Gestational diabetes leads to down-regulation of CDK4-pRB-E2F1 pathway genes in pancreatic islets of rat offspring

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ARTICLE INFO	ABSTRACT
	Objective(s): The link between a hyperglycemic intrauterine environment and the development of diabetes later in life has been observed in offspring exposed to gestational diabetes mellitus (GDM), but the underlying mechanisms for this phenomenon are still not clear. Reduced β-cells mass is a determinant in the development of diabetes (type 1 and type 2 diabetes). Some recent studies have provided evidence that the CDK4-pRB-E2F1 regulatory pathway is involved in β-cells proliferation. Therefore, we postulated that GDM exposure impacts the offspring's β-cells by disruption in the CDK4-pRB-E2F1 pathway. *Materials and Methods:* Adult Wistar rats were randomly allocated in control and diabetic group. The experimental group received 40 mg/kg/body weight of streptozotocin (STZ) on day zero of gestation. After delivery, diabetic offspring of GDM mothers and control dams at the age of 15 week were randomly scarified and pancreases were harvested. Langerhans islets of diabetic and control groups were digested by collagenase digestion technique. After RNA extraction, we investigated the expressions of the kir 6.2 and CDK4-pRB-E2F1 pathway genes by quantitative real-time PCR. *Results:* GDM reduced the expression of CDK4-pRB-E2F1 pathway genes in Langerhans islets cells of offspring. CDK4, pRB and E2F1 pathway genes were downregulated in diabetic islets by 51%, 35% and 84%, respectively. Also, the expression of Kir 6.2 was significantly decreased in diabetic islets by 88%. *Conclusion:* We suggest that the effect of gestational diabetes on offspring's β-cells may be primarily caused by the suppression of CDK4-pRB-E2F1 pathway.

Introduction

Gestational diabetes mellitus (GDM) is defined as "impaired glucose tolerance appearing specifically during pregnancy" (1). GDM develops by excessive insulin resistance, inadequate β-cell compensation, reduced β-cell function, or any combination of these (2). A large number of epidemiological studies demonstrated that diabetes in pregnant women is associated with an increased risk of maternal and neonatal morbidity (3). Infants of a diabetic mother (IDMs) have been shown to be prone to the development of complex diseases, including obesity, and metabolic and cardiovascular complications, during childhood and adulthood (4, 5). In animal models of GDM, it has been shown that IDMs overtly develop diabetes throughout life (6, 7). Furthermore, population-based studies have also demonstrated that IDMs have an increased risk for type 2 diabetes in later childhood and as adults (8).

Insulin-producing β-cells in the endocrine pancreas play a pivotal role in maintaining glucose homeostasis. Adult β-cells can dynamically respond to systemic increases in insulin demand by expanding their functional mass. Compensatory changes in β-cells mass are controlled by increases in cell size (hypertrophy) or increase in number of cells (hyperplasia) (9, 10). Recent findings have shown that defect in these mechanisms is a key feature of the pathogenesis of diabetes (11, 12). Similarly, IDM mature animals show β-cells dysfunction and decreased insulin secretion in response to glucose (7). However, the mechanisms responsible for β-cells malfunction in IDMs are unknown. Therefore, understanding how β-cells proliferate and function is important and may lead to development of new therapeutic strategies for diabetes.

Strikingly, recent studies have shown a link between the cell cycle regulators and the risk of type 2 diabetes (13, 14). Cyclin-dependent kinases (CDK) "a family of serine/threonine protein kinases" phosphorylate a number of substrates like retinoblastoma protein (pRB) mainly implicated in cell

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cycle progression. Association of E2Fs with pRB proteins causes active repression of E2Fs. Subsequent phosphorylation of pRB by the CDK results in the release of E2F. Upon activation, E2F1 is able to turn on genes required for progression through G1 into the S phase of cell cycle (15). In addition to activation of cell cycle, E2F directly contributes to insulin secretion through the regulation of Kir6.2 (also referred as Kcnj11) expression. It has been proven that Kir6.2 channels play a pivotal role in the regulation of insulin secretion. Recent studies have shown that expression of kir6.2 is directly regulated by E2F1. So, CDK4-pRB-E2F1 pathway directly contributes to both proliferation and regulation of insulin secretory capacity of β-cells (16).

