 can cleave Bid to form truncated Bid (tBid). tBid triggers a release of Cytochrome C from mitochondria. Cytochrome C further activates caspase9, leading activation of effector caspases and execution of apoptosis (8,9,10). In addition, caspase8 can also directly target effector caspases, depending on cell type and pathological conditions. In hyperglycemia-induced NTDs, caspase8 has been shown to play a key role in apoptotic regulation (5). It is activated by hyperglycemia in the embryos. A blockade of caspase8 activity significantly reduces NTD rate in the embryos exposed to high glucose (5).

Maternal diabetes disturbs intracellular signaling involving the protein kinase C (PKC) family (11,12,13,14,15). PKCs are serine/threonine protein kinases that can be activated by diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). The PKC family comprises of 12 isoforms that are grouped as: (1) classical, Ca^{2+} -dependent PKC α , β and γ ; (2) novel, Ca^{2+} -independent PKC η , θ , δ and ϵ and (3) atypical, DAG independent PKC λ , μ , ζ , ι (16). Among these isoforms, PKC α , PKC β 2, and PKC δ have been found to be associated with NTDs in diabetic embryopathy (17). In addition, PKC β 2 has been shown to be responsible for apoptosis through increased oxidative stress (18). Therefore, it is hypothesized that PKC β 2 plays a role in diabetic embryopathy via regulating apoptosis. In the present study, the role of PKC β 2 in apoptosis and caspase regulation has been investigated in embryos exposed to hyperglycemia.

Materials and Methods

Embryo culture

The use of animals was approved by Institutional Animal Care and Use Committee of University of Maryland Baltimore. Female mice (C57BL/6J) were mated in the afternoon. The next day was designated as embryonic (E) day 0.5 if a vaginal plug was present.

Mouse embryos were dissected in cold phosphate-buffered saline (PBS, pH 7.4). The parietal yolk sac was removed, and the visceral yolk sac was left intact. Embryos were cultured for 48 hours in a bottle containing 5 ml heat-inactivated rat serum at 38°C with rotation. Embryos were cultured in euglycemic (CON; 8.3 mM glucose) and hyperglycemic (HG, 22 mM) media in the presence or absence of PKC β 2 inhibitor (3-(1-(3-Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione; Calbiochem). The bottles were gassed with 5% O_2 /5% CO_2 /90% N_2 at first 24 hours and then in 20% O_2 /5% CO_2 /75% N_2 for another 24 hours. At the end of culture, embryos were harvested for examination of NTDs and protein assays.

Western Blot

The neural tube tissues of the brain and the upper part of spinal cord of the embryos were dissected using a pair of fine scissors. The ectoderm was carefully removed. Tissues were homogenized in RIPA lysis buffer (Millipore) containing protease inhibitor cocktail (Thermo Scientific) and, then, centrifuged at a speed of 14000rpm/min for 20 min to obtain supernatants. After denaturing for 3 min at 95°C, equal amount of the proteins were loaded on 8% SDS-PAGE, separated electrophoretically, and blotted to Immobilon-P nylon membranes (Millipore Corporation). The membranes were incubated with primary antibodies [anti- β -actin (Sigma-Aldrich), anti-Cytochrome C (Santa Cruz Biotechnology), anti-caspase8 (Alexis Biochemicals), anti-caspase3, and anti-Bid (Cell Signaling) in TBST (20 mM Tris-HCl, 136 mM NaCl, 0.1% Tween 20, pH 7.6) containing 5% nonfat milk. Detection of specific proteins was carried out using peroxidase-conjugated secondary antibodies and a chemiluminescent substrate (Amersham Biosciences). The bands of fluorescence were captured and fluorescence intensity was analyzed using UVP Bioimaging Systems (Upland) with VisionWork software. Data were expressed as ratio of the fluorescence intensity of target proteins to that of β -actin.

