Responses of mitochondrial biogenesis and function to maternal diabetes in rat embryo during the placental period

M. P. Alcolea, I. Lladó, F. J. García-Palmer, and M. Gianotti

Grup de Metabolisme Energètic i Nutrició, Universitat de les Illes Balears i Centro de Investigación Biomédica en Red (Network Biomedical Research Center) Fisiopatología Obesidad y Nutrición, Instituto de Salud Carlos III, Palma de Mallorca, Spain

Submitted 22 February 2007; accepted in final form 6 June 2007

Alcolea MP, Lladó I, García-Palmer FJ, Gianotti M. Responses of mitochondrial biogenesis and function to maternal diabetes in rat embryo during the placental period. Am J Physiol Endocrinol Metab 293: E636–E644, 2007; doi:10.1152/ajpendo.00120.2007.—Mitochondria are cellular organelles that have been reported to be altered in diabetes, being closely related to its associated complications. Moreover, mitochondrial biogenesis and function are essential for proper embryo development throughout the placental period, occurring during organogenesis, when a great rate of congenital malformations have been associated with diabetic pregnancy. Thus, the aim of the current work was to investigate the effect of the diabetic environment on mitochondrial function and biogenesis during the placental period. For this purpose, we studied the oxidative phosphorylation system (OXPHOS) enzymatic activities as well as the expression of genes involved in the coordinated regulation of both mitochondrial and nuclear genome (PGC-1α, NRF-1, NRF-2α, mtSSB, and TFAM) and mitochondrial function (COX-IV, COX-1, and β-ATPase) in rat embryos from control and streptozotocin-induced diabetic mothers. Our results reflected that diabetic pregnancy retarded and altered embryo growth. The embryos from diabetic mothers showing normal morphology presented a reduced content of proteins regulated through the PGC-1α mitochondrialogenic pathway on gestational day 12. This fact was accompanied by several responses that entailed the activation of OXPHOS activities on the same day and the recovery of the content of the studied proteins to control levels on day 13. As a result, the mitochondria of these embryos would reach a situation close to control on day 13 that could allow them to follow the normal mitochondrial schedule throughout the gestational period in which the mitochondrial differentiation process is critical. Nevertheless, malformed embryos from diabetic mothers seemed to show a lower adaptation capability, which could exacerbate their maldevelopment.

The altered embryo-fetal development induced by the oxidative environment of diabetic pregnancy is an important complication leading to a high rate of birth defects in the offspring of diabetic mothers, representing a major cause of mortality and morbidity in these infants, and also to diseases that will be evident later in the adult life of the newborn (11, 17, 25). The organogenic period is especially more susceptible to diabetes-related teratogenesis due to the immaturity of free radical scavenging mechanisms (25, 52) as well as to the critical processes taking place, such as the closure of the neural tube and the formation of many embryonic organs (10, 31). However, the pathological mechanisms involved in maternal diabetes-induced embryo maldevelopment are not fully understood.

Throughout organogenesis, embryo mitochondria acquire a critical role owing to the switch from glycolytic to oxidative metabolism that takes place (2, 44), coinciding with the establishment of the choioallantoic circulation on gestational day 12, when oxygen becomes more available to the embryo (1, 19). This metabolic onset entails a mitochondrial biogenic process that implies important changes in mitochondrial function, morphology, and expression, which leads to embryo mitochondria reaching a more differentiated stage (4, 28, 43, 51).

Mitochondriogenesis is a complex event that includes both mitochondrial proliferation and differentiation processes (16, 34). Numerous protein factors have been reported (13, 40, 50) to be involved in mitochondrial biogenesis in mammals so far. In short, the family of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) coactivates different transcription factors in response to energy requirements (40). Among these transcription factors, nuclear respiratory factor-1 and -2 (NRF-1 and NRF-2) regulate the expression of most nuclear genes involved in mitochondrial biogenesis such as genes encoding subunits of respiratory complexes, mitochondrial transcription factors A, B1, and B2 (TFAM, TFB1M, and TFB2M, respectively), mitochondrial replication factors such as mitochondrial single-strand DNA-binding protein (mtSSB) and mitochondrial RNA-processing endoribonuclease, and other proteins that are necessary for mitochondrial function (13, 40, 50).

