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SOD1 overexpression in vivo blocks hyperglycemia-induced specific PKC isoforms-substrate activation and consequent lipidperoxidation in diabetic embryopathy

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Abstract

Objective—Oxidative stress plays a causative role in diabetic embryopathy. We tested whether mitigating oxidative stress, using superoxide dismutase 1 (SOD1) transgenic mice, would block hyperglycemia-induced specific PKC isoform activation and its downstream cascade.

Study Design—Day 8.5 (E8.5) embryos from non-diabetic WT control (NC), diabetic mellitus WT (DM) and diabetic SOD1-Tg mice (DM-SOD1-Tg) were used for detection of phosphorylated (p-) PKC α/β II and p-PKC δ , and levels of two prominent PKC substrates, p-MARCKS and RACK1, and lipidperoxidation markers, 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA).

Results—Levels of p-PKC α/β II, p-PKC δ , p-MARCKS, 4-HNE and MDA were significantly elevated in the DM group compared with those in the NC group and the DM-SOD1-Tg group. The NC and DM-SOD1-Tg groups had comparable levels of these protein and lipidperoxidation markers. RACK1 levels did not differ among the three groups.

Conclusions—Mitigating oxidative stress by SOD1 overexpression blocks maternal hyperglycemia-induced activation of specific PKC isoforms and downstream cascades.

Keywords

SOD1 transgenic mice; PKC isoforms; MARCKS; lipidperoxidation; diabetic embryopathy

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INTRODUCTION

Pregestational diabetes is a significant risk factor for congenital malformations including major structure birth defects, neural tube defects (NTDs)¹. Maternal hyperglycemia increases the production of reactive oxygen species and impairs the intracellular antioxidant capability leading to oxidative stress²⁻⁹. Multiple evidence^{8, 10-13} support that hyperglycemia-induced oxidative stress causes malformations. Both *in vivo* and *in vitro* treatments with variety of antioxidants^{8, 10-13} can effectively reduce hyperglycemia-induced NTDs. Overexpression of the antioxidant enzyme, SOD1, in transgenic mice ameliorates maternal diabetes-induced NTDs¹³. Although oxidative stress appears to be a central mechanism underlying hyperglycemia-induced malformations, it is unclear whether the downstream intracellular signals mediate oxidative stress as well.

Oxidative stress activates multiple kinase signaling pathways. The Protein Kinase C (PKC) family consists twelve isoforms that involve diverse physiological and pathophysiological functions including cell proliferation, differentiation and apoptosis¹⁴. In diabetic embryopathy, prolonged PKC activation is associated with maternal diabetes-induced NTDs¹⁵. We have further reported that hyperglycemia specifically activates PKC α / β II and PKC δ ¹⁶. Specific pharmacological inhibitors to PKC α , PKC β II or PKC δ have been shown to significantly reduce hyperglycemia-induced NTDs¹⁶, strongly implicating that activation of these specific PKC isoforms plays a causative role in the induction of NTDs by hyperglycemia. PKC activation results in lipidperoxidation which leads to cell membrane damage¹⁷. PKC activation has been linked to altered AA metabolism during lipidperoxidation¹⁸. In diabetic embryopathy, hyperglycemia-induced lipidperoxidation alters cell membrane lipid metabolism by shifting arachidonic acid (AA) metabolism from prostaglandin E2 to isoprostanes¹⁹. The loss of membrane AA destabilizes the cell membrane structure and function. Conversely, AA supplementation has been shown to reduce the incidence of diabetic embryopathy^{11, 12, 20}. In the present study, we will determine if oxidative stress-induced specific PKC isoforms activation triggers lipidperoxidation which, in turns, intensifies the degree of oxidative stress in embryos exposed to hyperglycemia.

