Diabetes-Induced Fetal Growth Retardation is Associated with Suppression of NF- κ B Activity in Embryos

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■ Abstract

BACKGROUND: Mechanisms underlying diabetes-induced fetal growth retardation remain largely undefined. Two events such as the persistent activation of apoptosis or suppression of cell proliferation in embryos might directly result in fetal growth retardation. Evidence implicating the transcription factor NF- κ B in the regulation of the physiological and teratogen-induced apoptosis as well as cell proliferation suggests that it may be a component of mechanisms underlying this pathology. To address this issue, this study was designed to test: 1) whether diabetes-induced fetal growth retardation is preceded by the modulation of NF- κ B activity in embryos at the late stage of organogenesis and 2) whether apoptosis is altered in these embryos. **METHODS:** The embryos and placentas of streptozotocin-induced diabetic mice collected on days 13 and 15 of pregnancy were used to evaluate the expression of NF- κ B, I κ B α and phosphorylated (p)-I κ B α proteins by Western blot analysis and NF- κ B DNA

binding by an ELISA-based method. The detection of apoptotic cells was performed by the TUNEL assay and the expression of a proapoptotic protein Bax was evaluated by the Western blot. **RESULTS:** The embryos of diabetic mice were significantly growth retarded, whereas the placental weight did not differ in diabetic or control females. Levels of NF- κ B and p-I κ B α proteins as well as the amount of NF- κ B DNA binding was lower in embryos of diabetic mice as compared to those in controls. However, neither excessive apoptosis nor an increased Bax expression was found in growth-retarded embryos and their placentas. **CONCLUSION:** The study herein revealed that diabetes-induced fetal growth retardation is associated with the suppression of NF- κ B activity in embryos, which seems to be realized at the level of I κ B degradation.

Keywords: diabetes · pregnancy · intrauterine growth retardation · apoptosis · NF- κ B · Bax

Introduction

Diabetic pregnancy is associated with complications such as early and late embryonic death, inborn anomalies in newborns as well as fetal growth disorders [1, 2]. Nowadays, studies addressing mechanisms underlying these embryopathies seem mainly to be focused on embryonic death or the formation of inborn structural anomalies, while much less is known about

those underlying diabetes-induced fetal growth disorders.

Excessive apoptosis is presently seen as a pivotal event in the pathogenesis of diabetes-induced embryonic death and structural anomalies [3] and an association between this apoptosis and diabetes-induced fetal growth retardation has also been suggested [4]. On the other hand, the inhibition of cell proliferation in embryos during organogenesis may also be an event cul-

minating in growth-retarded fetuses. In this situation, the transcription factor NF- κ B seems to be a good candidate for studies addressing the mechanisms of this pathology.

NF- κ B is a collective name for transcription factors belonging to the Rel family comprising of several related proteins [5]. In most cell types, NF- κ B exists in a latent form in the cytoplasm bound to several NF- κ B inhibitor proteins (I κ Bs) [6]. NF- κ B may be activated by distinct stimuli [7], mainly, through a signaling cascade including the activation of the I κ B kinases (IKKs), phosphorylation and degradation of I κ Bs leading to the release of NF- κ B dimers, which then move to the nucleus, bind to target genes and stimulate their transcription [6].

NF- κ B is presently considered as a key molecule controlling the apoptosis process acting mainly to prevent cell death [8, 9]. It acts transcriptionally in embryos during organogenesis NF- κ B and one of its major components, p65 (RelA), has proved to be indispensable in protecting the embryonic liver against TNF α -induced physiological apoptosis [10]. Recently, studies performed in a number of labs including our work have provided evidence implicating NF- κ B in the regulation of the response of embryos to teratogens such as thalidomide [11], cyclophosphamide [12, 13], alcohol [14] and phenytoine [15] as well as to diabetes-generated embryotoxic and teratogenic stimuli [16, 17]. There is also evidence demonstrating that some teratogens initiate the process of mal-development by affecting proliferation in target cell population [18]. NF- κ B was shown to have the potential to positively regulate cell cycle progression by means of the transcriptional regulation of cyclin D [9, 19]. However, unlike its role in the regulation of teratogen-induced apoptosis, the involvement of NF- κ B in the regulation of cell proliferation in embryos exposed to developmental toxicants is yet to be revealed.