Mitochondria have been reported to be target organelles that are altered during the development of diabetic complications (29, 33, 39, 41). In this sense, diabetes-induced oxidative stress is known to cause defects in the expression, function, and structure of mitochondria (23, 33, 41, 42), which in turn might further increase reactive oxygen species production and, thus, the diabetic complications. However, despite the fact that mitochondrial biogenesis is a critical process in embryo development (4, 30, 48), nothing is known about the effect of maternal diabetes on this process and about its relationship to the embryopathies associated with this pathology.

The aim of the current work was to establish whether the teratogenic environment, present in the experimental model of maternal streptozotocin-induced type 1 diabetes, alters the
normal mitochondrial biogenic process taking place in rat embryo during the placentaion period. For this purpose, we investigated rat embryo mitochondrial function and biogenesis on gestational days 12 and 13 by measuring oxidative phosphorylation system (OXPHOS) enzymatic activities and the expression of genes involved in the coordinated transcriptional regulation of both nuclear and mitochondrial genome (PGC-1α, NRF-1, NRF-2α, and TFAM) as well as genes related to mitochondrial replication (mtSSB) and function [cytochrome c oxidase subunit (COX)-IV, COX-I, and ATP synthase β-subunit (β-ATPase)] (12). In parallel, we studied the different mRNA expression between malformed embryos and those showing a normal morphology from diabetic rats on gestational days 11, 12, and 13 to gain further insight into diabetes-associated embryopathy.

MATERIALS AND METHODS

Materials. All enzymes, substrates, and coenzymes were obtained from Sigma-Aldrich (St. Louis, MO). Routine chemicals and materials were supplied by Bio-Rad (Hercules, CA), Merck (Darmstadt, Germany), Panreac (Barcelona, Spain), Pronadisa (Madrid, Spain), Roche Diagnostics (Basel, Switzerland), and Sigma-Aldrich.

Lightcycler-FastStart DNA Master SYBR Green I for real-time PCR and oligonucleotide primer sequences were supplied by Roche Diagnostics. Chemicals for mRNA reverse transcription were obtained from Applied Biosystems.

Antibodies for COX-I were obtained from Molecular Probes (A-6403), for COX-IV from MitoSciences (MS407), and for β-ATPase and PGC-1α from Santa Cruz Biotechnology (sc-16690 and sc-5816). Rabbit antiserum against TFAM was kindly provided by Dr. H. Inagaki (15). Chemicals for immunoblot development were supplied by Amersham (Little Chalfont, UK).

Animals. Animal experiments were performed in accordance with the general guidelines of our institutional ethics committee and European Union (86/609/EEC) regulations and were approved by the ethics committee. Three-month-old virgin female Wistar rats (Charles River, Barcelona, Spain) with an initial weight of ~250 g were made diabetic with a single intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich), with 45 mg/kg freshly dissolved in sodium citrate buffer. Treatments were performed 1 day after overnight mating. When intravaginal sperm was found, STZ injection. Vaginal smears were examined for sperm the morning after the STZ injection. Uteri were removed and conceptuses quickly dissected into a sterile culture dish with physiological saline solution. After the STZ injection. Uteri were removed and conceptuses quickly dissected into a sterile culture dish with physiological saline solution.

Isolation of samples. Pregnant rats were killed by decapitation on gestational day 11, 12, or 13, and nonpregnant rats were killed 12 days after the STZ injection. Uteri were removed and conceptuses quickly dissected into a sterile culture dish with physiological saline solution. The embryos were examined for gross malformations according to a scoring system (Brown and Fabro, 5, 6), which included the morphological analysis of the brain spheres, neural tube, heart, optic and otic vesicles, and limb buds as well as the axial curvature, crown-rump length, and number of somites. Embryos not conforming to normal morphology in any of the aforementioned structures were classified as being malformed. Normal embryos did not show morphological alterations and exhibited the correct body flexure and closure of both the anterior and posterior neural pore. Due to the low success in obtaining pregnant diabetic rats (~30%) and the large amount of gestational day 11 embryos needed (from 3 to 4 pregnant rats) to pool large enough samples to be able to perform mitochondrial measurements, these embryos were included only in the study of the relative mRNA levels, owing to the low amount of sample needed for this determination. Thus, all day 11 embryos, all malformed embryos, and between one and three normal ones per mother on gestational days 12 and 13 were rapidly frozen in nitrogen liquid and stored at −70°C for RNA extraction.