Indeed, no study has investigated the gene expression of kir 6.2 and CDK4-pRB-E2F1 factors in adult offspring of diabetic rats. Thus, the purpose of the present investigation was to evaluate the expression changes of these genes in pancreatic islands extracted from offspring of streptozotocin-induced mildly hyperglycemic rats. This experimental study was performed to evaluate the effect of gestational diabetes on expression of CDK4-pRB-E2F1 pathway genes in pancreatic islets of rat offspring.

Materials and Methods

All animal procedures followed the guidelines set by the Institutional Animal Care and Use Committee at the Golestan University of Medical Sciences, Gorgan, Iran.

Generation of the diabetic rat model

Adult Wistar rats aged 10-12 weeks were used in this study. The rats were kept in a temperature-controlled environment (21±2 °C) on 12-hr light/dark cycles and allowed free access to standard rat chow and water. Female rats were separately placed with a proven breeder male overnight for breeding. Vaginal plaque was mentioned daily as a positive sign of pregnancy and the day on which vaginal plaque was observed, considered as day 0 of pregnancy. Dams randomly divided into two control and diabetic groups. A total of 20 dams were made diabetic by single IP injection of freshly prepared streptozotocin (STZ) solution (40 mg/kg body weight) in sterile saline solution (0.85%) on day zero of gestation (17); 7 dams were injected with equivalent volume of normal saline as control group. Four days later, blood glucose levels were checked using a glucometer (ACCU-CHEK® Active Glucometer, Roche Diagnostics, Germany). If glucose levels were between 120-250 mg/dl, the rats were selected and used as GDM. Mildly hyperglycemic dams in the current series of experiments were ~40%, or 8 out of 20 STZ- injected rats. The dams were allowed to deliver spontaneously. After offspring reached puberty, their blood glucose level

was measured with a glucometer. Totally six diabetic offspring from GDM mothers at the age of 12 and 15 weeks were selected. Offspring from normal dams were used as controls. Rats were sacrificed, and pancreases harvested and processed for isolation of Langerhans islets.

Isolation of langerhans islets

Islets were isolated from pancreases of diabetic and control group rats by a modification of the collagenase digestion technique (18). This involved cannulation of the common bile duct and the sequential administration of 2 ml digest solution containing 0.2 mg/ml liberase TL (Roche, Cat# 05 401 020 001) and 10 pg/ml DNase (Takara) in serum free RPMI 1640 medium. The organ was then incubated at 37 °C for 15 min and dispersed using pipetting action. At the end of the incubation, the tubes moved to ice and 10 ml RPMI 1640 with 10% serum added. The tissue was dissociated by shaking the tubes vigorously. The tubes were centrifuged for 2 min at 800 RPM and 4 °C. The islets were separated by centrifugation on a Ficoll gradient (Histopaque1077, Sigma-Aldrich10771) and collected from the histopaque/media interface with a disposable 10 ml serological pipette and resuspended in serum containing RPMI. The islets were then isolated by passage through a 100 μm cell strainer (BD Falcon) and handpicked with a Pasteur pipette using a dissecting microscope. The clean islets stored at −80 °C until further extraction.

RNA extraction

RNA was isolated from islets using the genabioscience RNA extraction kit according to the manufacturer's instructions. Residual DNA was digested with 10 U RNase-free DNase (DNase I, TaKaRa) in the presence of 20 units of RNase inhibitor at 37 °C for 20 min. After heat inactivation for 10 min at 75 °C in 2 mM EDTA, total RNA solution was removed for quantification. Concentration and purity of the DNase I-treated samples was measured using a NanoDrop ND-1000 spectrophotometer (A260/A280>1.8 and A260/A230>1.6). The integrity and stability of the RNAs was confirmed by demonstrating the intact 28s and 18s bands on gel electrophoresis.

