RESEARCH

Dexamethasone during pregnancy impairs maternal pancreatic β-cell renewal during lactation

Caio Jordão Teixeira[1], **Junia Carolina Santos-Silva**[1], **Dailson Nogueira de Souza**[1], **Alex Rafacho**[2], **Gabriel Forato Anhe**[1] and **Silvana Bordin**[3]

[1]Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas, Campinas, Brazil
[2]Department of Physiological Sciences, Center of Biological Sciences, Federal University of Santa Catarina, Florianópolis, Brazil
[3]Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil

Correspondence should be addressed to S Bordin: sbordin@icb.usp.br

Abstract

Pancreatic islets from pregnant rats develop a transitory increase in the pancreatic β-cell proliferation rate and mass. Increased apoptosis during early lactation contributes to the rapid reversal of those morphological changes. Exposure to synthetic glucocorticoids during pregnancy has been previously reported to impair insulin secretion, but its impacts on pancreatic islet morphological changes during pregnancy and lactation have not been described. To address this issue, we assessed the morphological and molecular characteristics of pancreatic islets from rats that underwent undisturbed pregnancy (CTL) or were treated with dexamethasone between the 14th and 19th days of pregnancy (DEX). Pancreatic islets were analyzed on the 20th day of pregnancy (P20) and on the 3rd, 8th, 14th and 21st days of lactation (L3, L8, L14 and L21, respectively). Pancreatic islets from CTL rats exhibited transitory increases in cellular proliferation and pancreatic β-cell mass at P20, which were reversed at L3, when a transitory increase in apoptosis was observed. This was followed by the appearance of morphological features of pancreatic islet neogenesis at L8. Islets from DEX rats did not demonstrate an increase in apoptosis at L3, which coincided with an increase in the expression of M2 macrophage markers relative to M1 macrophage and T lymphocyte markers. Islets from DEX rats also did not exhibit the morphological characteristics of pancreatic islet neogenesis at L8. Our data demonstrate that maternal pancreatic islets undergo a renewal process during lactation that is impaired by exposure to DEX during pregnancy.

Key Words

- lactation
- glucocorticoids
- pancreatic β-cell
- pregnancy

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Introduction

Although randomized controlled trials have shown that antenatal corticosteroid therapy yields consistent benefits to preterm newborns (1, 2), recent observational studies have noted that this strategy correlates with maternal hyperglycemia in nondiabetic women (3, 4). Concordantly, experiments with rats showed that exposure to dexamethasone (DEX) during the last third of pregnancy impaired maternal glucose-induced insulin secretion *in vivo*, leading to glucose intolerance prior to delivery (5).

Increased glucose oxidation and glucose-stimulated insulin secretion (GSIS) by pancreatic islets are the basis of the functional adaptation of the endocrine pancreas that takes place during pregnancy (6, 7). This functional adaptation depends on the action of hormones such as placental lactogen, growth hormone and prolactin

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(8, 9, 10). Interestingly, DEX was also reported to abrogate the upregulation of GSIS induced by prolactin in pancreatic islets *in vitro* (11, 12).

Undisturbed pregnancies in humans and rodents are also distinguished by morphological changes in the pancreatic islets. The most evident morphological adaptations described in the human endocrine pancreas are an increase in the pancreatic β-cell fractional area and an increase in the number of small islets (13). Pancreatic islets of pregnant rodents, however, undergo an increase in size with a parallel increase in pancreatic β-cell proliferation and mass (6, 14, 15). The endocrine pancreas of pregnant rats show evident plasticity, which allows the morphological structures to return to the nonpregnant state just after delivery. Increased apoptosis and reduced proliferation account for the reversal of pancreatic β-cell mass as early as 3–4 days after delivery (15, 16).

Therefore, the present study was conducted to evaluate yet unknown putative effects of antenatal DEX therapy on the morphological adaptation of the maternal endocrine pancreas to pregnancy. We also assessed whether the treatment of pregnant rats with DEX impacted the physiological reset of the maternal endocrine pancreas that occurs after delivery.

