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CASPASE-8 - A KEY ROLE IN THE PATHOGENESIS OF DIABETIC EMBRYOPATHY

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Abstract

Maternal diabetes causes neural tube defects in embryos which are associated with increased apoptosis in the neuroepithelium. Many factors, including effector caspases, have been shown to be involved in the events, however, the key regulators have not been identified and the underlying mechanisms remain to be addressed. Caspase-8, an initiator caspase, has been shown to be altered in diabetic embryopathy, suggesting a role as an upstream apoptotic regulator. Using mouse embryos as a model system, this study demonstrates that caspase-8 is required for the production of hyperglycemia-associated embryonic malformations. Caspase-8 was shown to be expressed in the developing neural tube. Its activity, as evidenced by enhanced cleavage, was increased by hyperglycemia. These changes were associated with increased formation of the active cleavage of Bid. Inhibition of caspase-8 activity in high glucose-challenged embryos reduced the rate of embryonic malformation and this was associated with decreased apoptosis in the neuroepithelium of the neural tube. Inhibition of caspase-8 activity also reduced hyperglycemia-induced Bid activation and caspase-9 cleavage. These data suggest that caspase-8 may control diabetic embryopathy-associated apoptosis via regulation of the Bid-stimulated mitochondrion/caspase-9 pathway.

Keywords

diabetic embryopathy; neural tube; embryo; caspase-8; Bid; apoptosis

Introduction

Diabetes mellitus in pregnancy causes abnormal development of the embryo and fetus (Eriksson et al., 2000; Reece and Eriksson, 1996; Zhao and Reece, 2005). The congenital birth defects caused by maternal diabetes are commonly manifest in the central nervous system as neural tube defects (NTDs). These malformations are believed to form a failure in closure of the neural folds during the early stage of embryogenesis (Loeken, 2005; Zhao and Reece, 2005). In rodents, which share similar mechanisms of diabetic embryopathy with humans, cell death (apoptosis) in the neuroepithelium of the neural tube is a hallmark of maternal diabetes-induced NTDs (Fine et al., 1999; Reece et al., 2005; Zhao et al., 2008).

Apoptosis is precisely controlled by a host of factors that function as an apoptotic cascade (Taylor et al., 2008). Key enzymes that execute cell demise at the late stages of apoptosis are

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a group of cysteine proteases, known as effector (or executioner) caspases, including caspase-3, -6, and -7 (Boatright and Salvesen, 2003; Riedl and Salvesen, 2007; Salvesen and Riedl, 2008). Activity of these effector caspases is controlled by initiator caspases, including caspase-8 and -9 (Riedl and Salvesen, 2007).

Initiator caspases are activated via different mechanisms and regulate different apoptotic pathways. Bcl-2 family proteins, including pro-apoptotic (e.g., Bid, Bak, and Bax) and anti-apoptotic (e.g., Bcl-2 and Bcl-xL) members play a key role in this process (Garrido et al., 2006). Caspase-8 is activated by the death-inducing signaling complex (DISC) which is formed by extracellular ligand-bound death receptors (Degterev and Yuan, 2008; Riedl and Salvesen, 2007). DISC recruits pro-caspase-8 and cleaves it into smaller molecules to form active dimers. Bid is a 22 kDa HB3 domain only member of the Bcl-2 family that is activated by being cleaved into a truncated 15 kDa form (tBid), which is catalyzed by caspase-8. tBid, in turn, interacts with the mitochondria to open pores composed of Bak and Bax on the mitochondrial outer membrane. Cytochrome C is then released from these pores into the cytosol (Esposito, 2002; Yin, 2006). Cytochrome C release leads to the formation of the apoptosome which is comprised of a complex of cytochrome C and apoptotic protease activating factor (Apaf)-1 (Riedl and Salvesen, 2007). Caspase-9 is activated by interaction with the apoptosome complex. In addition, caspase-8 also directly activates the effector caspases (Degterev and Yuan, 2008).

The processes that lead to diabetic embryopathy in the embryos of diabetic mothers are not well understood. However, it is clear that caspase-3 and -6 activity and Bax levels are increased, implying alterations in the apoptotic processes (Gareskog et al., 2007; Reece et al., 2005; Sun et al., 2005; Yang et al., 2008b). However, knowledge is limited regarding the processes that account for this increased apoptosis. As a key upstream regulator, caspase-8 is also activated by maternal diabetes (Toder et al., 2002), suggesting a key role in apoptotic regulation in diabetic embryopathy. In the present report, we demonstrate that caspase-8 is an essential factor in hyperglycemia-induced embryonic malformations. Our data also suggest that caspase-8 is required for activation of downstream caspases and is essential for the neuronal malformations observed in diabetic embryopathy.