Beside the differential activation mechanisms of the twelve PKC isoforms, individual PKC isoforms exerts distinct physiological and pathophysiological functions via substrate specificity. Limited number of PKC substrate has been identified. Among the known PKC substrates, Myristoylated Alanine-Rich Protein Kinase C Substrate (MARCKS) is a prominent PKC substrate that primarily resides in neural tissues²¹. Furthermore, it has been reported that MARCKS are specific substrate of PKC β II²² and PKC δ ²³. Another prominent PKC substrate is the Receptor for Activated C Kinase 1 (RACK1) which is originally discovered through its binding to active PKC β II and other classic PKC isoforms²⁴. RACK1 participates in multiprotein signaling complexes and can enhance PKC-dependent JNK activation²⁵, which plays a causative role in the induction of diabetic embryopathy²⁶. Therefore, in the present study, we will assess the activation and levels of these two PKC substrates with a goal to define the role of PKC substrates in oxidative stress-mediated teratogenicity in diabetic embryopathy.

The connection between oxidative stress and PKC activation has not been explored. Because both SOD1 overexpression *in vivo*¹³ and PKC inhibition *in vitro*¹⁶ reduce hyperglycemia-induced malformations, in the present study we will use SOD1-Tg mice to test whether SOD1 overexpression *in vivo*, blocks hyperglycemia-induced specific PKC isoforms activation and its downstream cascade.

Material and methods

Animals and reagents

C57BL/6J mice (median body weight 22 g) were purchased from Jackson Laboratory (Bar Harbor, Maine). Streptozotocin (STZ) from Sigma was dissolved in sterile 0.1 M citrate buffer (pH 4.5). Sustained-release insulin pellets were purchased from Linplant (Linshin, Canada). SOD1-Tg mice in C57BL/6J background were revived from frozen embryos by the Jackson Laboratory (Stock number: 002298).

Mouse models of diabetic embryopathy

The procedures for animal use were approved by the Institutional Animal Care and Use Committee of University of Maryland School of Medicine. Eight-week old Wildtype (WT) and SOD1-Tg mice were intravenously (iv) injected daily with 75 mg/kg STZ over two days to induce diabetes. Once a level of hyperglycemia indicative of diabetes (>250 mg/dl) was achieved, insulin pellets were subcutaneously implanted in these diabetic mice to restore euglycemia prior to mating. The mice were then mated with male mice of the same respective genotype. On Day 5 of pregnancy (E5), insulin pellets were removed to permit frank hyperglycemia (>250 mg/dl glucose level), so the developing conceptuses would be exposed to a hyperglycemic environment at E7 onwards. WT non-diabetic female mice with vehicle injections and sham operation of insulin pellet implants served as non-diabetic controls. On E8.5, mice were euthanized, and conceptuses were dissected out of the uteri for analysis. To avoid any redundancy, data of malformation incidences was not collected because it has been published elsewhere⁹. The typical malformations observed in embryos from diabetic WT mice are NTDs.

Western Blotting

Western Blotting was performed as described by Yang, et al.²⁷. Briefly, embryonic samples were sonicated in 80 μ l 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 μ g) were resolved by SDS-PAGE and transferred onto an Immobilon-P (Millipore). Membranes were incubated for 18 h at 4 °C with the following primary antibodies at 1:1000 to 1:2000 dilutions in 5% nonfat milk: anti-p-PKC α / β II (#9375), anti-p-PKC δ (#9374), anti-p-MARCKS (#2741), anti-RACK1 (#4716) and anti-SOD1 (human specific, #4266), all from Cell Signaling; anti-MDA (EMD, #442730); anti-4-HNE (Chemicon, #AB5605). Membranes were exposed to goat anti-rabbit, anti-mouse (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 were 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 $p < 0.05$.

