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Identification of novel cell survival regulation in diabetic embryopathy via phospholipidomic profiling

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ABSTRACT

Diabetes mellitus in early pregnancy causes birth defects by disturbing metabolic homeostasis and increasing programmed cell death in the embryo. Over-activation of phospholipase Cβ3 and γ1 suggests disturbed phospholipid metabolism, which is an important in regulation of cell signaling and activity. Metabolomic examinations reveal significant changes in the profile of phospholipid metabolism. Among the metabolites, levels of phosphatidylinositol bisphosphate (PIP_2) are increased. PIP_2 effector PTEN (phosphatase and tensin homolog deleted on chromosome 10) is activated. Activation of protein kinase Bα (PKBα, or AKT1) and mTOR (mechanistic target of rapamycin) is decreased. Inhibition of PLCs and PTEN suppresses over-generation of reactive oxygen species and inhibition of PLCs prevents fragmentation of mitochondria in neural stem cells cultured in high glucose. These observations suggest that maternal hyperglycemia disrupts phospholipid metabolism, leading to perturbation of mitochondrial dynamics and redox homeostasis and suppression of the PKB-mTOR cell survival signaling in the embryos.

1. Introduction

Diabetes mellitus in early pregnancy causes congenital birth defects in newborn infants, a complication known as diabetic embryopathy [1]. The abnormalities in the central nervous system, including exencephaly and spina bifida, are a result of incomplete closure of the neural tube during embryogenesis, and are, therefore, known as neural tube defects (NTDs) [2].

Maternal hyperglycemia perturbs intracellular metabolic homeostasis and organelle function, generating intracellular stress conditions in the embryo [3]. Under the stress conditions, molecular cascades are activated to cause excessive programmed cell death (apoptosis) in the neural epithelium, resulting in failure in neural tube closure [4].

Phospholipid metabolism is an important process in regulation of cell signaling and activities [5]. It is regulated by members of the phospholipase (PL) A, C, and D families [5,6]. Activation of cytosolic PLA_2 ($cPLA_2$) and peroxidation of arachidonic acid (AA) to generate isoprostanes (e.g., 8-iso-$PGF_{2\alpha}$) have been shown to be involved in diabetic embryopathy [7,8]. Phospholipid metabolism is a complex process, involving multiple factors and metabolic pathways. Therefore, systematic characterization of global metabolic profile is essential for identification of novel intracellular signaling in regulation of cell apoptosis in diabetic embryopathy.

PLCs are important enzymes to generate second messengers to regulation intracellular signaling [6]. They act on phosphatidylinositol (PI) monophosphate and PI biphosphate (PIP_2) [6]. Cleavage of PIP_2 generates second messengers, diacylglycerol (DAG) and inositol triphosphate (IP_3) [9]. PIP_2 can be phosphorylated to become PI triphosphate (PIP_3) [9,10]. PIP_3 activates protein kinase Bs (PKBs), also known as AKTs. PKBs activate mTOR (mechanistic target of rapamycin) to regulate cell survival and proliferation [11]. PIP_2 itself can activate PTEN (phosphatase and tensin homolog deleted on chromosome 10). PTEN dephosphorylates (deactivates) PIP_3 and PKBs, and thus, suppresses the PKB-mTOR cell survival signaling [12].

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The PKB-mTOR system controls cell survival and apoptosis by regulating pro- and anti-apoptotic factors in mitochondria [13]. Perturbation of the apoptotic regulators disturbs mitochondrial morphological dynamics and membrane activities, leading to generation of high levels of reactive oxygen species (ROS) and, resultant oxidative stress [14,15].

In this study, we characterized the global profiles of phospholipid metabolism in diabetic embryopathy, demonstrated effects of metabolites on mitochondrial dynamics and intracellular redox homeostasis, and identified a novel signaling cascade, involving PIP_2, PTEN, PKBs, and mTOR, which regulate cell survival in diabetic embryopathy.

2. Materials and methods

2.1. Diabetic animal model

The use of animals was approved by the Institutional Animal Care and Use Committee of University of Maryland, Baltimore. Generation of diabetic mice was described previously [16]. Briefly, female mice (C57BL/6J) were induced diabetic (DM; blood glucose ≥250 mg/dl or 14 mM) via intravenous injection of streptozotocin (in citrate buffer; 65 mg/kg body weight). Non-diabetes (ND) control mice were injected with citrate buffer. The female mice were paired with normal male mice after euglycemia (~8 mM) was restored by subcutaneous implantation of insulin pellets. Insulin implants were removed at embryonic (E) day 5.5 (E5.5) to become hyperglycemic again from E6.5 before neurulation begins [17]. At E10.5, embryos were collected for examination of NTDs (open neural tube) and metabolic and molecular changes.