Materials and Methods

Embryo culture

This use of animals in was approved by the Institutional Animal Care and Use Committee of University of Maryland Baltimore. 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 in Hank's saline. The parietal yolk sac was removed using a pair of fine forceps and the visceral yolk sac was left intact. Embryos (5–6/bottle) were cultured in 5 ml of rat serum at 38°C in 30 rev/min rotation in the roller bottle system (Sturm and Tam, 1993; Zhao and Rivkees, 2003). Embryos were treated with D-glucose (22 mM) with or without caspase-8 inhibitor II (5–10 μ M; EMD Biosciences). Embryos cultured in 8 mM glucose were used as euglycemic control. For examining the development of the embryos at the morphological level, the embryos were cultured for 48 hours. For assessing gradual changes in caspase-8 activation at the molecular level, the embryos were cultured for 6, 18, 24, and 48 hours.

At the end of culture, NTDs were characterized as opened neural tube either in the brain or spinal cord regions. A malformation rate was calculated as a percentage of the embryos with NTDs in total number of embryos. For molecular assays, the neural tubes were isolated in cold PBS (pH 7.4) for protein extraction.

Whole mount cell death assay

At the end of culture, the embryos were dissected out of the yolk sacs. After washing with phosphate-buffered saline (PBS; pH 7.4), the embryos were stained with 2 μ M calcein AM for living cells and 4 μ M EthD-1 for dead cells (Invitrogen). The stained embryos were observed under a fluorescent dissecting microscope (Zeiss). Images of whole embryo specimens were captured using a digital camera.

Immunocytochemistry

Mouse embryos at E9.5 and E10.5 were dissected out of the uteri in cold PBS (pH 7.4) and immediately frozen on dry ice in Tissue-Tek medium. Tissue sections in 8 μ m thickness were cut using a cryostat and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 minutes. Tissues sections were incubated with antibodies against caspase-8 (Cell Signaling) at 4°C overnight, and, then, with secondary antibodies conjugated with fluorescein at room temperature for 1 hour. After counterstained with propidium iodide, the sections were observed under a fluorescent microscope. Images of the specimens were captured using a digital camera.

Western blot

Isolated neural tube tissues from 4–5 embryos were homogenized in a lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM DTT, 5 mM EDTA) containing protease inhibitor cocktails (Thermo Scientific). The tissue homogenates were centrifuged at 13,000 rpm for 15 minutes at 4°C, and the supernatants were collected.

Samples containing 40–50 μ g of protein were resolved in 15% SDS-polyacrylamide gel using electrophoresis and blotted onto Hybond ECL nitrocellulose membranes (Amersham Biosciences) using a mini-PROTEAN blotting apparatus (Bio-Rad). The membranes were probed with antibodies against caspase-8 (Alexis Biochemicals; Cell Signaling), caspase-3, caspase-9, and Bid (Cell Signaling). The same membranes were stripped using Restore Western Blot Stripping Buffer (Thermo Scientific) and probed again with antibodies against β -tubulin (Santa Cruz Biotechnologies) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Millipore) to ensure equal loading of protein samples. Signals were detected using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnologies) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Chemiluminescent signals were captured using a UVP Bioimage EC3 system (UVP).

Data process and analysis

Montages of the digital images were produced using Adobe Photoshop. Fluorescence intensities of the bands on Western blots were measured using VisionWorksLS software (UVP) associated with the imaging system. The difference between the control and high glucose treatment was analyzed using student *t*-test, with the *p* value <0.05 indicating a significant difference.

Results

Localization of caspase-8 in the neural tube

To investigate the role of caspase-8 in diabetic NTDs, the first question was asked whether caspase-8 is present in the developing neural tube. Localization of caspase-8 protein was examined in embryos of normal mice using Immunocytochemistry. Immunoactivity of caspase-8 was detected in the neuroepithelium and ectoderm of the embryos at E9.5 and E10.5 (Fig. 1).

