Maternal hyperglycemia leads to fetal cardiac hyperplasia and dysfunction in a rat model

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1Department of Obstetrics and Gynecology, University of Turku and Turku University Hospital, Turku, Finland; 2Institute of Biomedicine, Department of Physiology, University of Oulu, Oulu, Finland; 3The Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Turku, Finland; 4Department of Pathology, Turku University Hospital, Turku, Finland; 5Department of Pediatrics, University of Turku, Turku, Finland; 6Department of Medicine, Turku University Hospital, Turku, Finland; 7Turku Center for Disease Modeling, University of Turku, Turku, Finland; 8Department of Obstetrics and Gynecology, Kuopio University Hospital and University of Eastern Finland, Kuopio, Finland; and 9Department of Obstetrics and Gynecology, Oulu University Hospital, Oulu, Finland

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Lehtoranta L, Vuolteenaho O, Laine VJ, Koskinen A, Soukka H, Kytö V, Määttä J, Haapsamo M, Ekholm E, Räsänen J. Maternal hyperglycemia leads to fetal cardiac hyperplasia and dysfunction in a rat model. Am J Physiol Endocrinol Metab 305: E611–E619, 2013. First published July 9, 2013; doi:10.1152/ajpendo.00043.2013.—Accelerated fetal myocardial growth with altered cardiac function is a well-documented complication of human diabetic pregnancy, but its physiopathology is still largely unknown. Our aim was to explore the mechanisms of fetal cardiac remodeling and cardiovascular hemodynamics in a rat model of maternal pregestational streptozotocin-induced hyperglycemia. The hyperglycemic group comprised 107 fetuses (10 dams) and the control group 219 fetuses (20 dams). Fetal cardiac function was assessed serially by Doppler ultrasonography. Fetal cardiac to thoracic area ratio, newborn heart weight, myocardial cell proliferative and apoptotic activities, and cardiac gene expression patterns were determined. Maternal hyperglycemia was associated with increased cardiac size, proliferative, apoptotic and mitotic activities, upregulation of genes encoding A- and B-type natriuretic peptides, myosin heavy chain types 2 and 3, uncoupling proteins 2 and 3, and the angiogenetic tumor necrosis factor receptor superfamily member 12A. The genes encoding Kv channel-interacting protein 2, a regulator of electrical cardiac phenotype, and the insulin-regulated growth factors (15).

ACCELERATED FETAL MYOCARDIAL GROWTH with increased interventricular septal thickness is a well-documented complication of human diabetic pregnancy, but its mechanisms are still incompletely understood (16, 32). The myocardial remodeling is associated with altered fetal cardiac diastolic function during the third trimester (48). In newborns of diabetic mothers, circulating atrial natriuretic peptide concentrations are elevated and correlate with maternal first-trimester glycosylated hemoglobin values even with good maternal glycemic control and normal placental hemodynamics (11). Animal studies yield conflicting results on fetal myocardial responses to maternal diabetes. In mice, maternal diabetes results in the dilation of cardiac ventricles and reduced total ventricular myocardial area (13), whereas in a rat model of gestational diabetes the fetal heart/body weight ratio and total nuclear area in cardiac tissues are increased, suggesting fetal cardiac hypertrophy (23).

Cardiac growth increases either by cellular hypertrophy or hyperplasia. Hypertrophy and nonmuscle cell hyperplasia are the main mechanisms after birth, whereas during fetal life hyperplasia predominates (25). During mammalian fetal life, normal cardiac development requires insulin-like growth factor I (IGF-I) and insulin for heart mass growth via cell division (3, 41). Circulating insulin levels are elevated in human fetuses of diabetic mothers and in animals with streptozotocin (STZ)-induced maternal hyperglycemia (5, 36). In human diabetic pregnancies, glucose transfer across the placenta induces fetal hyperinsulinemia (36) and elevates serum levels of various growth factors (15).

Insulin has mitogenic activity, albeit weaker than that of the structurally related IGF-I (45). In normally functioning pancreatic β-cells, hyperglycemia increases insulin secretion. We hypothesized that fetal heart growth is accelerated due to cardiomyocyte hyperplasia in hyperglycemic pregnancies. The specific aims were to analyze fetal cardiac size and cell turnover activity as well as expression of genes involved in cardiac remodeling and function. Furthermore, we investigated how maternal hyperglycemia affects fetal cardiac function.

METHODS

Animals, induction of hyperglycemia, and data acquisition. The rats were purchased from the University of Turku Central Animal Laboratory and housed in specific pathogen-free conditions in room air with a 14:10-h light-dark cycle, and they had free access to food and water. The study protocol was submitted and approved by the University of Turku Laboratory Animal Care and Use Committee (permission no. 1664/06). The animal care conformed to the European Community Council Directions 86/609/EC and followed the council’s principles of laboratory animal care.