In order to address the role of NF- κ B in mechanisms underlying diabetes-induced fetal growth retardation, we first tried to evaluate whether this effect is preceded by an alteration of NF- κ B activity in embryos at the late stage of organogenesis and whether apoptosis is altered in these embryos. Also, since intrauterine growth retardation is often associated with placental dysfunctions [20], placentas of these embryos were also used as targets in this study. NF- κ B (p65) DNA-binding and the expression of NF- κ B (p65), I κ B α and phosphorylated (p)-I κ B α proteins were evaluated to characterize NF- κ B activity. We also characterized the expression of Bax, a proapoptotic protein, which was suggested to be acting in apoptotic signaling pathways

underlying diabetes-induced embryotoxic and teratogenic effects [21-23].

Material and methods

Animal models

Six to eight week old ICR mice were obtained from the Tel Aviv University animal facility. Females of the same weight were randomly distributed between control and experimental groups. The animals were kept on a 14hr light/10hr dark cycle with food and tap water *ad libitum*. Females were caged with males for 3 hr, from 07:00 to 10:00 (dark period). The presence of a vaginal plug (11:00) was designated as day 1 of pregnancy.

To induce diabetes, females were injected i.p. with 240mg/kg streptozotocin (STZ) (Sigma, St. Louis, MI) in buffer acetate (pH 4.2) before mating. The blood glucose level (BGL) was measured with a commercially available glucometer (MediSense®, Abbott Laboratories, Bedford, MA, USA) in blood samples obtained from the orbital sinus on day 3 of pregnancy and then on days 13 or 15 of pregnancy. As the BGL in intact ICR female mice is estimated to be 6.7 ± 0.93 mmol/l ($M \pm SD$, $n = 22$) [24], STZ-treated females having a $BGL \geq 9.5$ mmol/l (mean ± 3 SD) on day 3 of pregnancy were considered as diabetic. As prominent fetal growth retardation was previously observed only in females having a $BGL \geq 25$ mmol/l [24], only females with that or higher BGL on days 13 and 15 of pregnancy were used in the study. Females injected with the buffer were used as controls.

The pregnant females were divided into two groups according to the times of testing, i.e. days 13 and 15 of pregnancy. Eight pregnant females from each experimental and control group were used to evaluate the number of implantation sites, resorptions and the weight of embryos and placentas. The experiments on the animals were approved by the Ethics Committee for Animal Use of Tel Aviv University.

Evaluation of Apoptosis

The detection of apoptotic cells in tissue sections of embryos and placentas collected on days 13 or 15 of pregnancy was performed by the Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method as described elsewhere [13]. Briefly, three or four embryos having no external anomalies (e.g., encephalocele, anophthalmia) and their placentas obtained from different litters of diabetic and control females, were fixed in formaldehyde, dehy-

drated in alcohol and xylene and embedded in paraffin. Tissue sections (7 μ M) were prepared and after deparaffinization, the nuclei were labeled with biotinylated-dUTP (Clontech Laboratories, Palo Alto, CA) by using the TdT enzyme (Promega, Madison, WI). Then, sections were incubated with Streptavidin-HRP (Zymed Laboratories, South San Francisco, CA) and the apoptotic cells were detected by incubation with Diaminobenzidine (DAB Substrate Kit, Zymed Laboratories, South San Francisco, CA) in the presence of hydrogen peroxide. The slides were counterstained with hematoxylin, mounted in glycerol-gelatine and visualized under a light microscope.

Western blot analysis

The head and trunk (without viscera) of embryos collected on days 13 or 15 of pregnancy and the placentas were used to evaluate the expression of Bax, NF- κ B (RelA), I κ B and phosphorylated (p)-I κ B proteins. In order to prepare whole-cell extracts, embryonic samples and placentas were homogenized in ice-cold RIPA buffer containing 1% NP40, 0.1% SDS, 0.5% Sodium Deoxycholate and 14.3% protease inhibitors (Complete; Roche Diagnostics, Mannheim, Germany) in phosphate-buffered saline (PBS) and the resulting homogenates were centrifuged for 10 min at 10,000 $\times$ g at 4°C, aliquated, and stored at -70°C until required. A NE-PER® Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology, Rockford, IL) was used to prepare cytoplasmic extracts. Protein concentration was measured by the Bio-Rad protein assay method (Bio-Rad Laboratories, Hercules, CA). Samples containing 50 μ g of protein were fractionated on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The membranes were blocked, probed using polyclonal antibodies against NF- κ B, I κ B α , and Bax or monoclonal antibodies against p-I κ B α (0.4-2 μ g/ml; Santa Cruz Biotechnology, Santa Cruz, CA) and visualized using Horseradish peroxidase (HRP)-conjugated secondary antibodies (0.2-0.4 μ g/ml; Santa Cruz Biotechnology, Santa Cruz, CA). After washing in TBS-Tween, the membranes were incubated with