Activation of caspase-8 by high glucose

Caspase-8 activation, as indicated by a presence of the 18 kDa form, was examined using Western blot assay. A moderate increase in the 18 kDa fragment was detected in the embryos treated with high glucose for 18 hours. A significant increase in caspase-8 activation was observed at the 24 hour point (Fig. 2). At the same time, Bid cleavage into tBid was also seen in the embryos treated with high glucose (Fig. 2).

Caspase-8 inhibition in neural tube defects

The involvement of caspase-8 in diabetic embryopathy was addressed by inhibiting its activity in the embryos exposed to a high concentration of glucose *in vitro*. Embryos at the neural fold stage (E8.0) were cultured in euglycemic (8 mM) and hyperglycemic (22 mM) levels of glucose for 48 hours. High concentration of glucose caused higher rate of neural tube defects (71.4%), as previously reported (Reece et al., 1996; Yang et al., 2008a; Zhao et al., 2008). Caspase-8 inhibitor at 2, 5, 10, and 20 μ M exerted no effect on the development of the embryos cultured in euglycemia; whereas 5, 10, and 20 μ M reduced the malformation rates in the embryos cultured in high glucose (data not shown). In subsequent experiments, only 5 μ M of caspase-8 inhibitor was used. Caspase-8 inhibition significantly reduced malformation rates (9.5%), to the similar level in control groups cultured in normal glucose (5%; Table 1).

Effect of caspase-8 inhibition on apoptosis

It has been characterized that increased apoptosis in the neuroepithelium is associated with neural tube malformations caused by hyperglycemia (Fine et al., 1999; Zhao et al., 2008). To address whether caspase-8 blockade also reduces apoptosis, cell death was first examined in whole mount embryos. The level of cell death was significantly decreased in the embryos treated with caspase-8 inhibitor while cultured in high concentration of glucose, in comparison with ones cultured in high glucose alone (Fig. 3). It was similar to control level (Fig. 3).

To determine if the cell death was apoptotic, levels of caspase-3 activation (cleavage) was examined. In correlation with the results of whole mount cell death assay, caspase-3 cleavage was also inhibited by caspase-8 inhibitor (Fig. 4).

Bid and caspase-9 cleavage

Caspase-8 is involved in a number of pathways leading to apoptosis. To investigate whether caspase-8 regulates apoptosis in diabetic embryopathy through activating the Bid/mitochondrion/caspase-9 pathway, cleavage of Bid and caspase-9 was examined in the embryos treated with high glucose and caspase-8 inhibitor. Significant increases in the cleaved Bid (tBid; 15 kDa) and caspase-9 (37 kDa) were observed in the embryos cultured in a high concentration of glucose, in comparison with normal control. In the embryos exposed to high glucose, treatments with caspase-8 inhibitor significantly reduced the levels of tBid and cleaved caspase-9, compared with the embryos incubated in high glucose alone (Fig. 5).

Discussion

Neural tube defects in diabetic embryopathy are believed to be caused by excessive cell death in the neuroepithelium during neurulation (Gareskog et al., 2007; Phelan et al., 1997; Zhao et al., 2008). The cell demise appears to be associated with the apoptotic pathways involving effector caspase-1, and -3 (Forsberg et al., 1998; Gareskog et al., 2007; Reece et al., 2006). These effector caspases can be cleaved (activated) by a large number of proteases. However, the responsible proteases have not been identified in diabetic

embryopathy. Data in this report strongly suggest that caspase-8 is most likely involved in activation of the effector caspases.

Caspase-8 can induce apoptosis through directly cleaving effector caspases or stimulating the mitochondria/caspase-9 pathway (Degterev and Yuan, 2008; Salvesen and Riedl, 2008). The latter has been implicated in diabetic embryopathy with the observations of altered Bcl-2, Bax, and effector caspases (Forsberg et al., 1998; Gareskog et al., 2007; Phelan et al., 1997; Reece et al., 2005; Reece et al., 2006; Sun et al., 2005; Torchinsky et al., 2003; Yang et al., 2008b; Zabihi et al., 2007).

Bax and Bak form complexes (pores) on the outer membrane of the mitochondrion (Antignani and Youle, 2006; Sharpe et al., 2004). Upon activated by tBid, the pores open to release cytochrome C, which, in turn, activates caspase-9 in the cytoplasm (Degterev and Yuan, 2008). In this study, an increase in the level of tBid was observed in the embryos exposed to high glucose, suggesting a potential role for tBid in the triggering of the mitochondria-dependent apoptotic pathways in hyperglycemia-induced apoptosis. Further experiments of caspase-8 inhibition clearly demonstrate the action of caspase-8 in activating the tBid/mitochondrion/caspase-9 pathway.