2.2. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)

Details are given in Supplementary Materials and Methods.

2.3. Ultra performance liquid chromatography (UPLC) and electrospray quadruple time-of-flight mass spectrometry (QTOF-MS)

The neural tubes of the embryos were isolated in cold phosphate-buffered saline (pH 7.4) and individually collected. Protein-free samples were prepared from tissue homogenates in 50% methanol containing internal standards (10 μl of 1 mg/ml debrisoquine and 50 μl of 1 mg/ml 4-Nitrobenzoic acid) and assayed using UPLC-QTOF-MS, using Acquity UPLC (Xevo; Waters Corporation) and G2-QTOF-MS systems (Waters Corporation). Detailed procedures are given in Supplementary Materials and Methods.

2.4. Western blot assay

Detailed procedures are given in Supplementary Materials and Methods. Primary antibodies to the following proteins were used in this study: PLCβ3, phospho-PLCβ3 (Ser1105), PLCγ1, phospho-PLCγ1 (Tyr783), PTEN, phospho-PTEN (Ser380/Thr382/383), non-phospho-PTEN, PKBα, phospho-PKBα (Ser473), mTOR, phospho-mTOR (Ser2448) (Cell Signaling Technology, Beverly, MA) and β-actin (Abcam, Cambridge, MA).

2.5. Cell-based ROS assay

Neural stem cells (NE-4C; American Type Culture Collection), derived from E9 mouse embryos, were plated on 96-well plates (2×10^4 cells/well) in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) containing 10% fetal bovine serum and a normal concentration of D-glucose (NG; 6 mM) at 37 °C for 16 h. The cells were treated with high glucose (HG; 33 mM) containing PLC inhibitor U73122 (HG+U73122) or PTEN inhibitor SF1670 (HG+SF1670), along with control groups [NG + vehicle (VEH, 0.1% dimethyl sulfoxide), HG+VEH, and L-Glucose (6 mM D-glucose, 27 mM L-glucose)+VEH]. After 24 h of treatment, the cells were loaded with fluorescent dyes, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; 5 μM), Hoechst 33342 (2 μM), and propidium iodide (1 μg/ml; Life Technologies) for 10 min at 37 °C. After washing twice with FluoroBite DMEM (Life Technologies), the levels of fluorescence were measured using a Biotek Synergy microplate reader at 480 nm, 360 nm, and 540 nm. The ratio of the fluorescent values between H_2DCFDA (480 nm) and Hoechst (360 nm) indicates the level of ROS per a number of cells. The ratio between the values of propidium iodide (540 nm) and Hoechst (360 nm) indicates cell viability. The experiments were repeated three times with six duplicates in each experiment.

2.6. Mitochondrial morphology imaging

The neural stem cells (NE-4C) were plated on chambered glass slides (4×10^4 cells/chamber) and cultured in NG for 16 h. The cells were treated with NG+VEH, HG+VEH, and HG+U73122 at 37 °C for 24 h. The cells were loaded with MitoTracker Red (1 μM; Life Technology) at 37 °C for 10 min to label mitochondria, washed with FluoroBite DMEM, fixed with 4% paraformaldehyde in PBS, and examined under a fluorescent microscope.

2.7. Statistical analyses

Ratios of fluorescence intensity at two different wavelengths and ratios of band density of interest to that of β-actin on Western blots, presented as Mean ± standard deviation (SD), were analyzed using the Student t-test. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. NTDs and apoptosis in embryos of diabetic mice

Maternal hyperglycemia disrupts neural tube fusion during the period of neurulation (E8.5 to E11.5) [17]. We examined NTDs at E10.5, which is the late stage of neurulation. The neural tube of the embryos of the ND group was closed (Fig. 1A). However, in the DM group, the neural tube was still open in the brain and/or spinal cord region (Fig. 1B).

We also examined apoptosis in the neural tissues of the embryos. In the ND group, very few TUNEL-positive signals were seen in the neural tube (Fig. 1C). In the DM group, much higher levels of TUNEL-positive apoptotic bodies were present in the neural epithelium of the open neural tube (Fig. 1D).

3.2. Phospholipid metabolism in embryos of diabetic mice

To gain systematic insights into the impact of phospholipid metabolism, we examined profiles of lipid metabolome of the embryos of ND and DM groups, using a high resolution UPLC-MS. Comparisons of the profiles showed evident differences between the ND and DM groups (Supplementary Figs. S1 and 2). Statistical and bioinformatic analyses revealed metabolites that showed significant increases or decreases in the DM group, compared with those in the ND group (Table 1). Significant perturbations were seen in lipids, phospholipids, amino acids, and co-enzyme As (Table 1; Supplementary Table S1).