The remaining normal embryos, on gestational days 12 and 13, were homogenized in isolation buffer (300 mM sucrose, 5 mM MOPS, 5 mM KH2PO4, 1 mM EDTA, 0.01% bovine serum albumin, pH 7.4) and used for mitochondrial isolation. For this purpose, two rat litters on day 12 and one litter on day 13 were used to pool the embryos to obtain large enough samples in each of the seven to 10 experiments carried out.

Determination of glucose and insulin. Blood glucose was measured in pregnant and nonpregnant rats from trunk blood by Accutrend GCT glucose meter (Roche Diagnostics). Insulin was determined in plasma samples using a double-antibody rat insulin ELISA kit (Mercodia, Uppsala, Sweden). All measurements were performed according to the manufacturer’s protocols.

Isolation of mitochondria. The mitochondrial fraction was obtained according to Justo et al. (20). In brief, nuclei and cell debris were first removed by centrifugation at 700 g for 10 min at 4°C (Sigma-3K30). The resulting supernatant was subjected to 10 min of centrifugation at 3,000 g, rendering the mitochondrial fraction studied. The pellets were resuspended in the isolation buffer. An aliquot of both homogenate and isolated mitochondria was used to determine cytochrome c oxidase activity, and the recovery percentage of the isolated mitochondria was calculated. The remaining samples were frozen at −70°C for subsequent analysis.

Citratase synthase (EC 4.1.3.7) activity was determined in homogenate and mitochondrial fraction, using a microtiter plate spectrophotometer (Bio-Tek Instruments), by following the increase in absorption of the 5-thio-2-nitrobenzoate ion at 412 nm and 30°C as previously reported (32). Total protein content was measured in both homogenate and mitochondrial fraction (27). Total DNA content was tested in homogenate samples (47).

Real-time RT-PCR analysis. Total RNA was isolated from frozen embryo samples using TRIzol reagent and quantified using a spectrophotometer set at 260 nm. One microgram of the total RNA was reverse transcribed to cDNA at 42°C for 60 min with 25 U MuLV reverse transcriptase in a 10-μl volume of retrotranscription reaction mixture containing 10 mM Tris·HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl2, 2.5 μM random hexamers, 10 U RNase inhibitor, and 500 μM each dNTP. Each cDNA sample was diluted 1/10, and aliquots were frozen (−70°C) until the PCR reactions were carried out.

Real-time PCR was done for seven target genes: PGC-1α, NRF-1 and NRF-2 DNA-binding α-subunit, mtSSB, TFAM, COX-IV, and COX-I. All oligonucleotide primer sequences were obtained from Primer3 and tested with IDT OligoAnalyser 3.0 (Table 1). Finally, a Real-time RT-PCR analysis. Total RNA was isolated from frozen embryo samples using TriPure isolation reagent and quantified using a spectrophotometer set at 260 nm. One microgram of the total RNA was reverse transcribed to cDNA at 42°C for 60 min with 25 U MuLV reverse transcriptase in a 10-μl volume of retrotranscription reaction mixture containing 10 mM Tris·HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl2, 2.5 μM random hexamers, 10 U RNase inhibitor, and 500 μM each dNTP. Each cDNA sample was diluted 1/10, and aliquots were frozen (−70°C) until the PCR reactions were carried out.