ECL reagents (Santa Cruz Biotechnology, Santa Cruz, CA) and exposed to an x-ray film. To control the loading, the membranes were stripped and re-probed using an antibody for β -actin (Santa Cruz Biotechnology, CA, USA). Results were reproduced in 4 independent experiments with different samples obtained from different litters.

In order for the optical densitometry analysis to be performed, films were scanned on an UMAX PowerLook III scanner (eTailElectronics, Jamestown, ND) attached to a computer and TINA v.2.07d Software was used to measure the density of bands representing the tested proteins and actin. The level of the expression of a given protein was expressed as a ratio of the intensity of a given band to the intensity for actin.

Evaluation of NF- κ B DNA binding using the ELISA-based method.

Females were sacrificed either on day 13 or 15 of pregnancy. Head and trunks of 4 - 6 embryos were collected, pooled, homogenized and nuclear extracts were prepared using A NE-PER® Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Protein concentration was measured by the Bio-Rad protein assay method (Bio-Rad Laboratories, Hercules, CA). Samples were frozen in aliquots in liquid nitrogen and stored at -70°C until required.

Table 1. Diabetes-induced inhibition of embryonic growth

Parameters	Day 13 of pregnancy		Day 15 of pregnancy	
	Control	Diabetic	Control	Diabetic
Pregnant females (no.)	8	8	8	8
Implantation sites ¹	11.30 $\pm$ 0.80	11.10 $\pm$ 0.90	11.30 $\pm$ 0.80	11.30 $\pm$ 0.80
Percentage of resorptions (arcsine, M $\pm$ SD)	8.90	25.80	11.80	33.30
	22.50 $\pm$ 5.40	35.40 $\pm$ 5.60	25.00 $\pm$ 4.80	40.20 $\pm$ 5.80 ²
Embryonic weight (g)	0.09 $\pm$ 0.00 ³	0.06 $\pm$ 0.00 ^{2,3}	0.36 $\pm$ 0.03	0.23 $\pm$ 0.03 ²
Placental weight (g)	0.06 $\pm$ 0.00 ³	0.06 $\pm$ 0.00 ³	0.10 $\pm$ 0.02	0.09 $\pm$ 0.03

Legend: Data are means $\pm$ SE. ¹ Per litter. ² Values differ significantly ($p \leq 0.05$) from those in control. ³ SE < 0.01.

NF- κ B DNA-binding activity was evaluated using an ELISA-based EZ-detected™ Transcription Factor Kit for NF- κ B p65 (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Briefly, 10 μ l of nuclear extracts containing 40 μ g proteins were added to the wells covered with oligonucleotide containing the NF- κ B consensus binding site. The active transcription factor bound to the consensus sequence was incubated with a specific primary antibody (NF- κ B p65) and then with a secondary HRP

conjugated antibody. After incubation, a chemiluminescent substrate was added to the wells and the resulting signal was detected using a Kodak Image Station 440CF (Kodak, Rochester, NY) attached to a computer. All samples were run in triplicates. A wild-type consensus oligonucleotide, provided as a competitor for NF- κ B binding, and a mutated consensus oligonucleotide with no effect on NF- κ B binding was used in order to monitor the specificity of the assay. TINA v.2.07d Software was used to perform the optical densitometry analysis. The results were reproduced in 3 independent experiments with different samples obtained from different litters.

Figure 1. Representative TUNEL-stained sections of the 15-day-old embryonic brain. Sections shown are: **A.** Control embryo. **B.** Embryo of a diabetic female (X30).