Real-time RT-PCR analysis

For real-time RT-PCR, the cDNA was synthesized from 1 μg of DNaseI-treated total RNA using prime script RT reagent kit (Takara) with random hexamer and oligo dT primers following the manufacturer's protocol. The forward and reverse PCR primers for the 7 genes were designed in accordance to the real-time PCR conditions, using perlprimer software (Bio-Rad, USA), and the sequences are listed in Table 1. For each genes, the cDNA amplified by specific primers using Taq Polymerase Kit (TaKaRa), and correct product was confirmed by running on gel electrophoresis.

Table 1. Real-time PCR primer name, sequences, size, GenBank accession number and PCR condition

Genes	Forward primer	Reverse primer	PCR product size	GenBank accession no.	TM (°C)
β-actin	AAGATCAAGATCATTGCTCCTC	CTCAGTAACAGTCCGCCT	169	NM_031144.3	60
CDK4	AATGTTGTACGGCTGATGG	GGTCTATATGCTCAAACACTAAGG	85	NM_053593.2	60
pRB	TGGCAGAAATGACTTCTACTC	CACATCCATGAGACACGA	147	NM_017045.1	59
E2F1	CCAGGGAAAGGTGTGAAATCTC	GCTCCAAGAAGCGTTTGGT	82	NM_001100778.1	63
Kir 6.1	CCAAGCCCAAGTTTAGCA	ACATACCGTACTTCACTTTCC	103	NM_031358.3	59

Real-time RT-PCR was performed using the SYBR-Green PCR Master Mix kit (TaKaRa) in the thermo Cycler (ABI, 7300). The cycling conditions were 95 °C for 30 sec followed by 40 cycles at 95 °C for 5 sec, 55 °C for 30 sec and 72 °C for 1 min. We used rat β-actin as internal control and non- diabetic offspring islets cDNA as calibrator. Amplification specificity was confirmed by gel electrophoresis. The relative expression level of mRNA between the diabetic and non-diabetic samples was determined with the comparative CT (cycle threshold) method. First, both CT values of target gene from control islets cDNA (n=3) and diabetic islets cDNA (n=3) were normalized by CT value of the internal control (ΔCT). Then, the former was subtracted by the latter, namely ΔΔCT. The value of $2^{-\Delta\Delta CT}$, the fold change of gene expression, Real-time serial data were statistically analyzed. Every Real-time PCR experiment was repeated with three samples and each sample was run in duplicate.

Data analysis

Data were presented as mean±standard deviation (SD). Relative target gene expression and blood glucose level was analyzed with one-way ANOVA using SPSS 16.0 statistical analysis software. The differences between groups were compared using Unpaired T test and *P*<0.05 was chosen as the level of significance.

Results

Glucose level

Fasting blood glucose concentration was significantly increased in IDM rats (Figure 1). By 12 weeks of age, about 40% of the IDMs developed mild hyperglycemia and at 15 weeks of age, glucose levels were markedly elevated in IDMs compared to controls (*P*<0.001).

qRT-PCR results

We assessed whether the expression levels of cell cycle regulator genes (CDK4-pRB-E2F1) were affected by gestational diabetes in adult offspring. Our group also evaluated the effects of GDM on mRNA expression of Kir6.2. Figure 1A is depicted the melt curves for 5 different amplicons. Gel electro-phoresis of amplified products is shown in Figure 2B.