In female Sprague-Dawley (SD) rats, hyperglycemia was induced with an intraperitoneal injection of 35 mg/kg STZ prior to mating...
Blood glucose levels were obtained 2 days after injection using an Elite glucometer (Bayer, Leverkusen, Germany). Pre-gestational glucose samples were collected from conscious animals, whereas gestational samples were collected under anesthesia. Both were obtained from the tail vein. Samples were drawn before noon. If necessary, one to four additional doses of 15 mg/kg STZ were given at 2-day intervals. Ten rats with a glucose level exceeding 15 mmol/l comprised the study group (maternal hyperglycemia). Twenty healthy female SD rats served as controls. All female rats were caged overnight with a male. The day of a positive vaginal smear was designated as day 1 of gestation. Blood glucose concentrations of pregnant rats were measured on the days of ultrasonographic examination and from hyperglycemic dams also on the 1st gestational day (GD). The control group consisted of 219 fetuses and the hyperglycemic group 107 fetuses.

Ultrasonography was performed serially on GD 13 and 14, 16 and 17, and 19–21 under isoflurane-induced (3–4%) anesthesia in an oxygen-air mixture into a chamber and maintained with 1.5–2% isoflurane-oxygen-air mixture via a mask. The depth of anesthesia was monitored by assessment of withdrawal reflexes, and full unconsciousness was evidenced by loss of toe and tail-pinch reflexes. After the last ultrasonovascular examination on GD 19–21, the unconscious dam was humanely euthanized in a CO2 chamber, and euthanization was evidenced by loss of toe and tail-pinch reflexes. The maternal abdomen was opened with a V-shaped incision, and the uterine horns and fetuses were identified according to their location on the ultrasonography. The fetuses were decapitated instantly using special sharp blades. The number of fetuses in hyperglycemic dams varied from nine to 12 and in control dams from 10 to 16. From each dam, fetuses were randomly assigned for histological analyses, microcomputed tomography (µCT), or gene expression analyses. The placenta, fetal heart, and lungs were recovered and processed for analysis.

Newborn heart weights were gathered from a separate set of dams following identical protocols, as described above. After spontaneous delivery at term (GD 22), the newborns were anesthetized with a rapid intraperitoneal injection of pentobarbital sodium (200 mg/kg) and euthanized by cervical dislocation, and their hearts and bodies were weighed.

Histological analysis. Heart/lung blocks were fixed for 1–3 days in 10% buffered formalin and embedded in paraffin. All samples were cut into 5-µm sections, stained, and examined under UV light with a ×40 magnification. Hematoxylin and eosin (H & E)-stained samples were used for general morphological examination. Setting a grid as a standard area inside the myocardial wall near the apex of the heart (excluding epithelium, endothelium, and septal wall), the mitotic and apoptotic myocardial cells were counted (4) independently by two investigators (J. Laine and L. Lehtoranta). Mitotic cells were identified in H & E- and Gömöri silver-stained samples by their dividing chromatin figures (metaphase and anaphase are most clearly identified) and counted (no. of mitotic cells/mm² tissue in the central area of the left ventricle). The peripheral (i.e., endocardial and epicardial) areas of the myocardium and the trabeculae were excluded. Apoptotic cells in H & E-stained myocardial samples were identified by their dense, hyperchromatic, and fragmented nuclei, eosinophilic cytoplasm, and a clear halo separating the apoptotic myocardial cell from their surroundings. (22) The number of erythroblasts per 100 erythrocytes (ERBL/ERYT) ratio was calculated. The proliferative activity of the myocardial wall near the apex was measured by labeling with the Ki-67 antigen. Ki-67 is expressed in the nuclei of all cells in the G1, S, G2, and M phases (10). Because Ki-67 staining is very sensitive in fetal myocardial cells, we conducted several experiments to titer the correct dilution for the primary antibody. We used a dilution of 1:1,000 based on our preliminary experiments, which clearly labeled all mitotic nuclei and some round nuclei (prophase and telophase). Dark brown color of the nucleus was regarded as a positive staining result. Very light brown staining found occasionally in the normal appearing nuclei was counted as no staining. The Ki-67-stained samples were analyzed by a single investigator (L. Lehtoranta) blinded to the study group. The apoptotic myocardial cells were assessed by terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay by a single investigator blinded to the experimental group (V. Kytö) (19). Gömöri silver staining method was used to count the number of mitotic cells and measure cardiomyocyte width from the narrowest dimension attained crossing the nucleus from its central point by a single investigator blinded to the experimental group (J. Laine) (2). Finally, the number of cell nuclei, using the standard grid as a reference area, were counted from TUNEL assay and Gömöri silver-stained samples.

µCT imaging. µCT was performed on a 1072 Desktop X-ray microtomograph (SkyScan, Kontich, Belgium). Fetal heart and lungs were prepared as thorax blocks, fixed in 4% formaldehyde in PBS for 48 h, and embedded in paraffin, and excess material was removed. Samples were scanned with ×45 magnification, 6.51-µm pixel resolution, 3.9-s exposure time (50 kV, 150 µA), 0.45 rotation step (180°), and a 0.25-mm aluminum filter. Image reconstruction was performed with volumetric reconstruction software (NRecon version 1.4.4, SkyScan) and three-dimensional image volume analysis with CT Analyzer software (version 1.7.0.5, SkyScan). Left and right ventricular chamber and free wall and interventricular septal volumes were measured (Fig. 1).

