Gestational diabetes influences the expression of hypertrophic genes in left ventricle of rat's offspring

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ABSTRACT

Objective(s): Gestational diabetes increases the risk of congenital heart disease in the offspring, but the molecular mechanism underlying this process remains unclear. Therefore, the current study was conducted to assess the effects of induced gestational diabetes on expression of some involved genes in cardiac hypertrophy in the offspring of diabetic rats.

Materials and Methods: Diabetes was induced in 40 adult Wistar rats by intraperitoneal injection of 45 mg/kg of streptozotocin. The day of appearance of the vaginal plug was assumed as day zero of gestation for inducing diabetes. After pregnancy, the offspring was maintained until they reach the age of 12 weeks. Then, their hearts were excised and were sectioned for molecular study. We analyzed the expression pattern of some hypertrophic genes by the quantitative real-time RT-PCR.

Results: The mRNA expression levels of all studied genes including c-jun, c-fos, c-myc, alpha-myosin heavy chain (α-MHC), atrial natriuretic factor (ANF) and β-MHC, which are important in cardiomyocyte hypertrophy, were higher in the offspring of the diabetic group compared to controls. Significant differences were found for β-MHC and c-myc with $P<0.01$ and for α-MHC and c-fos with $P<0.05$.

Conclusion: Gestational diabetes upregulates expression of c-jun, c-fos c-myc, α-MHC, ANF and β-MHC genes that are involved in cardiac hypertrophy in the offspring of diabetic rats.

Introduction

Gestational diabetes mellitus (GDM), affecting approximately 7% of pregnant women, is associated with a fivefold increase in the risk of cardiovascular malformations in their infants (1, 2). Metabolic disorders related to the gestational diabetes lead to long-term effects, including obesity, insulin resistance and diabetes in the offspring (3-5). Pathologic pregnancies, such as gestational diabetes, are accompanied by a heightened oxidative stress level (6). Studies have shown that infants of diabetic mothers have more complex congenital heart anomalies. In addition, poor glycemic control in the first trimester of pregnancy may lead to the presence of congenital diseases. The frequency of congenital malformations in infants of diabetic mothers is 10 times higher than the infants of non-diabetic mothers. So, hyperglycemia seems to be the primary teratogen, which has adverse impacts on the development of the cardiovascular system (7-9).

Despite clarifying the effects of GDM on fetal development by animal researches, the molecular mechanisms by which the teratogenicity of maternal diabetes causes congenital cardiovascular malformation remain undefined. During fetal development, cells from the cardiac neural crest cells (CNCC) migrate to the heart through the occipital somites to increase cardiac ganglia. High glucose level in GDM inhibits this migration to create congenital heart defects in the fetus. The migration and differentiation of the CNCC into cardiac ganglia are influenced by various developmental control genes (10-12). However, many studies have proven that maternal diabetes significantly affects the structure and function of fetal heart (3, 4). In addition, cardiac hypertrophy is associated with over-regulation of two groups of genes; the early response genes (c-fos, c-myc and c-jun) and the fetal genes such as beta-myosin heavy chain (β-MHC), atrial natriuretic factor (ANF) and skeletal alpha-actin (SKA), which are considered and employed as hypertrophic markers (13,14). Various procedures are available for the animal study of gestational diabetes. Streptozotocin (STZ) is the most commonly used drug for induction of diabetes in laboratory animals. This method provides a similar model of gestational diabetes in animals as in human (15). The aim of the current study was to investigate the effects of STZ-induced gestational diabetes on expression of some involved genes in cardiac hypertrophy in the left ventricular cardiomyocytes of rat offspring.

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Materials and Methods

Ethical approve

All animal experiments were approved by Institutional Animal Care and Use Committee of the Golestan University of Medical Sciences, Gorgan, Iran. (IR.goums.REC.1395.157).

Experimental animals

In this experiment, 40 adult females and 10 male Wistar rats aged 10-12 weeks were housed in a cage on a 12 hr light/dark cycle at 22–24 °C with food and water *ad libitum*. All of the experimental procedures were conducted in accordance with the National Institutes of Health's Guide for the care and use of laboratory animals.

Generation of the diabetic rat model

Female rats were separately placed with a male overnight for breeding. The day of appearance of the vaginal plug was assumed as day zero of gestation. Dams were randomly divided into two control and diabetic groups. Diabetes was induced by an intraperitoneal injection of 45 mg/kg body weight STZ, which dissolved in normal saline (0.85%) on day zero of gestation (16). Non-fasting blood glucose level 120 mg/dl or higher was considered as GDM models, and Dams without injection of STZ and with normal blood glucose level before and during pregnancy were considered as control group. After pregnancy, the offspring were maintained until they reach the age of 12 weeks. Then, they were anesthetized intraperitoneally with ketamine (100 mg/kg body wt.) and Xylazine (10 mg/kg body wt.), while they fasted overnight. For each experiment, hearts were excised and the left ventricle was isolated for molecular study.