TUNEL Assay

Embryos were dissected, embedded in OCT tissue embedding medium (Sakura Scientific), and frozen on dry ice. Sections were cut in 20 μ m. The sections were incubated with fluorescein-conjugated dUTP and terminal transferase for 60 min at 37 °C (Roche), counterstained with DAPI, and observed under a fluorescent microscope.

Statistical Analysis

Data are presented as Mean $\pm$ SE. Student's *t* test was used to compare significance between two groups. The criterion for statistical significance was $P<0.05$ in all tests.

Results

PKC β 2 inhibition significantly reduces NTDs

After 48 hours of culture in hyperglycemic condition, E8.5 embryos developed to the stage similar to E10.5 *in vivo* (Figure. 1). Embryos treated with high glucose exhibited open neural tubes, a characteristic of NTD (Figure. 1). The malformation rates (58.3 $\pm$ 8.4%) in the high glucose-treated group were significantly higher than those in euglycemic control (10 $\pm$ 6.1%). Addition of PKC β 2 inhibitor in hyperglycemic treatment significantly reduced malformation rates (27.9 $\pm$ 3.3%; $p<0.05$; Table 1).

Decreases in apoptosis by PKC β 2 inhibition

High levels of apoptosis, detected using TUNEL assay, were high in the neuroepithelium of the embryos treated in high glucose (Figure. 2A). Treatment with PKC β 2 inhibitor significantly decreased apoptotic levels (Figure 2B)

Decreases in Caspase8 activation by PKC β 2 inhibition

As previously reported, caspase8 activation is indicated by the presence of a cleaved form (18 kDa) (5). In the current experiments, a dramatic increase of the cleaved caspase8 was observed in embryos treated with high glucose, compared with the euglycemic group (Figure 3). The level of cleaved caspase8 was significantly reduced when embryos were treated with PKC β 2 inhibitor, compared with that in the hyperglycemic group ($p<0.05$). It was similar to that in the euglycemic control group.

Effect of PKC β 2 inhibition on activation of proapoptotic protein Bid

It is reported that increases in Bid cleavage is associated with the elevation of caspase8 activation in the embryos cultured in high glucose (5). Consistent with this finding, tBid was increased in the hyperglycemia-treated embryos (Figure 4). However, PKC β 2 inhibitor treatment significantly suppressed it to the level similar to that in the control group ($p < 0.05$).

Cytochrome C release in response to PKC β 2 inhibition

Bid activation leads to a release of Cytochrome C from mitochondria (8,19,20). To examine if this pathway is regulated by PKC β 2, the levels of Cytochrome C in the cytosol were quantified using Western blot assay. Increases in Cytochrome C were observed in the embryos treated with high glucose, compared with those in the euglycemic group (Figure 5). In response to PKC β 2 inhibitor treatment, the levels of Cytochrome C were significantly decreased, compared with those in the hyperglycemic group ($p < 0.05$).

Inhibition of caspase 3 activity by PKC β 2 inhibition

Cytochrome C activates caspase9 which, in turn, cleaves caspase3 (7). The levels of caspase3 cleavage were increased in hyperglycemic groups, compared with those in the euglycemic group (Figure. 6). A treatment with PKC β 2 inhibitor significantly reduced caspase3 activation, when compared with the hyperglycemic group (Figure 3; ($p < 0.05$)).

Discussion

It has been shown that PKC β 2 plays an important role in hyperglycemia-induced NTDs (17). In the current report, PKC β 2 is further demonstrated to stimulate apoptosis in the neural tube via activating caspase8, which, in turn, activates an apoptotic pathway through Bid, Cytochrome C, and caspase3. These results reveal a key role of PKC β 2 and associated molecular pathway leading to apoptosis in NTDs in diabetic embryopathy.

Evidence has shown that different PKC isoforms in different types of tissue are activated during hyperglycemia (21,22). In a diabetic state, total DAG has been reported to be increased in variety of tissues, including the neural tubes of embryos (17,23,24,25). In addition, PKCs are the targets of oxidative stress (26) and activated PKCs can trigger the production of reactive oxygen species (27), leading to a vicious cycle which can increase oxidative stress leading to NTDs. In addition to DAG, different PKC isoforms are activated by other factors. For example, PKC α and PKC β are activated by DAG in a Ca²⁺-dependent manner; whereas PKC δ is activated independent of Ca²⁺ (16). Therefore, the increased activity of PKC α , PKC β , and PKC δ may be differentially regulated in diabetic NTDs.