Quantitative reverse transcription-polymerase chain reaction. Total ribonucleic acid (RNA) was purified from 15 hyperglycemic and 14 control fetal whole heart samples using Qiagen RNeasy reagents. cDNA first strand was synthesized from RNA using Moloney murine leukemia virus reverse transcriptase. The PCR reactions were performed with an ABI 7300 Real-Time PCR System using TaqMan chemistry. The standard curve method was used for quantification, and the results were normalized to 18S RNA measured from the same samples (20). To facilitate comparisons, all individual values in both the control and the experimental groups were divided by the average of the control group. Thus the control group always has the value 1.0 ± SD. The primers and bifunctional fluorogenic probes (6-carboxyfluorescein and tetramethylrhodamine) were designed with Primer Express software (Applied Biosystems). They are listed in Table 6. The markers were selected according to their function and association with the so-called fetal gene expression program (14).

Ultrasonography. After induction of anesthesia the dam was placed in a dorsal position, and the lower abdomen was shaved. A heat pad was used to maintain body temperature at 38°C. Ultrasonography was performed using Acuson Sequoia 512 equipment (Mountain View, CA) on a 13-MHz linear probe (12).

![Fig. 1. A microcomputed tomography scan of the fetal heart. The volume of cardiac muscle and cavities were drawn by hand. Papillary muscles were excluded from muscle and included in cavity volume area for heart volume analysis.](http://ajpendo.physiology.org/).
The fetuses were localized in each uterine horn starting from the top. After identifying the fetal heart by color Doppler, the sample volume of pulsed Doppler was adjusted to cover the entire heart. The high-pass filter was set at its minimum. The fetal heart was examined from different directions to minimize the angle between the Doppler beam and the inflow (IF) and outflow (OF) regions of the heart to obtain their maximal velocities. The maximal IF and OF velocities were recorded using a sweep speed of 100 mm/s.

The ultrasonographic examinations were videotaped and analyzed offline. Fetal heart rate (FHR) and time-velocity integral (TVI) of the OF waveform were measured. The OF mean velocity (Vmean), which is directly proportional to volume blood flow, was calculated with the following formula: Vmean = FHR × TVI. From the IF waveform the TVIs of the E- (early filling) and A-waves (filling during atrial contraction) were measured, and their ratio was calculated. The proportions (%) of the isovolumetric relaxation (IRT) and contraction (ICT) times of the cardiac cycle were calculated. The IRT was measured as the time period between the end of ejection and the onset of filling, and the ICT represents the period between the end of ventricular filling and the onset of ejection. The index of myocardial performance (IMP), which describes the combined systolic and diastolic function of the heart, was calculated by the following formula: IMP = (ICT + IRT)/ejection time (43). The cardiac (CA) and thoracic (TA) areas were measured from a cross-sectional cardiac four-chamber view, and the CA/TA ratio was calculated.

Statistical analysis. All data were analyzed using mixed-model measurements, with the dam as a random effect. Analyses tested mean differences in the outcome variables. Repeated measurements of ultrasonographic data were analyzed using time, maternal hyperglycemia, and interaction between time and maternal hyperglycemia as independent variables. Morphological, μCT, and mRNA data were analyzed using hyperglycemia as an independent variable. Correlation between parameters was examined using Spearman’s correlation coefficient. The statistical analyses were performed using SAS (version 9.2; SAS Institute, Cary, NC). When required by the statistical distribution, a logarithmic scale or √x conversion was utilized. All P values <0.05 were considered statistically significant. The results are presented as means ± SD or confidence interval (CI) 95%.

### RESULTS

**Maternal glucose levels.** STZ-treated dams had consistent pregestational and gestational hyperglycemia. The control dams were euglycemic (Table 1).

**Maternal hyperglycemia and fetal cardiac structure.** Maternal hyperglycemia was associated with increased CA/TA ratio. In the hyperglycemic group, newborn mean heart weight was ~29% and heart/body weight-ratio ~21% greater than in the control group. (Table 2).

To gain insight into whether the increase in heart size in the fetuses of hyperglycemic dams was due predominantly to hypertrophy or hyperplasia, we measured rates of mitosis and apoptosis in the myocardium. In the hyperglycemic dams, the cell turnover and activity in the fetal myocardium were increased (Fig. 2). Furthermore, the number of proliferative cells and apoptoses were increased, indicating accelerated myocardial turnover and growth (Table 3). The expression of a marker of cardiomyocyte proliferation, tumor necrosis factor receptor superfamily member 12a (Tnfrsf12a), was upregulated (Table 4). Cell width and nuclei numbers per area did not differ between the groups. These results suggest that, in the hyperglycemic dams, fetal myocardium shows hyperplastic features.