Materials and methods

Experimental design

The experimental procedures were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and approved by the State University of Campinas Committee for Ethics in Animal Experimentation (protocol No. 3973-1). Female Wistar rats were obtained at 4 weeks of age from the Animal Breeding Center at the University of Campinas (CEMIB, Campinas, Sao Paulo, Brazil) and kept under a 12-h light–dark cycle at 22±2°C and allowed *ad libitum* access to standard rat chow and water. At 12 weeks of age, females were housed in individual cages with one male for 3 days. The concomitant presence of spermatozoa and estrous cells in a vaginal lavage indicated day 0 of gestation. Pregnant rats were isolated until the last day of lactation. Age-matched virgin females were maintained in the same animal care facility under the same housing conditions. On the 14th day of pregnancy, rats were assigned to two groups that received either a vehicle (CTL) or dexamethasone (DEX) (0.1 mg/kg body mass; Achê Pharmaceutical Laboratories, Guarulhos, SP, Brazil) in the drinking water for 6 days. On the day of delivery, the number of pups was adjusted to six per lactating mother, and the remaining neonates were killed by decapitation. The mothers were used for experimental procedures on the 20th day of pregnancy (P20) or on the 3rd (L3), 8th (L8), 14th (L14) and 21st (L21) days of lactation. On the day of the experiments, rats were killed with an i.p. injection of a lethal dose of sodium thiopental (80 mg/kg body mass) followed by decapitation.

Immunohistochemistry

The intact pancreas was carefully excised, cleared of fat and lymph nodes, weighed, immersed in 4% (wt/vol) paraformaldehyde fixative solution for 24 h and embedded in paraffin for a posterior immunoperoxidase reaction. Serial sections (5 µm thick and 200 µm apart from each other) were mounted onto aminopropyltriethoxysilane-coated glass slides. After paraffin removal, sections were rehydrated and washed with 0.05 M Tris buffered saline (TBS) (pH 7.4) and incubated with 0.01 M Tris-EDTA buffer containing 0.05% Tween-20 (pH 9.0) for 24 min at 98°C for antigen retrieval. Endogenous peroxidase activity was blocked with a 0.3% solution of hydrogen peroxide before permeabilization with TBS containing 0.1% Tween-20 and 5% bovine serum albumin (BSA) at room temperature. Sections were incubated with either polyclonal guinea pig anti-insulin (1:400; Dako North America, Inc.; cat. no. A0564) or rabbit monoclonal anti-Ki-67 (1:75; Spring Bioscience, Pleasanton, CA, USA; cat. no. M3064) antibodies diluted in TBS containing 3% BSA overnight at 4°C. Subsequently, sections were washed with TBS and incubated either with HRP-conjugated anti-guinea-pig IgG (1:1000; Invitrogen; cat. no. 614620) or HRP-conjugated anti-rabbit IgG (Nichirei Bioscience, Tokyo, Japan; cat. no. 414191F) for 2 h at room temperature. Insulin- and Ki-67-positive cells were detected with 3,3′-diaminobenzidine (Sigma Chemical) solution. All slides were counterstained with Ehrlich's hematoxylin and mounted for observation by microscopy.

Pancreas morphology

Two sections of each pancreas were randomly selected for analysis of endocrine pancreas morphology. All islets of the sections were captured in images under a final magnification of 20× using a light microscope (Olympus BX51TF) coupled to a digital camera (Olympus DP72). The pancreatic islet area was obtained by manual tracing of all islets on the sections. Pancreatic β-cell and

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non-β-cell areas were obtained by manual tracing of insulin-positive and insulin-negative cells from all islets on the sections. Images analysis was performed with ImageJ software (http://imagej.nih.gov/ij). The relative mass of islets, β-cells and non-β-cells was calculated by dividing their respective total areas by the total pancreatic section area; the islet, β-cell and non-β-cell mass (mg) was then estimated by multiplying their relative mass by the total pancreas mass (17).