Caspase-8 activation has been extensively characterized in DISC induced by members of the TNF α family (Degterev and Yuan, 2008; Riedl and Salvesen, 2007). However, TNF α does not seem to promote the effect of maternal diabetes on embryonic development (Torchinsky et al., 2004), suggesting other molecular pathways in caspase-8 activation. For example, stress-response c-Jun N-terminal kinases (JNKs) have been shown to be involved in caspase-8 activation (Cho and Choi, 2002). Interestingly, we recently showed that *jnk2* plays a role in diabetic embryopathy (Yang et al., 2007). The relationship between JNKs and caspase-8 remains to be addressed.

In addition to JNKs, caspase-8 is also activated by other factors, including reactive oxygen species (ROS), which play a key role in diabetic embryopathy (Kim and Chung, 2007; Perez-Cruz et al., 2007). Caspase-8, on the other hand, plays a part in the production of ROS in mitochondria via tBid (Webster et al., 2006). Such positive feedback regulation may result in an augment in apoptosis in the embryo. Based on the observations that caspase-8 is sufficient to mediate the effect of maternal hyperglycemia on embryonic neural tube malformations, it is more important to investigate whether the molecular pathways involving caspase-8 consist of the crucial molecular system in mediating the effect of maternal hyperglycemia on embryonic development.

The intracellular signaling in maternal diabetes-induced embryonic apoptosis is a complex network. Identification of the dominant pathways and key factors will be the main focus of future studies, in order to discover interventional targets for preventing maternal diabetes-associated birth defects. A number of factors, such as protein kinase C family, JNKs, and caspase-8, have been found to be essential in diabetic embryopathy, and potential molecular pathways have been suggested (Reece et al., 2006; Yang et al., 2007; 2008a; Zhao et al., 2008). Delineation of the relationship among these pathways will provide an important insight into the molecular mechanisms of diabetic embryopathy.

Acknowledgments

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Figure 1. Localization of caspase-8 in the neural tube. (A) Immunocytochemistry of E10.5 mouse embryos with an anti-caspase-8 antibody. (B, D) Propidium iodide staining showing the structure of the tissues. (C) Negative control (without primary antibody). Dot lines indicate the boundary between neuroepithelium (ne) and ectoderm (ed). Scale bar = 100 μ m, applied to all images.

Figure 2.

Activation of caspase-8 and Bid by high glucose. Western blot assays of caspase-8 (18 kDa) and Bid (tBid) cleavage in the neural tubes of the embryos cultured in control (CON) and high glucose (HG) for 24 hours. GAPDH is used to control equal loading of the samples.

Figure 3.

Effects of caspase-8 inhibition on the development and apoptosis of the embryos cultured in high glucose. (A,D) Normal glucose control (CON). (B,E) High glucose treatment (HG). Open anterior neural tube is evident. (C, F) High glucose + caspase-8 inhibitor (Casp8-I; 5 μ M). (A,B,C) Calcein AM staining for living cells. (D,E,F) EthD-1 staining for apoptotic cells. Arrow heads indicate the neuroepithelium of the midbrain. fb, forebrain; hb; hindbrain; mb, hindbrain. Scale bar = 500 μ m, applied to all the images.

Figure 4. Effect of caspase-8 inhibition on caspase-3 activation. Western blot assay of caspase-3 cleavage. CON, normal glucose control; HG, high glucose treatment; Casp8-I, caspase-8 inhibitor (5 μ M). GAPDH is used to control equal loading of the samples.

Figure 5.

Effect of caspase-8 inhibition on cleavage of Bid and caspase-9 in the embryos cultured in high glucose. CON, normal glucose control; HG, high glucose treatment; Casp8-I, caspase-8 inhibitor (5 μ M). GAPDH is used to control equal loading of the samples.

Table 1

Effect of caspase-8 inhibition on NTDs in high glucose-treated embryos

	Total embryos	Malformed embryos	MR (%)
Control (glucose 8 mM)	20	1	4.25 ± 4.25
High glucose (HG; 22 mM)	21	15	74.25 ± 4.23
HG + Casp8-I (5 μM)	21	2	8.50 ± 4.91 *

MR, malformation rate (% of malformed in total embryos).

* significant different from the HG group (*t*-test; *p*>0.05, *n* = 4)