**Maternal hyperglycemia and fetal cardiac gene expression patterns.** To find out whether maternal hyperglycemia influences cardiac gene expression, we measured mRNA concentrations of several markers. The expression of atrial (Nppa) and B-type natriuretic peptide (Nppb), adult skeletal myosin heavy chain 2 (Myh2), and embryonic skeletal myosin heavy chain 3 (Myh3) were upregulated in the fetuses of hyperglycemic dams. The expression of α-cardiac myosin heavy chain 6 (Myh6), encoding the fast cardiac myosin that dominates in the mature myocardium, and Z-line actin-capping protein alpha1 (Capz1), a regulator at the end of sarcomeric actin, was not different between the groups.

**Genes regulating fetal cardiac function.** Maternal hyperglycemia was not associated with changes in the fetal cardiac expressions of adenoreceptor-β1 (Adrb1), sarcoplasmic reticulum Ca²⁺-ATPase (Atp2a2), or hyperpolarization activated cyclic nucleotide-gated potassium channel 2 (Hcn2), a channel expressed in atrial pacemaker cells. However, the expression of Kv channel-interacting protein 2 (Kcnip2), a key regulator of electrical cardiac function, was downregulated (Table 4).

**Genes affecting fetal cardiac metabolism.** Maternal hyperglycemia was associated with upregulation of the fetal cardiac metabolism.

### Table 1. Maternal glucose concentrations

<table>
<thead>
<tr>
<th>Variable</th>
<th>n₀</th>
<th>Control</th>
<th>n₀</th>
<th>Hyperglycemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregestag</td>
<td>10</td>
<td>25.0 ± 5.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GD 13 and 14</td>
<td>20</td>
<td>6.2 ± 1.2</td>
<td>9</td>
<td>29.6 ± 4.6</td>
</tr>
<tr>
<td>GD 16 and 17</td>
<td>20</td>
<td>4.4 ± 0.8</td>
<td>9</td>
<td>29.3 ± 3.3</td>
</tr>
<tr>
<td>GD 19–21</td>
<td>20</td>
<td>4.0 ± 0.8</td>
<td>10</td>
<td>29.6 ± 4.5</td>
</tr>
</tbody>
</table>

Data are given as means ± SD and in mmol/l. GD, gestational day; n₀, no. of dams.

### Table 2. Fetal heart size

<table>
<thead>
<tr>
<th>Variable</th>
<th>n =</th>
<th>Control</th>
<th>Hyperglycemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn heart</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, mg</td>
<td>9</td>
<td>2</td>
<td>30.3 ± 4.7</td>
</tr>
<tr>
<td>Heart/body weight ratio</td>
<td>9</td>
<td>2</td>
<td>0.53 ± 0.084</td>
</tr>
<tr>
<td>Ultrasonography (GD 20 and 21)</td>
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<td></td>
</tr>
<tr>
<td>CA/TA ratio</td>
<td>41</td>
<td>15</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>μCT volumes</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cardiac muscle, mm³</td>
<td>3</td>
<td>3</td>
<td>3.19 ± 0.69</td>
</tr>
<tr>
<td>Septum, mm³</td>
<td>3</td>
<td>3</td>
<td>0.60 ± 0.41</td>
</tr>
<tr>
<td>Left ventricle, mm³</td>
<td>3</td>
<td>3</td>
<td>0.57 ± 0.30</td>
</tr>
<tr>
<td>Right ventricle, mm³</td>
<td>3</td>
<td>3</td>
<td>0.77 ± 0.37</td>
</tr>
</tbody>
</table>

Data are given as means ± SD. n, No. of fetuses; μCT, microcomputed tomography; CA/TA, cardiac area/thoracic area ratio. Heart weight is given in mg. μCT volumes in mm³, *P ≤ 0.0001.
expression of uncoupling proteins 2 (Ucp2) and 3 (Ucp3), whereas the expression of insulin-responsive glucose transporter 4 (Slc2a4) was downregulated. The expression of Slc2a3, pyruvate dehydrogenase kinase isozyme 2 (Pdk2), a negative regulator of pyruvate metabolism, and cell cycle controller cyclin-dependent kinase inhibitor 1B (Cdkn1b) was similar in both groups (Table 4).

Maternal hyperglycemia and fetal hypoxia markers. Maternal hyperglycemia was associated with increased fetal ERBL/ERYT ratio (3.7 ± 4.5 in hyperglycemic pregnancies, n = 48; 0.6 ± 0.7 in euglycemic pregnancies, n = 58; P < 0.0001). ERBL/ERYT ratio had a negative correlation with OF Vmean [P = 0.038, b (95% CI) = −0.064 (−0.13, −0.0035)]. Surprisingly, the fetal cardiac expression of vascular endothelial growth factor A (Vegfa) was not upregulated, and another hypoxia marker gene, HIF-prolyl hydroxylase 3 (Egln3), was downregulated in the fetuses of hyperglycemic dams, thus contradicting the presence of myocardial hypoxia.