Quantitative real-time RT-PCR assay

Total RNA was extracted from the left ventricle tissue of control and diabetic offspring using the RNeasy mini kit (Jena Biosciences, Germany) according to the manufacturer's instructions. Concentration and purity of RNA samples were measured using a NanoDrop ND-1000 spectrophotometer (A260/A280>1.8 and A260/A230>1.6). For cDNA synthesis, 1 µg of total RNA was amplified using prime script RT reagent kit (TaKaRa). The forward and reverse PCR primers for the 8 genes (β-actin, c-fos, c-myc, c-jun, α-MHC, β-MHC, ANF, and SKA) were designed using oligo software, and the sequences are listed in Table 1. Real-time PCR was performed using the SYBR-Green PCR Master Mix kit
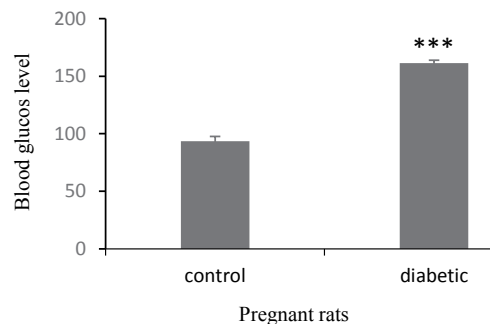

Figure 1. Blood glucose concentrations in gestational diabetes mellitus (GDM) and control pregnant rats. Values are means±SEM. *** (*P*<0.001)

(TaKaRa) in the thermocycler (ABI, 7300). Rat β-actin used as internal control and non- diabetic left ventricle cDNA used as calibrator. The relative expression level of mRNA between two groups was determined with the comparative CT method. Every real-time PCR experiment was repeated with three samples and each sample was run in duplicate.

Statistical analysis

Blood glucose level and relative mRNA expression were analyzed with one-way ANOVA using Prism statistical analysis software. Student's t-test was used to compare two independent groups and *P*<0.05 was chosen as the level of significance.

Results

Serum glucose levels in pregnant rats and their offspring

The serum glucose levels in diabetic pregnant rats compared to normal rats is depicted in Figure 1) The student t-test indicated a significant difference between two group regard to serum glucose (*P*<0.001).

Fasting blood glucose concentration was significantly increased in diabetic offspring (Figure 2). By 12 weeks of age, about 60% of the offspring of GDM developed diabetes. Glucose levels were significantly elevated in diabetic offspring compared to controls (*P*<0.001).

Results of quantitative real-time RT-PCR

We assessed whether the expression levels of hypertrophic genes including c-jun, c-fos, c-myc, α-MHC, ANF and β-MHC were affected by gestational diabetes in adult offspring. Figure 3 and 4 are depicted the gel electrophoresis of amplified products and real-time

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

| Genes	Forward primer	Reverse primer	product size (bp)	GenBank accession no.
β-actin	AAGATCAAGATCATTGCTCCTC	CTCAGTAACAGTCCGCCT	169	NM_031144.3
c-fos	ACGGAGAATCCGAAGGGAAAGGAA	TCTGCAACGCAGAC TTCTCGTCTT	125	NM_022197.2
c-myc	TGACGAGACCTTCGTGAAGA	ATTGATGTTATTTACACTTAAGGGT	895	NM_013194.2
c-jun	CATGGTCGCTCCCGCTGTGG	CGCTTCCTCTCCGCCTTGAT	363	NM_021835.3
α-MHC	CAAGGCAAACCTGGAGAAAG	GGGTATAGGAGAGCTTGCCC	206	XM_017599641.1
β-MHC	GAGGAGAGGGCGGACATT	ACTCTTCATTCAGGCCCTTG	88	XM_006251951.2
ANF	CAACACAGATCTGATGGATTTCA	CGCTTCATCGGTCTGCTC	112	NM_012612.2
SKA	CTGTCTCTCTATGCCTCCGG	CTTCTCTTTGATGTCGCGCA	228	NM_019212.2
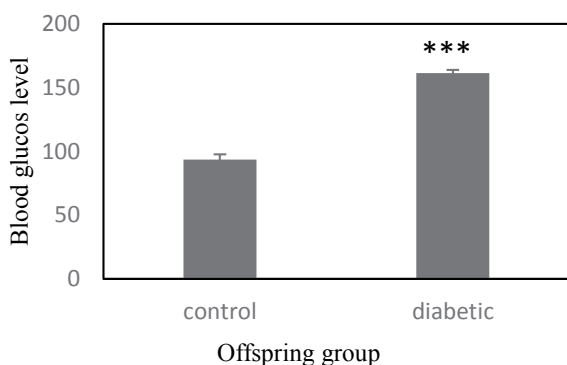
Figure 2. Serum glucose levels in offspring of diabetic rats compared to controls. Values are means± SEM.
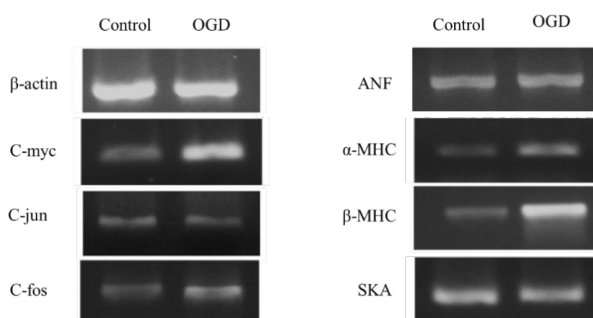*** (*P*<0.001)