Statistical analysis

The statistical analysis of data characterizing the reproductive performance of mice such as the number of implantation sites and resorptions as well as the weight of embryos and placentas was performed on a litter basis. The proportion of resorptions per litter was transformed to arcsine values by Freeman-Tukey's method as described elsewhere [25]. All indices were analyzed statistically using Student's *t*-test. The two-tailed level of significance of differences was = 0.05.

Results

The reproductive performance of diabetic mice

A significant decrease in the weight of diabetic mice and rats' fetuses has been repeatedly demonstrated in both our own and other previous studies. In this study, we tested embryos at the late stage of organogenesis and observed that the weight of embryos of diabetic mice at both times of testing was also significantly lower than the weight of embryos from non-diabetic females (Table 1). However, the weight of placentas of diabetic and control females was found to be comparable (Table 1). Also, as in our previous studies [17,

23], the number of implantation sites in diabetic females did not differ statistically from those in non-diabetic ones, whereas the proportion of resorptions was higher in the former (Table 1).

The evaluation of apoptosis

In studies addressing mechanisms of diabetes-induced structural anomalies, excessive apoptosis was observed in malformed mouse and rat embryos tested at the stage of early organogenesis (days 10-12 of pregnancy) [17, 23, 26-28]. Therefore, in this study, we tested embryos exhibiting no structural anomalies and revealed only single apoptotic cells in TUNEL-stained

sagittal sections of embryos of diabetic and control mice (Figure 1). Also, no excessive apoptosis was observed in placentas from diabetic mice (data not presented). Furthermore, the level of the expression of a proapoptotic protein Bax tested by Western blot analysis seemed to be lower in embryos of diabetic females than that in control embryos (Figure 2) and it

did not differ significantly in placentas of diabetic and control females.

Evaluation of the expression of NF- κ B (RelA), I κ B α , phosphorylated (p)- I κ B α proteins and NF- κ B DNA binding

Western blot analysis of whole-cell protein extracts revealed a prominent decrease in the level of the expression of NF- κ B (p65) in embryonic samples collected from diabetic mice on both day 13 and day 15 of pregnancy (Figure 2). At the same time, the level of this protein was practically equal in placental samples of diabetic and non-diabetic females (Figure 2). We continued to analyze embryonic samples and found that the level of p-I κ B α was also lower in embryos of diabetic mice compared to that in control embryos (Figure 3). I κ B α protein expression seemed not to differ in embryonic samples collected on day 13 of pregnancy from diabetic and non-diabetic mice and demonstrated a trend toward a decrease in samples collected from diabetic mice on day 15 of pregnancy (Figure 3). Finally, the evaluation of NF- κ B DNA binding by an ELISA-based method revealed that level of NF- κ B complex formation in embryonic samples from diabetic mice was lower than in samples from control embryos (Figure 4).

Figure 2. Expression of NF- κ B (p65) and Bax proteins in embryos and placentas of diabetic mice. Western blots illustrate the expression of p65 and Bax proteins in whole-cell extracts isolated from 13- and 15-day-old embryos (13E and 15E) and their placentas (13P and 15P). Data are representative of four independent experiments done with different samples. Bands were analyzed densitometrically and the graphical data are presented as the density of a given band relative to actin.

Discussion

Despite metabolic control, fetal growth disorders remain to be one of the main complications of maternal diabetes [29]. As yet, molecular mechanisms underlying this pathology remain to be clarified.

In this study, we observed that the embryos of diabetic mice were already significantly growth retarded on day 13 of gestation. Fifteen-day old embryos from these mice showed also a decrease in weight compared to that of the controls. In our previous studies, the weight of fetuses of STZ-induced diabetic mice registered at the end of gestation (days 18-19 of pregnancy) was approximately 25% lower than the weight of their non-diabetic counterparts [17, 24]. Together, this data allows us to suggest that the decrease in weight of 13-day old embryos is a pathogenic event for growth retardation demonstrated by fetuses at the end of gestation.