Real-time PCR was performed as previously described (4). The amplification program consisted of a preincubation step for denaturation of the template cDNA (10 min, 95°C) followed by 40 cycles consisting of a denaturation step (1 s, 95°C), an annealing step (12 s, 60°C for PGC-1α; 10 s, 58°C for mtSSB and COX-IV; 60°C for TFAM and 64°C for NRF-2α; 8 s, 55°C for COX-I; and 5 s, 56°C for NRF-1), and an extension step (12 s, 72°C for all, except for NRF-1 and COX-I, which were 8 s, 72°C, and 26 s, 72°C, respectively). After each cycle, fluorescence was measured at 72°C. A negative control without cDNA template was run in each assay. The real-time PCR efficiencies (e) were estimated on average of all sample efficiencies, which were calculated by the following formula: e = (F0/F)n/n0, where n and n0 were the crossing point values of F and F0, respectively.
mitochondrial proteins were incubated with mouse monoclonal antibodies for COX-1α. Blots with mitochondrial proteins were incubated with mouse monoclonal antibodies for COX-1α, mouse polyclonal antibodies for COX-IV, goat polyclonal antibodies for β-ATPase, and rabbit antisera against TFAM. Immunoblot development was performed using an enhanced chemiluminescence Western blotting analysis system. Film bands were quantified by densitometric analysis (Kodak 1D Image Analysis Software). The apparent molecular weights of the proteins detected were 91, 39, 14, 51, and 25 kDa for PGC-1α, COX-1α, COX-IV, β-ATPase, and TFAM, respectively.

**OXPHOS enzymatic activities.** OXPHOS enzymatic activities were measured in the mitochondrial fraction using a microtiter plate spectrophotometer (Bio-Tek Instruments). Cytochrome c oxidase (ferrocyanochrome c: oxygen oxidoreductase, COX, EC 1.9.3.1) activity was determined by following the oxidation of 3,3′-diaminobenzidine tetrahydrochloride at 450 nm and 37°C (9). ATPase (ATP phosphohydrolase, complex V, EC 3.6.1.3) activity was measured by following the oxidation of NADH at 340 nm and 37°C (38). The extinction coefficient used was 6.22 mM/cm.

**Electron microscope analysis.** The embryo mitochondria samples from both control and diabetic mothers of three independent experiments for transmission electron microscope (TEM) examination were obtained on gestational day 13 under exactly the same conditions as indicated in Isolation of mitochondria. They were carefully removed and placed in ice-cold fixative buffer (2.5% glutaraldehyde in 0.2 M trihydrated sodium cacodylate buffer, pH 7.2) for 2 h. The fixed pellets were then washed four times in 0.2 M trihydrated sodium cacodylate buffer and postfixed (1% osmium tetroxide) for 2 h. They were then dehydrated in graded aceton steps, stained with 2% uranyl acetate overnight, and embedded in Spurr’s resin. Semithin sections for light microscopy, ~0.5 mm thick, were cut with glass knives, stained with methylene blue, and examined under a light microscope (Olympus BX-60). Ultrathin sections for electron microscopy, ~50 nm thick, were stained with saturated lead citrate solution and examined by a Hitachi H-600 electron microscope at 75 kV. TEM micrographs were obtained at magnifications of ×10,000.

TEM micrographs were analyzed by Scion Image software for morphometric studies, with mitochondrial area and perimeter measured. Three ultrathin cuts were analyzed in each of the three independent samples, with a total number of 300 mitochondria per sample measured.

**Statistics.** All data are expressed as mean values ± SE. Differences between gestational days and diabetes induction were assessed by two-way analysis of variance followed by Student’s t-test post hoc for embryo measurements and by least significant difference post hoc for maternal plasma parameters. Since mitochondrial area and perimeter data did not resemble a theoretical normal distribution according to the Kolmogorov-Smirnov test, differences between groups were assessed by a nonparametric Kruskal-Wallis test. Statistical analyses were performed with the statistical software package SPSS 13.0 for Windows (Chicago, IL).

The statistical PCR data analysis was performed using the Relative Expression Software Tool (REST-MCS V.2, 2006). The statistical model was a pair-wise fixed reallocation randomization test (36, 37). Differences in expression between groups were assessed using the means for statistical significance by randomization tests.

Results were considered statistically significant at the P < 0.05 level in all cases.

**RESULTS**

**Maternal plasma glucose and insulin levels.** Glucose levels in both pregnant and nonpregnant STZ-treated animals (Table 2) were significantly higher than in control groups (from 3- to 4-fold), in agreement with Guijarro et al. (14). Due to our interest in the study of the embryos, mothers were not under fasting conditions at the moment they were killed to avoid alterations in the normal embryo development (1). For this reason, we calculated the ratio between insulin and glucose to normalize the differences between the animals due to food consumption. Our data reflect that the STZ-treated rats developed a diabetic condition, showing a reduction in both insulin levels (from 1.4- to 2-fold) and insulin/glucose ratio (from 3.3- to 5.3-fold) compared with controls. In addition, on gestational day 12, there was a significant increase in both insulin levels and insulin/glucose ratios, in agreement with previous reports (22, 35).