Although PKC α , PKC β , and PKC δ are activated in the embryos treated with high glucose, they may regulate apoptosis via different mechanisms. For example, PKC β 2 activates caspase8, as demonstrated by this study. Under hyperglycemic conditions, the glucose metabolite methylglyoxal can stimulate PKC δ in cisplatin-induced apoptosis (28). Studies have also shown that in apoptosis, activated PKC δ can lead to cleavage of caspase3, and inversely PKC δ can be activated by caspase3 (29,30). PKC δ has been also shown to be closely related to BAD alteration during apoptosis (31). PKC α is known to be related to apoptosis by inducing mitochondrial damage (32). These data indicate that different PKC isoforms might play distinct roles in apoptosis.

In conclusion, the data presented here indicate that PKC β 2 plays a role in NTDs in diabetic embryopathy via activating caspase8 and associated apoptotic cascade. Future work will be to investigate the possible interventional applications of PKC β 2 inhibition, as its inhibitors have been tested in other diseases. (33,34,35,36,37,38).

Acknowledgments

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Figure 1.
Development of the embryos in culture. (A) euglycemia; (B) hyperglycemia; open forebrain and midbrain; (C) hyperglycemia+PKC β I

Figure 2. Apoptosis in the embryos cultured in hyperglycemia and PKC β 2 inhibitor. (A), Hyperglycemia; (B) hyperglycemia + PKC β 2 inhibitor. TUNEL assay showing apoptotic cells (green). The nuclei are stained blue with DAPI.

Figure 3. Effect of PKC β 2 inhibition on caspase8 activation. (A) Western blot assay of caspase8 cleavage. β -actin serves as sample loading control. (B) Quantification of fluorescence intensity of the bands of cleaved caspase8. CON, euglycemic control; HG, hyperglycemia. PKC β I, hyperglycemia+PKC β 2 inhibitor. * $p < 0.05$ vs HG group.

Figure 4.

Effect of PKC β 2 inhibition on Bid cleavage. Treatment with PKC β 2 inhibitor significantly reduced tBid in neural tube of cultured embryos. (A) Western blot result of bid and tBid. (B) Quantification of fluorescence intensity of the bands of tBid. CON, euglycemic control; HG, hyperglycemia; PKC β I, PKC β 2 inhibitor. * $p < 0.05$ vs HG group.

Figure 5. Responses of Cytochrome C release to high glucose culture and treatment of PKC β 2 inhibitor. (A) Western blot result of Cytochrome C. (B) Quantification of fluorescence intensity of the bands of Cytochrome C. CON, euglycemic control; HG, hyperglycemia. PKC β I, PKC β 2 inhibitor.. * $p < 0.05$ vs HG group.

Figure 6.

The effect of PKC β 2 inhibition on caspase3 cleavage. The expression of cleaved caspase 3 was shown after normalized with actin densitometry. (A) Western blot result of cleaved caspase3. (B) Quantification of fluorescence intensity of the bands of cleaved caspase3. CON, euglycemic control; HG, hyperglycemia. PKC β I, PKC β 2 inhibitor.. * $p < 0.05$ vs HG group.

Table 1Effect of PKC β 2 inhibitor on hyperglycemia induced neural tube malformation

	CON	HG	HGBI
Total embryos	19	18	15
Malformed embryos	2	7	4
Malformation rate (%)	9.7 $\pm$ 6.2	61.1 $\pm$ 5.6	27.9 $\pm$ 3.3*
Number of cultures	6	6	5

CON: euglycemic group; HG, hyperglycemic group; HGBI, hyperglycemia +PKC β 2 inhibitor.

* significantly different from HG group (p<0.05)