We categorized the insulin-stained cell groups as EICs (<300 μm^2) or small (300–1999 μm^2), medium (2000–9999 μm^2), large (10,000–49,999 μm^2) or very large (≥50,000 μm^2) islets based on a previous investigation (18). An example of a section stained with anti-insulin antibody and the respective classification of its islets according to their sizes are shown as supporting information (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

We also assessed the percentage of ducts associated with islets, which is another parameter that indicates the formation of new pancreatic islets (19). Examples of islets that were considered to be associated with ducts are shown in representative images (Supplementary Fig. 2).

Pancreatic islet proliferation

Pancreatic islet cell proliferation was estimated as previously described (17). Briefly, the number of nuclei positive for Ki-67 was expressed as the percentage of the total number of nuclei per islet.

Pancreatic islet isolation and DNA fragmentation

DNA fragmentation was assessed as a parameter of apoptosis. In a separate set of rats, islets were isolated after perfusion and digestion of the pancreas with collagenase solution immediately after killing as previously described (20). DNA fragmentation was assessed as previously described (16). Briefly, 100 freshly isolated islets from each rat were dissociated in Ca^{2+}-free Krebs buffer (138 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM Hepes, 1.2 mM EGTA, supplemented with 3 mM glucose and 0.1% BSA, pH 7.4) at 37°C for 10 min. Cells were centrifuged at 1000 ***g*** and incubated with 200 μL of hypotonic solution containing 0.8% propidium iodide, 0.1% sodium citrate and 0.1% Triton X-100 at room temperature for 2 h. Fluorescence was measured with a FACSCalibur flow cytometer (Becton Dickinson) using the FL2 channel (orange/red fluorescence; 535/617 nm).

Protein extraction and immunoblotting

Pools of approximately 350 freshly isolated islets were processed for western blotting as previously described (16). The primary antibodies used were anti-TRB3 and anti-phospho-AKT (Ser473) (Santa Cruz Biotechnology). We used a secondary antibody conjugated with horseradish peroxidase (Bio-Rad) for chemiluminescent detection of the bands on X-ray-sensitive films. Optical densitometry analysis was performed using Scion Image software (Scion Corporation, Frederick, MD, USA). The results were normalized to the total amount of protein transferred to the membranes as indicated by Ponceau S staining.

RNA extraction and qPCR

Total RNA was extracted from a pool of approximately 500 freshly isolated islets using an RNeasy Plus Mini kit (Qiagen), and the concentration was estimated using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Aliquots containing 2 μg of RNA were subjected to reverse transcription using a high-capacity cDNA reverse transcription kit (Applied Biosystems). PCR reactions were conducted using KAPA SYBRβ FAST qPCR Master Mix (Kapa Biosystems, Inc., Boston, MA, USA) in a StepOnePlus Real-Time PCR System (Applied Biosystems). The specificity of the reactions was verified with melting curve analysis. The primer sequences used are as follows: *Cd163* sense 5′-ATGGAGTCACAGCGACTGCG-3′, and antisense 5′-GAGGAAGGCAATGAGAAGGACC′3; *Lta* sense 5′-TACAAGGACCGTGGGTACGCTC-3′, and antisense 5′-GTGTAAGTGGGAGATGCCGTCTG-3′; *Cd206* sense 5′-CTGGAAGACATCATACTGCAATG-3′, and antisense 5′-CAGTCTCGATGGAAACCAGG-3′; *Tnf* sense 5′-CTCTCTGCCATCAAGAGCC-3′, and antisense 5′-CACAGAGCAATGACTCCAAAG-3′; *Rpl37a* sense 5′-CAAGAAGGTCGGGATCGTCG-3′, and antisense 5′-ACCAGGCAAGTCTCAGGAGGTG-3′. Values of mRNA expression were normalized with the internal control gene *rpl37a*. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The results are presented as the mean ± standard error of the mean (s.e.m.). Comparisons were made with two-way ANOVA considering (i) time after mating and (ii) treatment during pregnancy. Tukey's multiple comparison test was used to indicate intragroup differences at different time

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points, and a Sidak multiple comparison test was used to indicate the differences between CTL and DEX at the same time points (GraphPad Prism, version 6.01). *P* values <0.05 indicated significant differences.