	week 12	week15
▢ control	93.5	95.25
▪ diabetic	161.33	273.83
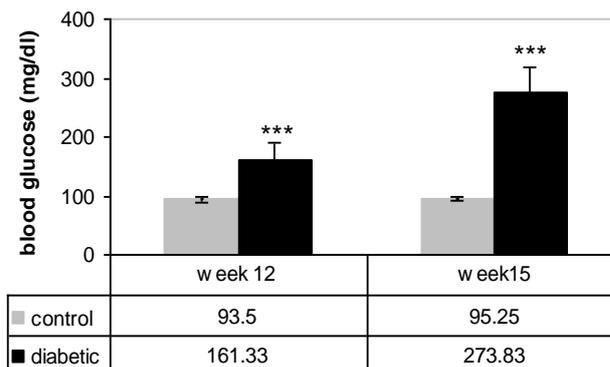
Figure 1. Blood glucose concentrations in IDMs (infants of a diabetic mother) and control animals (week 12 and week 15). Blood glucose level of offspring was obtained via tail vein and was measured with ACCU-CHEK glucometer. Values are means± SEM. *** *P*<0.001

The expression levels of pRB in isolated islets were not significantly different between the two groups, indicating that the expression of pRB was not disturbed by GDM. But analysis of the mRNA levels of CDK4 and E2F1 showed significant alterations in the diabetic group compared to controls (Figure 3). On the other hand, Kir6.2 mRNA expression severely reduced in IDMs, which followed the expected decrease in E2F1 protein (Figure 3).
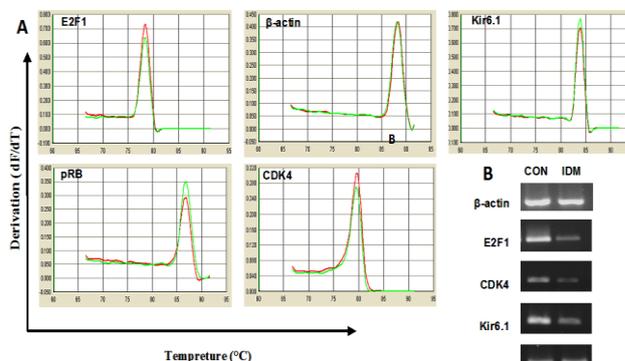
Figure 2. Real-time PCR melting curve for E2F1, CDK4, Kir6.2, pRB and β-actin as internal control (A). Agarose gel electrophoresis of PCR products following real-time SYBR green amplification (B)

	Kir6.1	CDK4	Prb	E2F1
■ control	1	1	1	1
▨ Diabetic	0.12	0.49	0.65	0.16
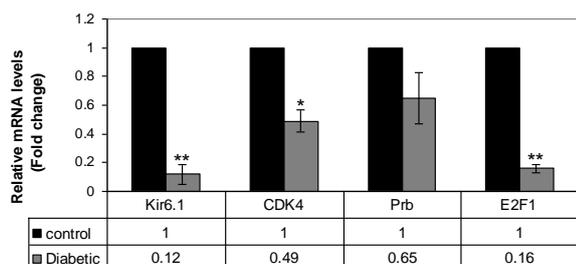
Figure 3. Real-time PCR analysis of Kir6.2, E2F1, CDK4 and pRB. mRNA levels were measured using gene specific primers (Table 1) and the values were normalized to β-actin. Data are presented as mean ± SD. Statistical significance was calculated using the t test (*$P<0.05$, **$P<0.01$, n=3). Significant *P*-values are indicated over the bar graphs

Discussion

Maintenance of appropriate insulin-producing β-cells growth and mass is critical for metabolic balance (19, 20); thus, the molecular mechanisms by which β-cells proliferate are the focus of recent studies. The first years of life in humans and the first postnatal months of life in rodents are crucial periods of islet β-cells growth that result in establishment of appropriate β-cells mass (21-24). Fetal hyperglycemia is a consequence of maternal mild hyperglycemia that may disturb β-cells growth during early postnatal period and causes diabetes in offspring later in life (25). Previous studies have demonstrated that CDK4-pRB-E2F1 pathway plays a crucial role in the control of glucose homeostasis. As a cell cycle regulatory pathway, CDK4-pRB-E2F1 genes not only regulate β-cells proliferation, but also control the expression of genes implicated in insulin secretion, such as Kir6.2 (15, 16).