Figure 3. Agarose gel electrophoresis of real-time PCR products following real-time PCR amplification (45 cycles for both gestational diabetes mellitus (GDM) and control group) for c-jun, c-fos, c-myc, alpha-myosin heavy chain (α-MHC), atrial natriuretic factor (ANF), beta-myosin heavy chain (β-MHC) and β-actin mRNA

PCR results, respectively. Our data showed that the mRNA expression levels of all studied genes that are important in cardiomyocyte hypertrophy were higher in the diabetic offspring compared to controls. Analysis of the mRNA levels of c-myc and β-MHC showed significant alterations in the diabetic group compared to controls (*P*<0.01**). Also, the expression levels of c-fos and α MHC in isolated left ventricle showed significant alteration in diabetic groups compared to controls (*P*<0.05*). Whereas, expression levels of c-jun, ANF, and SKA were not significantly different between two groups.

Dicussion

This experimental study investigated the effect of induced GDM on the expression of some important genes involved in cardiac hypertrophy in the rat offspring. In the present study, the expression of all studied genes in the offspring of diabetic rats was higher than controls. We observed more than 2-fold increase in c-myc and β-MHC expression in left ventricle tissue of diabetic offspring (*P*<0.01**). Furthermore, we also showed that GDM significantly upregulates the expression of c-fos and α MHC in left ventricle tissue of diabetic offspring (*P*<0.05*).

The cause of hypertrophic cardiomyopathy in infants of the diabetic mothers is not clear, but it is likely due to the excessive secretion of insulin by the fetus pancreatic cells under the influence of hypoglycemia (17). Menezes *et al.* in 2009 showed that the thickness of ventricular septum and the weight of heart in the offspring of STZ-

induced diabetic rat mothers were significantly higher than control group. Offspring of diabetic rats at birth had higher heart weight, heart dimensions and heart-to-body weight ratio than control group (18). Today, it has been found that cardiomyocytes response to pathological hypertrophic stimuli by changes in the expression of some genes. The first groups of genes that become active in this path are: c-fos, c-jun and c-myc. These genes induce the activation of AP-1 complex by heterodimerization (14). Several growth factors, such as insulin, can also induce the activation of AP-1 by increasing the amount of c-fos and c-jun mRNA. On the other hand, both c-fos and c-jun are able to self-regulate their own gene transcription through binding to the promoter region of AP-1 gene. Therefore, mutations in the C-terminal region of these genes prevent the attachment of these proteins to the promoter region of AP-1 (19, 20). Furthermore, Olson *et al.* in 1994 proved that the amount of c-fos and c-jun mRNA in adipose tissue and cardiac muscle of diabetic rats is higher than non-diabetic rats. It was pointed out that insulin has a negligible effect on the level of c-fos and c-jun mRNA. Also, previous studies have shown that the expression of c-fos and c-myc genes in rats with aortic stenosis is increased, and c-fos expression increases in response to mechanical stretch of ventricular wall (13). Studies have also shown that in diabetic patients suffering from heart problems, the expression of myc genes has changed. Since c-fos and c-jun have a role in controlling the intracellular signaling pathways, regulation of growth and differentiation, we hypothesized that the expression of these genes in the offspring of GDM may be changed compare to controls (14). Present study confirmed this hypothesis and the results showed that the expression of these genes is upregulated in the offspring of diabetic group compare to offspring of non-diabetic rats. On the other hand, myocyte hypertrophy is also associated with increased expression of some embryonic genes such as ANF, β-MHC and SKA. These genes need to activate the synthesis of proteins that are involved in contraction. Proteins coded by these genes are normally produced in fetal cardiomyocytes, but they are synthesized greater than normal in hypertrophic cardiomyocytes. Some regulatory proteins have been identified that induce the expression of some of these embryonic genes in response to α-adrenergic and endothelin-1 (21, 22). Our study also confirmed that the expression of ANF, β-MHC and SKA, which encode proteins that are involved in creation of cardiomyocytes hypertrophy, was significantly increased in offspring of GDM compare to normal group.

Conclusion

Our data indicated that gestational diabetes upregulates the expression of c-jun, c-fos, c-myc, α-MHC, ANF and β-MHC (the genes involved in cardiac hypertrophy) in the offspring of diabetic rats. Previous studies on type 1 and type 2 diabetes have reported that the expression of some genes, such as α-MHC and ANP is increased in cardiac hypertrophy. Since insulin failed to affect significantly the protein production of hypertrophic genes, it was concluded that these genes control the development during the embryonic

period, and changes in their expression are related to cardiovascular complications in adults. However, it seems that more extensive cellular and animal studies are necessary for analyzing the effect of insulin on hypertrophic changes of cardiac myocytes in infants of diabetic mothers.

Acknowledgment

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