Results

The expansion of pancreatic β-cell mass observed in pregnant rats was sustained until L3 in DEX rats

Pancreatic islet mass in CTL rats had a nonsignificant increase (72%) at P20 and remained unaltered throughout lactation. DEX rats exhibited an increase in pancreatic islet mass detected at P20 and L3 (105% and 121% greater than virgin rats, respectively; $P<0.05$). This increase in pancreatic islet mass was not accompanied by an increase in pancreas mass (Supplementary Fig. 3). Consequently, maternal treatment with DEX impacted pancreatic islet mass and caused a 95% increase at L3 compared with the CTL rats at L3 ($P<0.05$) (Fig. 1A).

In accordance with a previous study (15), we observed that the CTL rats experienced an increase in pancreatic β-cell mass at P20 (77% greater than virgin rats; $P<0.05$), which was no longer detected a few days after delivery at L3. Although restricted to pregnancy in CTL mothers, an increase in pancreatic β-cell mass in DEX-treated rats was observed at P20 and L3 (114% and 140% greater than virgin rats, respectively; $P<0.05$). Furthermore, pancreatic β-cell mass was 124% greater in DEX compared to CTL at L3 ($P<0.05$) (Fig. 1B).

No changes in non-β-cell mass were detected at the late stage of pregnancy and throughout lactation in CTL mothers. However, lactating mothers treated with DEX exhibited a transitory expansion of non-β-cell mass at L8 (86% greater than virgin rats; $P<0.05$) (Fig. 1C). Figure 2 shows representative images of pancreas sections immunostained for insulin.

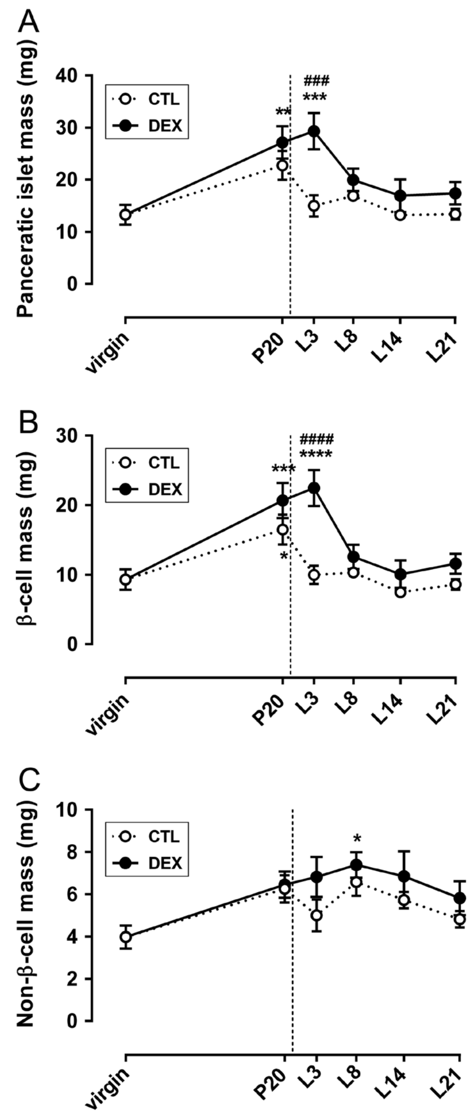

Figure 1
Rats that underwent undisturbed pregnancies (CTL) or received dexamethasone between the 14th and 19th gestational days (DEX) were killed on the 20th day of pregnancy (P20) or on the 3rd (L3), 8th (L8), 14th (L14) and 21st (L21) days of lactation. Virgin rats were also killed. Pancreata were removed for immunohistochemical detection of insulin. Sections were used for the calculation of pancreatic islet (A), pancreatic β-cell (B) and non-β-cell (C) mass. Data are presented as the mean ± S.E.M. (N = 4). *$P<0.05$ vs virgin; **$P<0.01$ vs virgin; ***$P<0.001$ vs virgin; ****$P<0.0001$ vs virgin; $^{###}P<0.001$ vs CTL at the same time point; $^{####}P<0.0001$ vs CTL at the same time point.