At the same time, we did not observe differences in the weight of placentas of control and diabetic females or excessive apoptosis in the placentas of the latter. This result somewhat contradicts studies in which the weight of placentas was found to have increased in diabetic females [30, 31]. Based on these studies, it has been suggested that placental dysfunctions may be involved in the pathogenesis of diabetes-induced fetal growth retardation. Interestingly, however, no placental pathology seems yet to have been found, except reduced uteroplacental blood flow [32], which could directly impact on fetal growth. Whether or not this factor affects diabetic mice being used in the present study remains unclear. Nevertheless, it is worth mentioning that 13-day old embryos were already severely growth-retarded, whereas the formation of placentas in mice is complete on approximately day 12 of pregnancy. Furthermore, in our previous studies of 9-day old mouse embryos cultured in media containing teratogenic concentrations of glucose [28], embryos examined 24 hours after the beginning of culturing exhibited growth retardation, regardless of whether or not they were malformed. This observation suggests that at least in mice with severe STZ-induced diabetes, the process of fetal growth retardation may start early, at the period of or shortly after their highest sensitivity to diabetes-induced teratogenic stimuli, which is considered to be days 8-9 of pregnancy [1, 2]. Together, these facts suggest that placental dysfunctions might contribute to but hardly initiate the process of growth retardation of murine embryos developing in a diabetic environment.

We also observed decreases in the levels of the NF- κ B (p65) protein and NF- κ B DNA binding in the growth-retarded embryos from diabetic mice. The expression of phosphorylated I κ B α protein also decreased suggesting that this suppression of NF- κ B activity might be realized at the level of I κ B degradation.

These results seemingly contradict considerable evidence demonstrating that diabetes is associated with activation of NF- κ B [33]. Importantly, however, diabetes-induced activation of NF- κ B was mainly observed in studies in adult cells exhibiting low constitutive NF- κ B activity in normoglycemic conditions, whereas embryos tested in the present study exhibited high constitutive NF- κ B activity. As multiple mechanisms acting in a cell type-dependent fashion are implicated in the regulation of NF- κ B activity [8, 9], the possibility that diabetes might suppress NF- κ B activity in embryos seems to be conceivable.

As previously mentioned, suppression of NF- κ B activity in embryos at the early stage of organogenesis

Figure 3. Expression of IκBα and phosphorylated (p)-IκBα proteins in embryos of diabetic mice. Western blots illustrate the expression of IκBα and p-IκBα proteins in cytoplasmic extracts isolated from 13- and 15-day-old embryos (13E and 15E). Data are representative of four independent experiments done with different samples. Bands were analyzed densitometrically and the graphical data are presented as the density of a given band relative to actin.

induced by a number of teratogens was suggested to be followed by the activation of the apoptotic process in embryonic structures that are subject to malformation [34]. At the same time, in this study, no excessive apoptosis was observed in growth-retarded embryos at the late stage of organogenesis. Besides, the expression of a proapoptotic protein Bax was found to have decreased in these embryos.

Bax, a member of the Bcl-2 family of proteins [35], plays a key role in the 'intrinsic' apoptosis pathway [36]. This pathway is initiated by various effectors including reactive oxygen species (ROS) [36]. Hyperglycemic conditions were shown to be associated with the excessive ROS production [37] and an increased Bax expression was detected in studies addressing mechanisms of diabetes-induced apoptosis in adult cells [38, 39]. In the light of this data, it is tempting to speculate that the decrease in the expression of Bax observed in

this study might reflect the suppression of apoptosis in growth-retarded embryos. Such a scenario seems to be logical enough under the premise that the growth retardation of the tested embryos might be due to the suppression of cell proliferation. In that case, suppression of apoptosis would be essential in preventing intrauterine death of growth-retarded embryos. Whether this scenario is correct and whether NF-κB plays a role in it is now being researched.

In conclusion, this study suggests that diabetes-induced fetal growth retardation may be accompanied by the suppression of NF-κB activity in embryos. Meanwhile, the pathogenesis of diabetes-induced complications such as retinopathy, endothelial dysfunction and nephropathy includes the sustained activation of NF-κB in target cell populations [33, 37], suggesting that anti-NF-κB therapy may be used for treatment of these complications [33,40]. The results of this paper appear to highlight the necessity to reveal the role of the NF-κB signaling in diabetes-induced embryopathies to estimate the risk of anti-NF-κB therapy for pregnant women.

Figure 4. NF-κB complex formation in embryos of diabetic mice. The amount of NF-κB DNA binding was measured by the chemiluminescence assay using an ELISA-based method directed against the p65 subunit of NF-κB in nuclear extracts isolated from 13- and 15-day-old embryos (13E and 15E). All samples were run in triplicates. Data are representative of three independent experiments done with different samples. The graphical data present the results of the densitometric analysis.

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