Maternal hyperglycemia and fetal cardiac function. To find out the functional significance of maternal hyperglycemia, we performed serial ultrasonographic examinations of the fetal hearts (Table 5). FHR was lower in the hyperglycemic dams throughout pregnancy. At GD 13 and 14, 98% of the fetuses of hyperglycemic dams displayed a holosystolic atrioventricular valve regurgitation (AVVR), whereas the incidence was only
6.26% in the control fetuses (Fig. 3). By GD 16 and 17 and 19–21, the AVVR had disappeared completely in both groups. The OF Vmean was significantly lower at GD 13 and 14 and the fetuses of hyperglycemic dams had increased cardiac size and cardiomyocyte mitotic and apoptotic activities. The gene expression of a marker of cardiomyocyte proliferation, Tnfrsf12a, was markedly upregulated, as was the expression of Nppa and Nppb, Myh2, Myh3, Ucp2, and Ucp3. On the other hand, there was a clear downregulation in insulin-resistant cardiac glucose transporter 2; Myh2, Z-line actin-capping protein-1; Slc2a3, facilitated glucose transporter 4; Slc2a4, insulin-responsive glucose transporter 4; Ucp2, uncoupling protein 2; Ucp3, uncoupling protein 3; Pdk2, pyruvate dehydrogenase kinase, isozyme 2; Egln3, hypoxia-inducible factor prolyl hydroxylase 3; Vegfa, vascular endothelial growth factor A; Tnfrsf12a, tumor necrosis factor receptor superfamily member 12a; Cdkn1b, cyclin-dependent kinase inhibitor 1B. mRNA concentrations are given as arbitrary units (AU). Expression of each gene in the diabetic group is adjusted to the expression in the control group as 1 AU. *P < 0.05; †P < 0.001; ‡P < 0.0001.

DISCUSSION

Our results reveal that maternal hyperglycemia is associated with major structural and functional changes in the fetal heart. In this rat model, the fetuses of hyperglycemic dams had increased cardiac size and cardiomyocyte mitotic and apoptotic activities. The gene expression of a marker of cardiomyocyte proliferation, Tnfrsf12a, was markedly upregulated, as was the expression of Nppa and Nppb, Myh2, Myh3, Ucp2, and Ucp3. On the other hand, there was a clear downregulation in insulin-resistant cardiac glucose transporter 2; Myh2, Z-line actin-capping protein-1; Slc2a3, facilitated glucose transporter 4; Slc2a4, insulin-responsive glucose transporter 4; Ucp2, uncoupling protein 2; Ucp3, uncoupling protein 3; Pdk2, pyruvate dehydrogenase kinase, isozyme 2; Egln3, hypoxia-inducible factor prolyl hydroxylase 3; Vegfa, vascular endothelial growth factor A; Tnfrsf12a, tumor necrosis factor receptor superfamily member 12a; Cdkn1b, cyclin-dependent kinase inhibitor 1B. mRNA concentrations are given as arbitrary units (AU). Expression of each gene in the diabetic group is adjusted to the expression in the control group as 1 AU. *P < 0.05; †P < 0.001; ‡P < 0.0001.

Table 3. Fetal cardiomyocyte cell cycle

<table>
<thead>
<tr>
<th>Variable</th>
<th>n0</th>
<th>n1</th>
<th>Control</th>
<th>n0</th>
<th>n1</th>
<th>Hyperglycemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H &amp; E</td>
<td>85</td>
<td>13</td>
<td>36.0 ± 27.5</td>
<td>46</td>
<td>10</td>
<td>55.0 ± 30.5</td>
</tr>
<tr>
<td>TUNEL</td>
<td>39</td>
<td>12</td>
<td>0.036 ± 0.023</td>
<td>23</td>
<td>10</td>
<td>0.11 ± 0.045</td>
</tr>
<tr>
<td>Mitosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H &amp; E</td>
<td>85</td>
<td>13</td>
<td>183.4 ± 79.6</td>
<td>46</td>
<td>10</td>
<td>254.3 ± 103.7</td>
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<tr>
<td>Ki-67</td>
<td>42</td>
<td>17</td>
<td>83.8 ± 37.1</td>
<td>25</td>
<td>10</td>
<td>200.3 ± 87.9</td>
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<tr>
<td>Gömöri</td>
<td>21</td>
<td>12</td>
<td>318.5 ± 176.3</td>
<td>20</td>
<td>10</td>
<td>427.2 ± 153.7</td>
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<td>Nuclear count</td>
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<tr>
<td>TUNEL</td>
<td>39</td>
<td>12</td>
<td>5,871.7 ± 457.9</td>
<td>23</td>
<td>10</td>
<td>5,709.8 ± 471.7</td>
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<tr>
<td>Gömöri</td>
<td>22</td>
<td>12</td>
<td>5,716.5 ± 1,037.4</td>
<td>21</td>
<td>10</td>
<td>5,756.8 ± 822.4</td>
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</table>

Data are given as means ± SD. H & E, hematoxylin and eosin. Results are given as no. of nuclei/mm² apart from TUNEL apoptotic cell counts, where the unit is width in μm. *P < 0.05; †P < 0.001; ‡P < 0.0001.