Islets of DEX mothers displayed increased rates of cellular proliferation at P20 and attenuated apoptosis at L3

We assessed cellular proliferation and apoptosis in pancreatic islets in order to investigate growth aspects underlying the differential changes in pancreatic β-cell mass in pregnant and lactating CTL and DEX rats. We detected an increase in Ki-67-positive cells at P20 in both CTL and DEX mothers (253% and 387% greater than virgin rats, respectively; $P<0.05$). Furthermore, the number of Ki-67-positive cells at P20 in DEX mothers was 39% greater than that for pregnant CTL rats ($P<0.05$) (Fig. 3A). The increase in the number of Ki-67-positive cells was limited to pregnancy in CTL and DEX mothers since throughout lactation, these values are similar to those for virgin rats. Figure 4 shows representative images of pancreas sections immunostained for Ki-67.

In accordance with previous studies (15, 16), pancreatic islets of early lactating CTL rats had an

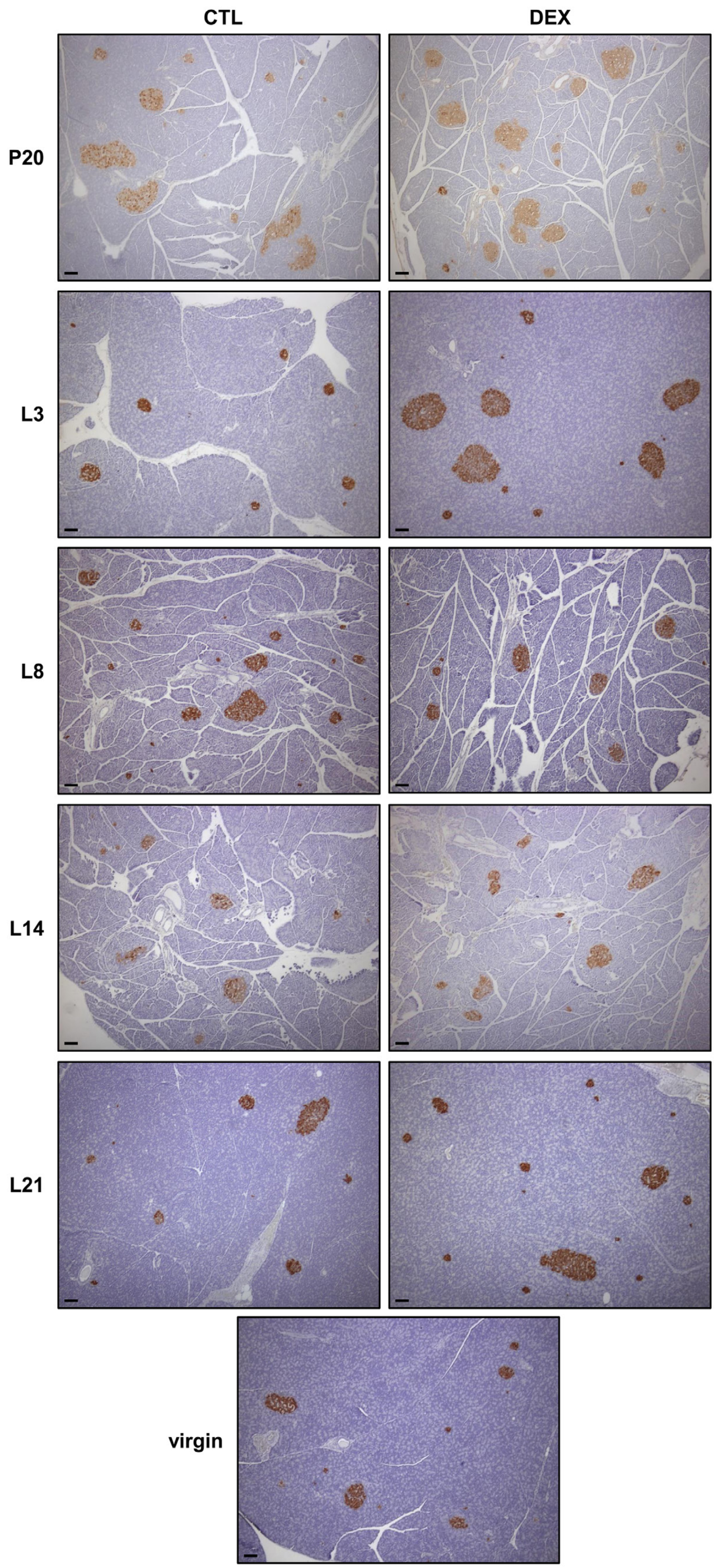

Figure 2
Representative images of pancreas sections used for immunohistochemical detection of insulin. Sections were obtained from virgin rats and rats that underwent undisturbed pregnancies (CTL) or received dexamethasone between the 14th and 19th gestational days (DEX). The rats were killed on the 20th day of pregnancy (P20) or on the 3rd (L3), 8th (L8), 14th (L14) and 21st (L21) days of lactation. Horizontal bars = 100 μm. The images were acquired under a final magnification of 5×.

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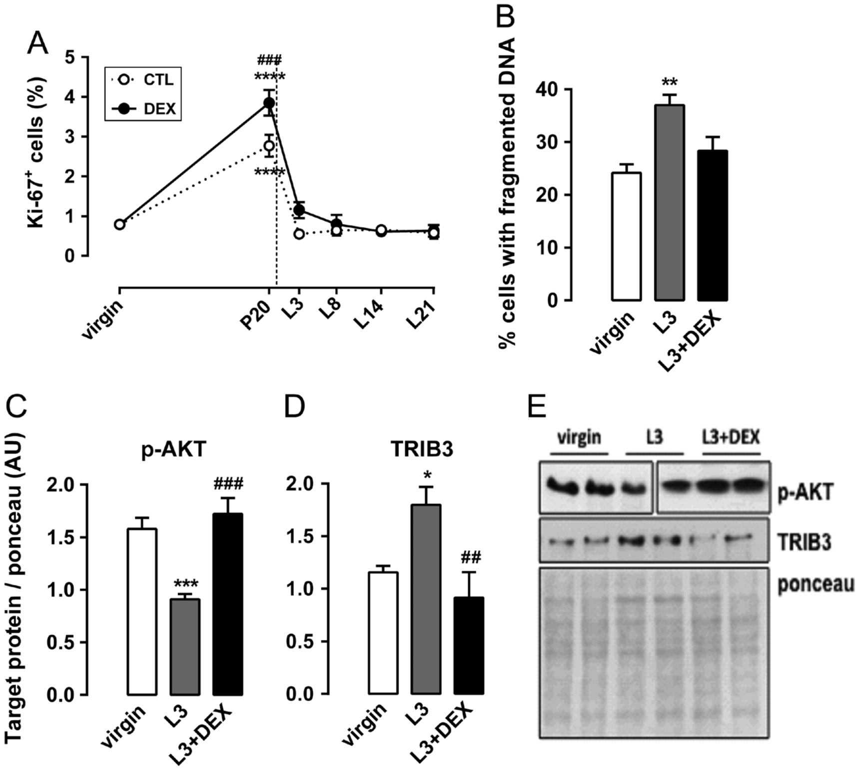