Results

SOD1 overexpression blocks maternal hyperglycemia-induced PKC α / β II and PKC δ activation

To investigate whether mitigating oxidative stress, using SOD1-Tg transgenic mice, blocks hyperglycemia-induced specific PKC isoform activation, E8.5 embryos from non-diabetic WT control (NC), diabetic mellitus WT (DM) and diabetic SOD1-Tg mice (DM-SOD1-Tg) of C57BL/6J background were used for determination of p-PKC α / β II and p-PKC δ levels. SOD1 overexpression did not affect diabetic status because mice in the DM and DM-SOD1-Tg groups had comparable glucose levels which were about 4-fold higher than that in the NC group (Table 1). Levels of p-PKC α / β II and p-PKC δ in the DM group were significantly higher than those in the NC group, and levels of p-PKC α / β II and p-PKC δ in the DM-SOD1-Tg group were significantly lower compared to those in the DM group (Fig. 1A and B). The results show that maternal hyperglycemia activates PKC α / β II and PKC δ , and that SOD1 overexpression blocks activation of these PKC isoforms.

SOD1 overexpression reduces maternal hyperglycemia-induced MARCKS phosphorylation

Because SOD1 overexpression blocks maternal hyperglycemia-induced PKC α / β II and PKC δ activation, we sought to determine the levels and activities of two downstream effectors MARCKS and RACK1 of PKC α / β II and PKC δ . Levels of p-MARCKS in the DM group was significantly higher than that in the NC group, and the level of p-MARCKS in DM-SOD1-Tg group was significantly lower than that in the DM group (Fig. 2A). In contrast, RACK1 levels did not differ among the three experimental groups (Fig. 2B). These results provide strong evidence that SOD1 overexpression blocks the maternal diabetes-induced PKC and the cascading sequelae by suppressing the activation of downstream substrates such as MARCKS.

SOD1 overexpression reduces maternal hyperglycemia-induced lipidperoxidation

Because PKC activation directly results in lipidperoxidation, we determined the levels of two major cytotoxic aldehydes, 4-HNE and MDA, which are prominent lipidperoxidation markers in other systems. Levels of 4-HNE were significantly higher in the DM group compared to those in the NC group and the DM-SOD1-Tg group (Fig. 3A). Similarly, levels of MDA in the DM group were significantly increased compared to those in the NC group and the DM-SOD1-Tg group (Fig. 3B). Quantification of specific sizes of protein modified by MDA showed significantly elevated levels in the DM group compared to the NC and DM-SOD1-Tg group (Fig. 3Ba and 3Bb). The NC and DM-SOD1-Tg groups had comparable levels of these two lipidperoxidation markers (Fig. 3A, B). These results demonstrate that maternal diabetes induces lipidperoxidation via PKC activation, and mitigating oxidative stress by SOD1 overexpression blocks lipidperoxidation.

Comment

In the present study, we have provided for the first time direct *in vivo* evidence that oxidative stress causes activation of PKC α / β II and PKC δ in diabetic embryopathy. The SOD1-Tg mouse line used in our study is a valid tool in suppressing hyperglycemia-induced oxidative stress. This transgenic line carries the human SOD1 gene and has been demonstrated that the protein products of the transgene expressed in mouse tissue have high enzymatic activities^{13, 28}. Hyperglycemia increases the production of superoxide, and SOD1 effectively reduces oxidative stress by converting superoxide into oxygen and hydrogen peroxide. As previously reported¹³, we have also found that SOD1-Tg mice do not exhibit any excess embryonic malformations. Our results demonstrate that SOD1 overexpression

effectively reduces diabetic embryopathy through blockade of PKC α / β II and PKC δ activation.

Enhanced apoptosis in vulnerable embryonic tissues such as the neural tube in embryos exposed to hyperglycemia leads to malformations^{6, 16}. Oxidative stress activates pro-apoptotic intracellular signaling in diabetic embryopathy^{6, 8, 9}. Because the activation of PKC α / β II and PKC δ mediates the teratogenicity of oxidative stress, we propose that PKC α / β II and PKC δ are pro-apoptotic intracellular signaling intermediates. Indeed, PKC δ induces apoptosis in certain cell types¹⁴. We and others have demonstrated that maternal diabetes-induced apoptosis is caspase dependent^{6, 29}. Dietary antioxidant supplementation blocks maternal hyperglycemia-induced caspase activation³⁰. Because activation of PKC α / β II and PKC δ is a downstream event of oxidative stress, inhibition of the activation of PKC isoforms would block caspase-dependent apoptosis. In a separate study (manuscript submitted), we have determined that inhibition of PKC β II blocks hyperglycemia-induced caspase activation and apoptosis. Future studies will aim to delineate the apoptotic mechanism underlying PKC α / β II and PKC δ activation leads to the induction of diabetic embryopathy.