In spite of several studies regarding the effects of diabetes I and II on pancreas structure and function, there is no investigation about the effect of GDM on cell cycle regulators expression in offspring's pancreatic islets. Thus, this study was designed to investigate the effects of hyperglycemic intrauterine environment on the expression of cell cycle regulator genes in Langerhans islets of adult diabetic offspring. We used STZ to induce mild hyperglycemia in pregnant Wistar rats. Consistent with some previous studies (6, 7), our offspring rats developed diabetes at 12 weeks of age.

In this study, we observed a 51% decrease for CDK4 expression in Langerhans islets of diabetic offspring (*P*<0.05). Many studies implicate CDK4 as a crucial factor for successful expansion of β-cells mass in various conditions. For instance, when cell cycle arrest is altered specifically in β-cells by deletion of CDK4 postnatally, β-cells mass decreases or fails to expand (13). Also Rieck *et al* showed that during the peak of β-cells DNA synthesis, CDK4 expression is induced in the islets of pregnant mice (26). Also, it has been shown that pharmacological inhibition of CDK4 activity on glucose tolerance in mice drama-

tically decreases the clearance of glucose in treated, compared to non-treated mice with IDCX, which is a specific CDK4 inhibitor (15). Correlated with previous studies, our observation links CDK to the risk of diabetes in offspring of GDM mothers.

Furthermore, we showed that GDM also causes reduction of pRB and E2F1 gene expression in pancreatic islets of diabetic offspring by 35% and 86% (*P*<0.01), respectively. Findings have shown that E2f1-/- mice have decreased pancreatic size and insulin secretion, as the result of impaired postnatal pancreatic growth. The results have also demonstrated that E2F1 was highly expressed in non-proliferating pancreatic β-cells, suggesting that E2F1, besides the control of β-cells number could have a role in pancreatic β-cells function (27). These findings provided enough evidence to propose that CDK4-pRB-E2F1 pathway genes are critical mediators of insulin secretion (16). Regarding the down-regulation of CDK4-pRB-E2F1 pathway genes in pancreatic islets of rat (IDMs), we can conclude that induction of diabetes in offspring of GDM is mediated by the reduction of CDK4 activity and subsequent E2F1 transcriptional activity.

In addition to evaluation of genes involved in β-cells proliferation, we also wanted to evaluate whether GDM affects β-cells function. So, we analyzed the expression of Kir6.2, a key component of the ATP-sensitive potassium channel involved in the regulation of glucose-induced insulin secretion in β-cells. Our data indicated that GDM causes reduction in Kir6.2 gene expression by 0.12 fold compare to control group. Recent *in vitro* and *in vivo* investigations have shown that E2F1 directly controls the expression of Kir6.2. Several studies on pancreas of E2f1-/- mice have shown that expression of Kir6.2 is downregulated that causes insulin secretion defects in these animals (15, 16, 27). Our data are in agreement with other reports and suggest that uncontrolled GDM may cause diabetes in offspring by repression of CDK4-pRB-E2F1 pathway in their pancreases.

Conclusion

Our data showed that down-regulation of CDK4-pRB-E2F1 pathway genes is related with development of diabetes in offspring of gestational diabetic rats. Furthermore, in this study, decreased Kir6.2 mRNA expression in diabetic offspring underscores a dual effect of the GDM on both proliferation, and function of β-cells. Taken together, this study may open up new ways for understanding the molecular basis of GDM and type 2 diabetes in the offspring.

However, many advances must be made to fully appreciate the exact molecular mechanism of inducing diabetes in offspring by gestational diabetes.

Acknowledgment

Conflict of Interest

The authors declare that there are no conflicts of interest.

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