**Rat embryo number, weight, and composition.** The number of embryos per rat, embryo weight, and total protein and DNA (tDNA) content per embryo (Table 3) were reduced in diabetic groups compared with control values, reflecting a retarded growth in embryos under a diabetic environment. Furthermore,
Measurements were carried out in embryos from C and D mothers. tDNA was measured in homogenate samples, and protein and CS activity were determined in both homogenate and isolated mitochondria. CS activity of control embryos on a 13 vs. 12; DE vs. C.

Table 3. *Maternal plasma glucose and insulin levels*

<table>
<thead>
<tr>
<th>Glucose, mg/dl</th>
<th>Gestational Days</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NP</td>
<td>11</td>
</tr>
<tr>
<td>C</td>
<td>138±5</td>
<td>137±6</td>
</tr>
<tr>
<td>D</td>
<td>522±44</td>
<td>479±57</td>
</tr>
</tbody>
</table>

Table 3. *Rat embryo number, weight, and composition on gestational days 12 and 13*

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Gestational Days</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>No./rat</td>
<td>14.6±0.7</td>
<td>12.2±0.8</td>
</tr>
<tr>
<td>Weight, mg</td>
<td>18.0±0.4</td>
<td>14.5±0.6</td>
</tr>
<tr>
<td>Protein, mg/embryo</td>
<td>19.9±1.4</td>
<td>14.0±1.9</td>
</tr>
<tr>
<td>tDNA, ng/embryo</td>
<td>129±5</td>
<td>84.7±5.4</td>
</tr>
<tr>
<td>CS, %/tDNA</td>
<td>100±5</td>
<td>121±8</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Protein, g protein/g tDNA</td>
<td>0.563±0.046</td>
</tr>
<tr>
<td></td>
<td>CS, %/CS/tDNA</td>
<td>100±11</td>
</tr>
<tr>
<td></td>
<td>Protein/CS, %protein/CS</td>
<td>100±13</td>
</tr>
</tbody>
</table>

Data represent the mean ± SE of 7–10 independent experiments. Parameters were measured in control (C) and diabetic (D) mothers on gestational days 11, 12, and 13 as well as in nonpregnant (NP) rats. G, gestational day effect; DE, diabetes effect. Two-way ANOVA (P < 0.05); post hoc analysis (LSD test, P < 0.05). *NP vs. 12; bNP vs. 13; c11 vs. 12; d11 vs. 13.

Mitochondrial protein content per cell (Table 3) was increased in embryos from diabetic rats compared with control rats in such a way that this parameter was maintained when expressed per embryo (data not shown), and this rise was more self-evident on gestational day 12. The mitochondrial protein content showed a reduction between the gestational days studied, a trend that was more apparent in diabetic groups, at least when expressed per citrate synthase activity.

Citrate synthase activity in both homogenate and isolated mitochondria samples (Table 3) did not show significant differences either between treatments or between gestational days. Since this activity is widely used as a mitochondrial mass indicator, cells of all the studied embryos were maintaining their mitochondrial content. On gestational day 12, the ratio between mitochondrial protein and citrate synthase activity was greater in embryos from diabetic mothers compared with those from controls, and it decreased in both control and diabetic groups, reaching similar values on day 13.

Relative mRNA levels of genes involved in mitochondrial biogenesis and function. The levels of mRNAs encoding proteins whose expression is known to be regulated through PGC-1α (21) were measured in normal embryos from control and diabetic mothers and in malformed embryos only from diabetic rats, due to the extremely low percentage of malformations found in control groups (Fig. 1). The relative mRNA levels of nuclear- (PGC-1α, NRF-2, mtSSB, and TFAM) and mitochondrial-encoded proteins (COX-I) significantly increased in embryos from the diabetic group compared with those from control mothers on gestational day 12; afterward, on day 13, PGC-1α and TFAM remained increased, whereas NRF-2, mtSSB, and COX-I did not differ from control values. In contrast, the nuclear-encoded NRF-1 and COX-IV mRNAs showed higher levels in diabetic groups than control groups throughout these 3 days of embryo development. The increased expression of the genes studied shown by the normal embryos under diabetic conditions compared with control ones was delayed in malformed embryos until gestational day 13 in most regulatory genes (PGC-1, NRF-1, NRF-2, mtSSB, and TFAM) and was not observed in the functional ones (COX-I and COX-IV).