PGE₂-like isoprostanes, such as 8-iso-prostaglandin F₂ (8-iso-PGF₂) and 8-iso-PGF₂ α , have been identified as lipidperoxidation biomarkers in diabetic embryopathy¹⁹. In the present study, we have further identified two additional lipidperoxidation biomarkers, MDA and 4-HNE in diabetic embryopathy. The protein adducts resulting from MDA or 4-HNE modifications are induced by oxidative stress. However, SOD1 overexpression abolishes maternal hyperglycemia-increased activities of these two biomarkers. MDA, a low-molecular-weight aldehyde, is a byproduct of oxidized polyunsaturated fatty acid such as AA. The increase in MDA in embryos exposed to diabetes provides further evidence that AA metabolism is altered in diabetic embryopathy. MDA cross-links to protein to form protein adducts which is cytotoxic to the cell³¹. Plasma MDA levels are robust indicators of oxidative stress in pathophysiological conditions^{32, 33}, suggesting that MDA may be a suitable peripheral biomarker in diabetic embryopathy. 4-HNE is also a cytotoxic aldehyde and is formed during oxidation of omega-6 fatty acids. 4-HNE reacts with several amino acid residues of proteins to form 4-HNE-protein adducts. 4-HNE can directly induce cell apoptosis³⁴, indicating that 4-HNE may directly participate in hyperglycemia-induced apoptosis leading to malformations. 4-HNE can activate c-Jun-N-Terminal kinase (JNK)³⁵, which plays a causative role in the induction of diabetic embryopathy²⁶, suggesting that 4-HNE may act as an intermediate in the link between PKC activation and JNK activation in hyperglycemia-induced malformations.

Our data provide strong evidence that the activity of MARCKS is increased in concert with the activation of PKC α / β II and PKC δ in embryos exposed to maternal hyperglycemia, whereas RACK1 levels are not affected. This is due to the lack of antibody against phosphorylated RACK1 which directly reflects the activity of RACK1³⁶. Nevertheless, we have determined that SOD1 overexpression blocks hyperglycemia-enhanced MARCKS activities concurrent with the inhibition of PKC α / β II and PKC δ activation. These findings implicate MARCKS as an important substrate of these PKC isoforms in diabetic embryopathy.

Non-phosphorylated MARCKS is associated with cell membrane via its myristoylation domain³⁷. Membrane-associated MARCKS binds to calmodulin, membrane lipids and F-actin filaments²¹. PKC phosphorylates the serine residues of MARCKS thus induces MARCKS translocation from the membrane to the cytosol³⁸. It will be of interest to examine if MARCKS membrane disassociation resulted from phosphorylation contributes to hyperglycemia-induced lipidperoxidation and subsequent cell membrane damage. PKC-

induced MARCKS phosphorylation may disrupt some of the physiological functions of MARCKS such as cell adhesion, membrane trafficking and cell motility³⁹. In addition, myristoylation and phosphorylation may be two counteractive events in regulation of MARCKS functions under physiological and pathophysiological conditions. Future studies may focus on the differential effects of MARCKS myristoylation and phosphorylation on hyperglycemia-induced oxidative stress, apoptosis and malformations.