Table 4. Fetal heart gene expression (gene/18S)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>Hyperglycemia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n0</td>
<td>n1</td>
</tr>
<tr>
<td>Kcnip2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atp2a2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrb1</td>
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</tr>
<tr>
<td>Hcn2</td>
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<tr>
<td>Natriuretic peptides</td>
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</tr>
<tr>
<td>Nppa</td>
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<td></td>
</tr>
<tr>
<td>Myosin and actin-associated</td>
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</tr>
<tr>
<td>Myh2</td>
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</tr>
<tr>
<td>Myh3</td>
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<td>Myh6</td>
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<td>Hypoxia</td>
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<td>Egln3</td>
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<tr>
<td>Vegfa</td>
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</tr>
<tr>
<td>Growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tnfrsf12a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdkn1b</td>
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</table>

Data are given as means ± SD. Kcnip2, kv-channel interacting protein; Atp2a2, sarcoplasmic reticulum Ca2+ -ATPase; Adrb1, β1-adrenergic receptor; Hcn2, hyperpolarization-activated cyclic nucleotide-gated potassium channel 2; Nppa, atrial natriuretic peptide; Nppb, brain natriuretic peptide; Myh2, adult skeletal myosin heavy chain 2; Myh3, embryonic skeletal myosin heavy chain 3; Myh6, α-cardiac myosin heavy chain 6; Capza1, Z-line actin-capping protein-a1; Slc2a3, facilitated glucose transporter 3; Slc2a4, insulin-responsive glucose transporter 4; Ucp2, uncoupling protein 2; Ucp3, uncoupling protein 3; Pdk2, pyruvate dehydrogenase kinase, isozyme 2; Egln3, hypoxia-inducible factor prolyl hydroxylase 3; Vegfa, vascular endothelial growth factor A; Tnfrsf12a, tumor necrosis factor receptor superfamily member 12a; Cdkn1b, cyclin-dependent kinase inhibitor 1B. mRNA concentrations are given as arbitrary units (AU). Expression of each gene in the diabetic group is adjusted to the expression in the control group as 1 AU. *P < 0.05; †P < 0.005; ‡P < 0.001; §P < 0.0001.
responsive glucose transporters \textit{Slc2a4} and \textit{Kcnip2}, a key regulator of electrical cardiac function. There were profound transient alterations in the cardiac function at midgestation, as assessed by ultrasonography. The fetuses of hyperglycemic dams had a much higher incidence of AVVR, lower OF Vmean, a greater proportional role of early rather than atrial filling of the ventricles, and a longer isovolumetric contraction period of the ventricles. At term gestation, these fetuses showed signs of diastolic cardiac dysfunction that were not present in the control fetuses. The changes in the fetuses of hyperglycemic dams were probably not due directly to increased hypoxia since the expression of two well-established hypoxia marker genes, \textit{Egln3} and \textit{Vegfa}, were similar in both groups.

The increased CA/TA ratio in the fetuses of hyperglycemic dams was associated with greater mitotic and apoptotic activity. There were no differences either in cell width or in the number of nuclei per area. Thus, our results suggest that the increase in cardiac size is caused predominantly by hyperplastic rather than hypertrophic growth. Increased apoptotic activity reflects a dynamic growth process with high cellular turnover. Natriuretic peptides have been shown previously to induce apoptosis in myocardial cells (46), and we found that gene expressions of \textit{Nppa} and \textit{Nppb} were markedly upregulated in the fetal hearts of hyperglycemic dams.

Enhanced cardiac hyperplastic growth in fetuses of hyperglycemic dams was associated with markedly altered cardiac gene expression patterns spanning contractile, electrical, and endocrine function as well as metabolism. Upregulation of natriuretic peptides and \textit{Myh3} and the downregulation of \textit{Slc2a4} may result from an exaggerated fetal gene expression pattern that may provide the fetal heart with a more economical use of oxygen and energy at the cost of lower cardiac performance (27). Upregulation of \textit{Ucp2} and \textit{Ucp3} seems to contradict this. On the other hand, their main role in the heart may be not to uncouple mitochondrial respiration but rather to protect mitochondria against lipid-induced oxidative stress. Upregulation of \textit{Ucp3} is present when mitochondrial fatty acid supply exceeds the oxidation capacity, as in insulin resistance (7, 24). It is possible that fetuses of diabetic mothers have insulin resistance due to strong and prolonged insulin secretion. The observed downregulation of \textit{Slc2a4} expression supports this conclusion.