Western blot analysis of proteins involved in mitochondrial biogenesis and function. On gestational day 12, the content of the proteins of the PGC-1α pathway involved in mitochondrial biogenesis (PGC-1α and TFAM) and function (COX-I, COX-IV, and β-ATPase) (Table 4) was significantly lower in embryos from diabetic rats compared with controls and subse-

Table 3. Rat embryo number, weight, and composition on gestational days 12 and 13
Maternal diabetes entails embryo maldevelopment, leading to increased perinatal pathological situations, such as microsomas and congenital malformations (3, 26), that are obvious from earlier stages of pregnancy according to our results and other previous works (10, 49). Along these lines, we observed both an increased percentage of malformed embryos (4-fold increase on gestational day 13) and retarded growth in normal embryos from diabetic mothers.

**DISCUSSION**

The altered embryo-fetal development induced by diabetic pregnancy during the organogenetic period is a complication that endangers the health of the offspring (11, 25). We (4) have previously reported that placentation, taking place during organogenesis, is a critical period in which embryo mitochondria differentiate to activate the oxidative metabolism needed for proper embryo development during the second half of pregnancy. Because mitochondrial dysfunction is related to diabetes complications (24, 33, 41, 42), in the present work we investigated whether diabetic pregnancy alters the mitochondrial differentiation process taking place in rat embryo during the placentation period. We found that, in the embryos from diabetic mothers showing normal morphology, the levels of the proteins of the PGC-1α pathway involved in mitochondrial biogenesis were decreased on gestational day 12 but subsequently increased on day 13, when most reached the control values. Thus, in light of the results, one possible explanation would be that these embryos developed several compensatory responses during the placentation period consisting of increased gene expression and activation of the OXPHOS activities, which could help them to follow an apparently normal mitochondrial biogenic schedule, at least throughout the period studied, despite showing a retarded growth compared with controls. Nevertheless, malformed embryos from diabetic mothers seem to not be able to respond to maternal diabetes to the same extent as their normal counterparts.

Morphology and morphometry of embryo mitochondria. On gestational day 13, embryo mitochondria from both control and diabetic mothers showed a similar morphology with few vesiculated cristae (Fig. 2), reflecting, in agreement with previous studies (20), that these mitochondria are not completely differentiated as yet. Neither the area nor the perimeter data of embryo mitochondria showed significant differences between control and diabetic groups (Table 6). These results indicate that mitochondrial morphology and morphometry of embryos under diabetic environment were not altered, at least on gestational day 13.
Mitochondriogenesis is a complex event requiring a strict regulation to synchronize the gene expression of mitochondrial and nuclear genomes (12, 40). PGC-1α coactivator plays a critical role in coordinating nuclear-mitochondrial transcriptional regulation through the coactivation of different transcription factors in response to mitochondrial signals, thereby regulating the expression of most genes involved in mitochondrial biogenesis and function (40). The measurement of the content of proteins whose expression is regulated through the mitochondrial genome (COX-I) as well as other downstream proteins, i.e., TFAM and OXPHOS subunits encoded by the nucleus, (COX-IV and β-ATPase) and by the mitochondrial genome (COX-I). In contrast, the mRNA levels of proteins of this mitochondrial pathway (PGC-1α, NRF-1, NRF-2, mtSSB, TFAM, COX-IV, and COX-I) increased in embryos from diabetic mothers on gestational day 11 or 12. Hence, the effect of maternal diabetes on the reduced content of PGC-1α-regulated proteins on day 12 would most likely be due to a posttranscriptional regulation such as, for instance, lower mRNA stability and/or translational efficiency. Besides, the increased transcription of genes regulated through PGC-1α mitochondrial biogenic pathway on day 12 could constitute a compensatory response that would lead to balancing, at least in part, their reduced protein levels, as pointed out by the concomitant rise in the content of the regulatory proteins (PGC-1α and TFAM) and functional OXPHOS subunits (COX-I, COX-IV, and β-ATPase) on gestational day 13, which in most cases reached the control levels. Although this suggestion would be in agreement with previous compensatory findings reported in the heart mitochondria of adult diabetic mice (41, 42), other possible explanations cannot be discarded in this regard. However, what our findings certainly suggest is that the reduced content of proteins of the PGC-1α pathway on day 12 was accompanied by the activation, at least at a transcriptional level, of this mitochondrial biogenic pathway in embryos from diabetic mothers showing normal morphology.