Figure 3
Rats that underwent undisturbed pregnancies (CTL) or received dexamethasone between the 14th and 19th gestational days (DEX) were killed on the 20th day of pregnancy (P20) or on the 3rd (L3), 8th (L8), 14th (L14) and 21st (L21) days of lactation. Virgin rats were also killed. Pancreata were removed for immunohistochemical detection of Ki-67. Sections were used to calculate the percentage of Ki-67$^+$ cells in pancreatic islets (A). Pancreatic islets from a different set of virgin, CTL at L3 and DEX at L3 were isolated and used for the measurement of DNA fragmentation (B) and western blot detection of phosphorylated AKT (p-AKT) (C) and TRIB3 (D). Detected proteins were normalized by Ponceau staining (E). Data are presented as the mean ± s.e.m. (N = 4 for immunohistochemical detection of Ki-67; N = 6–14 for DNA fragmentation; N = 6–12 for p-AKT; N = 5–7 for TRIB3). *P < 0.05 vs virgin; **P < 0.01 vs virgin; *** P < 0.001 vs virgin; ****P < 0.0001 vs virgin; $^{\#\#}P$ < 0.01 vs CTL at L3; $^{\#\#\#}P$ < 0.001 vs CTL at the same time point.

increased rate of DNA fragmentation at L3 (34% greater than that for virgin rats; P<0.05). However, this wave of DNA fragmentation was not observed in islets isolated from DEX rats at L3 (Fig. 3B).

According to our previous study, an increase in DNA fragmentation in rat pancreatic islets at L3 was correlated with a reduction in AKT phosphorylation and an increase in TRIB3 content (16). In the present study, we found consistent changes in AKT phosphorylation and TRIB3 protein content in pancreatic islets of CTL rats at L3 (43% lower and 54% greater than virgin rats, respectively; P<0.05). These changes were not observed in islets of rats treated with DEX at L3 (Fig. 3C and D).

Pancreatic islets of DEX mothers at L3 had increased expression of M2 macrophages relative to M1 macrophages and T lymphocytes

To understand the mechanism by which islets of DEX mothers exhibited reduced apoptosis at L3, we evaluated the expression of leukocyte markers that infiltrate pancreatic islets and affect pancreatic β-cell death.

We found that pancreatic islets of DEX mothers at L3 demonstrated increased *Cd206* expression relative to *Lta* and tumor necrosis factor-α (*Tnf*) (157% and 197% greater than virgin rats, respectively; P<0.05). These modulations were not observed in islets isolated from CTL mothers at L3 (Fig. 5A and B, respectively). No significant changes in *Cd163* expression relative to *Lta* and *Tnf* was observed in islets of CTL and DEX mothers isolated at L3 (Fig. 5C and D, respectively).

Parameters related to pancreatic islet neogenesis during lactation were absent in rats that received DEX during pregnancy

The percentage of EICs was increased in CTL rats at P20 (89% greater than virgin rats; P<0.05). Although the percentage of EICs returned to levels found in virgin rats at L3, a second increase in the percentage of EICs was observed at L8 (77% greater than that in virgin rats; P<0.05). DEX mothers also experienced a transitory increase in the percentage of EICs at P20 (91% greater than that in virgin rats; P<0.05) that was no longer observed at L3. However, a second increase in the percentage of EICs was not observed at L8. The percentage of EICs in DEX mothers exhibited a continuous decrease during lactation, reaching the lowest value at L14 (61% lower than the values of DEX at P20; P<0.05). In addition, the percentage of EICs in DEX mothers at L14 was 49% lower than that of CTL mothers at L14 (P<0.05) (Fig. 6A).

The percentages of small, medium, large and very large islets did not change at the late stage of pregnancy