One of the most interesting effects of maternal hyperglycemia on fetal cardiac gene expression was downregulation of \textit{Kcnip2}, a key regulator of cardiac electrical and contractile function. \textit{Kcnip2} has been shown previously to be downregulated in cardiac hypertrophy (18) and cardiac failure (26). \textit{Kcnip2} is a voltage-gated K\textsuperscript{+} channel-interacting protein that controls many aspects of electrical cardiac phenotype, including the balance of polarizing (Na\textsuperscript{+}) and depolarizing (I\textsubscript{so}) currents early in the ventricular action potential, and the intracellular calcium concentration (9, 40). It also binds calcium and is an integral subunit component of the native Kv4 channel complex. \textit{Kcnip2}-knockout mice have a complete loss of I\textsubscript{so} and a susceptibility to ventricular tachycardia by lengthening the plateau phase of the action potential (18). Attenuated \textit{Kcnip2} protein level increases the duration of cardiomyocyte action potential, which results in increased \textit{I}_{Ca,L} and intracellular Ca\textsuperscript{2+} concentration and ultimately increased contractility and systolic function (33). The cardiomyocytes of mice with type 2 diabetes have been shown to exhibit reduction in repolarizing outward K\textsuperscript{+} currents so that the duration of action potential is markedly increased (38). Thus the \textit{Kcnip2} downregulation we observed may be a protective mechanism against impending cardiac failure in the fetuses of hyperglycemic dams.

![Pulsed Doppler ultrasonography of fetal heart](image-url)
Table 6. Sequences of primers and bifunctional fluorogenic probes (5′-FAM, 3′-TAMRA) used in the quantitative RT-PCR analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
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<tbody>
<tr>
<td>Adrb1</td>
<td>Forward primer GATCGCCTTCTGTTCTTCTTTT</td>
</tr>
<tr>
<td></td>
<td>Reverse primer GAGTCGCCCTTTGCTGGTGG</td>
</tr>
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<td></td>
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<td>Atp2a2</td>
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<tr>
<td></td>
<td>Reverse primer GAGTCGCCCTTTGCTGGTGG</td>
</tr>
<tr>
<td></td>
<td>Probe TGGGCTACGGCGACCTGCGCCTCTT</td>
</tr>
<tr>
<td>Cgca1</td>
<td>Forward primer TCGAGAAGGCGCGACCTGCGC</td>
</tr>
<tr>
<td></td>
<td>Reverse primer GAGTCGCCCTTTGCTGGTGG</td>
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<tr>
<td></td>
<td>Probe TGGGCTACGGCGACCTGCGCCTCTT</td>
</tr>
<tr>
<td>Cdkn1b</td>
<td>Forward primer CCAGCCACGGCGACCTGCGC</td>
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<tr>
<td></td>
<td>Reverse primer GAGTCGCCCTTTGCTGGTGG</td>
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<td></td>
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<td>Egln3</td>
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<td></td>
<td>Reverse primer GAGTCGCCCTTTGCTGGTGG</td>
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<td></td>
<td>Probe TGGGCTACGGCGACCTGCGCCTCTT</td>
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<td>Myh3</td>
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<td>Nppa</td>
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<tr>
<td></td>
<td>Probe TGGGCTACGGCGACCTGCGCCTCTT</td>
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</table>

5′-FAM, 6-carboxyfluorescein; 3′-TAMRA, tetramethylrhodamine. All sequences are listed from 5′ to 3′.

dams. The expression of another key regulator of cardiomyocyte calcium metabolism, Atp2a2 (Serca2), was unaffected by maternal hyperglycemia. Studies on adult diabetic mice have revealed conflicting results with a reduction (47) or no difference (8) in Atp2a2 myocardial expression.

Ultrasound evaluation showed that in hyperglycemic dams FHR was lower throughout pregnancy, being the lowest at GD 13 and 14. Previously, lower heart rate has been observed in adult type 2 diabetic mice (6, 8, 37). Cardiac function and left ventricular pressure characteristics were comparable with those of healthy control mice at baseline conditions, but the inotropic and lusitropic responses to dobutamine stress test were attenuated in adult type 2 diabetic mice (8). Downregulation of adrenoceptor-β1 induced by diabetes was speculated to cause these cardiovascular changes (1). However, in our study, fetal Adrb1 expression was not affected by maternal hyperglycemia and is thus unlikely to contribute to the observed reduction in FHR.

In human diabetic pregnancies, fetal myocardial fractional shortening is decreased if the pulsatility of the aortic blood flow is increased or the total blood flow in the aorta is high (28). This could indicate decreased functional capacity of the fetal heart in diabetic pregnancy, especially at near-term gestation. In the present study, we found that the %ICT was greater in the fetuses of hyperglycemic dams at GD 13 and 14 and GD 16 and 17. Furthermore, we found increased %IRT at term, which is suggestive of fetal diastolic dysfunction. Therefore, we propose that maternal hyperglycemia may limit fetal cardiac functional capacity, especially under constraints such as hypoxemia.