Because the activation of the OXPHOS activities on gestational day 12 is coincident with the reduced content of OXPHOS subunits, this fact could also be understood as an additional response to further compensate this alteration. This idea would be reinforced by the fact that on gestational day 13, coinciding with the increase of most OXPHOS protein levels to control values, the activation seen in the OXPHOS activities studied per mitochondrion is not maintained but, rather, returns almost to control values on that day. Overall, the aforementioned results suggest that these likely compensatory responses could to some extent lead mitochondria from the diabetic group to achieve similar features compared with control ones on day 13, as shown by the similar morphology, morphometry, and content of embryo mitochondria from both groups, thereby

### Table 4. Rat embryo relative content of proteins involved in mitochondrial biogenesis and function on gestational days 12 and 13

<table>
<thead>
<tr>
<th>Regulation</th>
<th>Gestational Days</th>
<th>C</th>
<th>D</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGC-1α</td>
<td>12</td>
<td>100±14</td>
<td>30.0±10.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>99.4±12.0</td>
</tr>
<tr>
<td>TFAM</td>
<td>12</td>
<td>100±15</td>
<td>37.4±9.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>204±14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>12</td>
<td>100±10</td>
<td>58.5±5.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>143±10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>COX-I</td>
<td>12</td>
<td>100±30</td>
<td>31.3±16.6</td>
<td>266±38</td>
</tr>
<tr>
<td>COX-IV</td>
<td>12</td>
<td>100±26</td>
<td>45.6±9.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>180±11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data represent the mean ± SE of 7–10 independent experiments. Values on control day 12 were set as 100%. OXPHOS, oxidative phosphorylation system; β-ATPase, ATP synthase β-subunit. PGC-1α, TFAM, COX-I, COX-IV, and β-ATPase protein content were measured in embryos from C and D mothers. Two-way ANOVA (P < 0.05); Student’s t-test (P < 0.05). *13 vs. 12; #DE vs. C.

### Table 5. Rat embryo CIV and ATPase activities on gestational days 12 and 13

<table>
<thead>
<tr>
<th>CIV activity</th>
<th>Gestational Days</th>
<th>C</th>
<th>D</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>%CIV/dNA</td>
<td>12</td>
<td>100±16</td>
<td>157±27</td>
<td>91.4±10.8</td>
</tr>
<tr>
<td>%CIV/CS</td>
<td>12</td>
<td>100±13</td>
<td>182±20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>94.6±5.5</td>
</tr>
<tr>
<td>%CIV/COX</td>
<td>12</td>
<td>100±17</td>
<td>179±14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>92.0±2.7</td>
</tr>
<tr>
<td>%CIV/COX-IV</td>
<td>12</td>
<td>100±23</td>
<td>379±178</td>
<td>53.0±12.2</td>
</tr>
<tr>
<td>ATPase activity</td>
<td>12</td>
<td>IU/g tDNA</td>
<td>41.8±4.8</td>
<td>65.0±12.7</td>
</tr>
<tr>
<td>%ATPase/CS</td>
<td>13</td>
<td>100±11</td>
<td>199±11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.0±4.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>%ATPase/β-ATPase</td>
<td>13</td>
<td>100±17</td>
<td>248±20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58.1±6.6</td>
</tr>
</tbody>
</table>

Data represent the mean ± SE of 7–10 independent experiments. CIV, complex IV. Activities were determined in embryo mitochondria from C and D mothers. All values, except for ATPase/dNA data, were set as 100% on control day 12, expressing the other groups as a percentage. Two-way ANOVA (P < 0.05); Student’s t-test (P < 0.05). *13 vs. 12; #DE vs. C.
facilitating their normal developmental schedule, at least during the placentation period. Supporting this idea, the embryos showing malformations did not present the same responses as normal ones, at least at transcriptional level, reflecting that their lower individual adaptation capability could be one of the causes for their altered development. Nevertheless, although all of the results are consistent with the proposed interpretation, further studies would be necessary to ensure this.