In summary, our study demonstrates that hyperglycemia specifically induces PKC α / β II and PKC δ phosphorylation, and that oxidative stress is responsible for the activation of these specific PKC isoforms (Fig. 4). The activation of these PKC isoforms triggers lipidperoxidation and MARCKS phosphorylation, which may lead to cellular stress and consequently apoptosis and malformations (Fig. 4). SOD1 overexpression abolishes this PKC cascade activation, suggesting that the PKC cascade mediates the teratogenicity of oxidative stress in diabetic embryopathy. Our results provide mechanistic basis for the development of antioxidant and specific PKC α / β II and PKC δ inhibitors to prevent against human NTDs. Antioxidants have been shown effective in animal studies^{10–12} and we have proposed multi-vitamin clinical trails in diabetic pregnant women. Epidemiological studies^{1, 40} have shown that pregnant women who take periconceptional multi-vitamin have lower risks to have a baby with birth defects than those who do not. Development of safe and effective PKC isoforms specific inhibitor will be new area with much potential to prevent against maternal-diabetes-induced birth defects.

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Figure 1.

SOD1 overexpression blocks maternal hyperglycemia-induced p-PKCα/βII and p-PKCδ activation. The upper panels of **A** and **B** showed representative images of p-PKCα/βII and p-PKCδ Western blotting, respectively. The lower panels of **A** and **B** were the graphic data of densitometric analysis of protein levels of p-PKCα/βII and p-PKCδ, respectively. β-actin served as an equal protein loading control. Data expressed as Mean ± standard errors (SE) (n = 3). **NC**: non-diabetic WT control. **DM**: Diabetic WT and **DM-SOD1-Tg**: diabetic SOD1 Tg. * denotes significant difference ($p < 0.05$) and ** means significant difference ($p < 0.01$) when compared to the diabetic DM group.

Figure 2. SOD1 overexpression abolishes maternal hyperglycemia-induced p-MARCKS activations. The upper panels of **A** and **B** showed representative images of p-MARCKS and RACK1 Western blotting, respectively. The lower panels of **A** and **B** were the graphic data of densitometric. β -actin served as an equal protein loading control. Data expressed as Mean $\pm$ standard errors (SE) ($n = 3$). **NC**: non-diabetic WT control. **DM**: Diabetic WT and **DM-SOD1-Tg**: diabetic SOD1 Tg. * ($p < 0.05$) and ** ($p < 0.01$) denote significant difference when compared to the DM group.

Figure 3.

SOD1 overexpression blocks maternal hyperglycemia-induced lipidperoxidation. The upper panels of **A** and **B** showed representative images of 4-HNE and MDA Western blotting, respectively. The lower panel of **A** was the graphic data of densitometric analysis of the bands which were pointed by the arrow. In panel **B**, **Ba** and **Bb** pointed specific bands which were quantitatively graphed in the underneath bar graphs (**Ba** and **Bb**). β -actin served as an equal protein loading control. **NC**: non-diabetic WT control. **DM**: Diabetic WT and **DM-SOD1-Tg**: diabetic SOD1 Tg. Data expressed as Mean $\pm$ standard errors (SE) ($n = 3$). * denotes significant difference ($p < 0.05$) when compared to the DM group.

Figure 4.

A schematic shows the oxidative stress-induced PKC cascade in diabetic embryopathy. Maternal hyperglycemia induced-oxidative stress activates PKC α / β II and PKC δ , which trigger lipidperoxidation and MARCKS phosphorylation. Lipidperoxidation and translocation of p-MARCKS from the cell membrane result in cell membrane damage and cellular stress leading to apoptosis.

Table 1

Glucose levels in non-diabetic WT Control, diabetic WT and diabetic SOD1-Tg mice

Group	Average glucose level (mg/dl)
Non-diabetic WT Control (NC)	119.5 ± 5.5*
Diabetic Mellitus WT (DM)	428.5 ± 10.1
Diabetic SOD1-Tg (DM-SOD1-Tg)	419 ± 8.5

Data are presented as mean ± SE (n = 5). Average glucose level is the mean of the glucose levels at E7–E8.

* denotes significant differences compared to the DM and the DM-SOD1-Tg groups.