Almost all of the fetuses of hyperglycemic dams had holosystolic AVVR and decreased OF Vmean at GD 13 and 14. Later in pregnancy, the incidence of AVVR and OF Vmean were comparable with the control group. Thus any persistent anatomic abnormality in the atrioventricular valve structure could not explain the presence of AVVR in midgestation. The disappearance of AVVR and normalization of fetal OF Vmean might be signs of successful cardiac remodeling. A sudden change in cardiac loading conditions, especially in the afterload, often causes tricuspid regurgitation that resolves after the heart adjusts to a new loading environment (30). Cardiac development at GD 13 and 14 in rat pregnancy corresponds to 6–8 gestational weeks in human pregnancy. In human fetuses cardiac diastolic function improves, and cardiac output and
placental volume blood flow increase significantly between 6 and 8 gestational weeks (21). Simultaneously, the fetal heart adapts to swift alterations in cardiac loading. In human pregnancies with pregestational diabetes, fetal cardiac function deviates from controls at 11–14 wk gestation (44). Especially fetuses of mothers with poorly controlled diabetes demonstrate signs of abnormal diastolic and global cardiac performance. Furthermore, the first-trimester maternal glucose levels correlate to circulating concentrations of atrial natriuretic peptide in the newborn, suggesting that maternal hyperglycemia affects fetal cardiovascular development and contractile function (11).

Our results demonstrate that cardiac response to changing loading conditions is compromised in fetuses of hyperglycemic dams. This may partially explain the increased early fetal loss rate in diabetic pregnancies.

In human pregnancies with maternal diabetes, a well-documented complication is fetal hypoxia (42). In this study, the ERBL/ERYT ratio in the fetuses of hyperglycemic dams was higher than in the euglycemic controls, possibly reflecting the hypoxia-induced increase in erythropoiesis. However, fetal cardiac expressions of the two well-documented hypoxia markers Egln3 and Vegfa were not upregulated, contradicting myocardial hypoxia. Egln3 expression was actually downregulated in the hyperglycemic pregnancies. Thus enhanced erythropoiesis may have been able to prevent hypoxia of the myocardial tissue.

The main limitations of this study are the STZ-induced hyperglycemic model and the noninvasive measurement of cardiovascular functional parameters by ultrasonography. Although the STZ-induced hyperglycemic model does not manifest all of the disease processes present in an autoimmune type 1 diabetes, it has long been used successfully as a model to examine the pathophysiological consequences of diabetes in the myocardium (39). In the clinical setting, especially in early pregnancy, maternal hyperglycemia is a common problem in diabetic pregnancies that has been associated with fetal myocardial remodeling. In addition, unfortunately, in many cases the maternal glycemic control has not been optimized prior to pregnancy. Our model simulates the clinical disease in humans, but our results cannot be applied directly to the clinical situation. We wanted to ensure increased fetal cardiac growth by producing a severe hyperglycemic environment with STZ, which causes dose-related, highly specific damage to pancreatic β-cells and insulin deficiency (17). STZ itself is unlikely to be associated with any fetal heart toxicity because of its cellular uptake via glucose transporter 2 found only in pancreatic β-cells, renal cells, and hepatic cells of the rat (35). In addition, we avoided the direct effect of STZ on fetal organs by administering it before mating. The ultrasonographic indices used in the present study have been validated in animal experiments by comparing with invasive measurements (34). We did not distinguish between right and left ventricular IF and OF areas and thus were unable to determine whether AVVR originated from the mitral or tricuspid valve. However, in the fetus, the ventricles function in parallel against practically equal systemic pressure because the foramen ovale and ductus arteriosus are widely open. General anesthesia used for the ultrasonographic recordings may affect cardiac function, but because both groups are affected, differences can still be observed. Finally, we have shown with mouse fetuses that the intraobserver variabilities of Doppler-derived parameters are low, ranging for IF and OF TVIs from 6.0 to 6.5% and for time interval measurements from 2.5 to 15.9% (31).

In conclusion, this rat model of pregestational maternal hyperglycemia demonstrates that fetal cardiac enlargement and myocardial remodeling are caused primarily by hyperplasia. Furthermore, maternal hyperglycemia has profound effects on fetal cardiac gene expression patterns involving genes that control and regulate electrical, contractile, endocrine, and metabolic functions of the heart. Hyperplastic myocardial growth and altered cardiomyocyte gene expression have functional consequences, including decreased fetal heart rate, transient depression of cardiac output, and the presence of holosystolic AVVR. Our results suggest that fetal myocardial adaptation to maternal hyperglycemia increases fetal vulnerability to stress stimuli such as hypoxemia.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are reported by the authors.

AUTHOR CONTRIBUTIONS

L.L., O.V., M.H., and J.R. contributed to the conception and design of the research; L.L., A.K., H.S., and J.R. performed the experiments; L.L., O.V., J.L., A.K., V.K., J.M., M.H., E.E., and J.R. analyzed the data; L.L., O.V., J.L., A.K., H.S., V.K., E.E., and J.R. interpreted the results of the experiments; L.L., J.L., and J.R. prepared the figures; L.L., O.V., and J.R. drafted the manuscript; L.L., O.V., J.L., H.S., E.E., and J.R. edited and revised the manuscript; L.L., O.V., J.L., A.K., H.S., V.K., J.M., M.H., E.E., and J.R. approved the final version of the manuscript.

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