The enhanced oxidative stress in diabetes has been connected with the associated complications of this pathology, including diabetes-induced embryopathy (1, 11, 24), which could be one of the causes of the alterations in the mitochondrial process here observed to occur in rat embryos from diabetic mothers, coinciding with a developmental period that is highly susceptible to diabetes-related teratogenesis. In agreement with this suggestion, we observed an increased expression of the mRNA levels of the major antioxidant enzymes (manganese superoxide dismutase, catalase, and glutathione peroxidase) in the embryos from the diabetic group compared with control ones (data not shown).

On gestational day 12, a critical event for the rat embryo development takes place, i.e., the establishment of the chorioallantoic placenta circulation, which thoroughly changes the hormonal environment surrounding the embryo and makes maternal nutrients and oxygen more available to the embryo (1, 8, 18). Thus, the newly formed placental circulation provides, in a more efficient way, the essential nutrients needed for the embryo oxidative metabolism, which, in fact, has been reported (2, 44) to become drastically activated on this gestational day. This fact is accompanied by a mitochondrial oxidative metabolism activation is essential for the energy supply for the highly sensitive organogenic processes taking place during the studied period, such as the closure of the neural tube and the formation of many embryonic organs (10, 31), we hypothesize that such normalization of the mitochondrial biogenic schedule could certainly be a process that would facilitate to a certain extent normal development in those embryos, at least throughout the gestational days studied.

In conclusion, although the mitochondrial content remains unchanged in normal embryos from diabetic rats, the mitochondrial process is altered by the diabetic environment on gestational day 12, at least in part, through a reduction of the content of proteins regulated by the PGC-1α mitochondrial pathway. This fact is followed by several responses consisting of activating the gene transcription of the aforementioned pathway and the OXPHOS enzymatic activities that could compensate the aforementioned alterations. Likewise, on day 13, after the establishment of the chorioallantoic circulation, the content of PGC-1α-regulated OXPHOS subunits rose until almost reaching control levels, and hence, the OXPHOS activities would be normalized to control values. These likely adaptations could facilitate “normal” embryo development despite exposure to the teratogenic environment of maternal diabetes. This, all in all, might be the result of the opposite effect between the toxicity of the oxidative stress caused by hyperglycemia and the possible beneficial effect of the placenta. Finally, the different gene expression profile observed between normal and malformed embryos from diabetic mothers could reflect that the latter have a lower adaptation capability, which could exacerbate their maldevelopment. Further study is needed to establish whether alterations in the mitochondrial biogenic process are involved in diabetic pregnancy teratogenesis and its relation to oxidative stress.

Table 6. Morphometry of embryo mitochondria

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>D</th>
<th>Kruskal-Wallis Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area, ( \mu m^2 )</td>
<td>0.312±0.019</td>
<td>0.315±0.023</td>
<td>NS</td>
</tr>
<tr>
<td>Perimeter, ( \mu m )</td>
<td>1.98±0.06</td>
<td>2.07±0.07</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data represent the mean ± SE. Area and perimeter analyses were performed in embryo mitochondria from C and D mothers on gestational day 13. Kruskal-Wallis test \((P < 0.05)\).

ACKNOWLEDGMENTS

We are grateful to Hidetoshi Inagaki, from the National Industrial Research Institute of Nagoya (Japan), for kindly providing the rabbit antisera against mitochondrial transcription factor A protein. We thank Emilia Amengual for technical support.
GRANTS

M. P. Alcolea was funded by a grant from the Ministerio de Educación Cultura y Deporte of the Spanish Government. This investigation was sup- ported by Centro de Investigación Biomédica en Red (Network Biomedical Research Center) Fisiopatología Obesidad y Nutrición (CB06/03), Instituto de Salud Carlos III del Ministerio de Sanidad y Consumo, and Fondo de Investigaciones Sanitarias (PI042294 and PI042377), all from the Spanish Government.

REFERENCES