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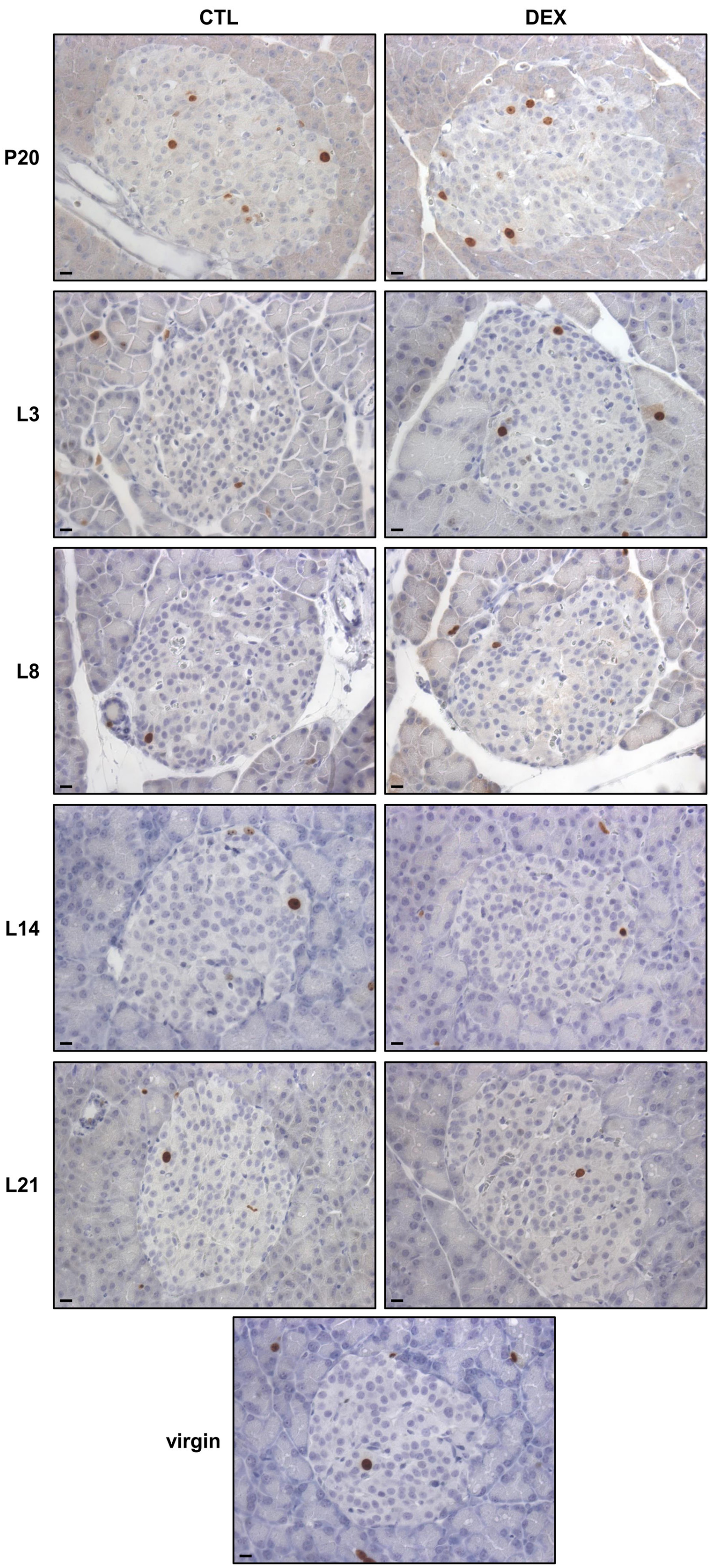

Figure 4
Representative images of pancreas sections used for immunohistochemical detection of Ki-67. Sections were obtained from virgin rats and rats that underwent undisturbed pregnancies (CTL) or received dexamethasone between the 14th and 19th gestational days (DEX). The rats were killed on the 20th day of pregnancy (P20) or on the 3rd (L3), 8th (L8), 14th (L14) and 21st (L21) days of lactation. Horizontal bars = 20 μm. The images were acquired under a final magnification of 40×.

and during lactation either in CTL or in DEX mothers (Fig. 6B, C, D and E, respectively).

Although the percentage of ducts associated with islets increased in CTL and DEX mothers at P20 (132% and 227% greater than virgin rats, respectively; $P<0.05$), the increase was significantly greater in pregnant DEX rats (40% greater than pregnant CTL; $P<0.05$). At L3, the percentage of ducts associated with islets in both CTL and DEX mothers decreased to values similar to those for virgins. In DEX mothers, these values remained similar to those for virgin rats throughout the entire lactation period. However, in CTL mothers, we observed a second transitory increase in the percentage of ducts associated with islets at L8 (190% greater than that for CTL mothers at L21; $P<0.05$) (Fig. 6F).

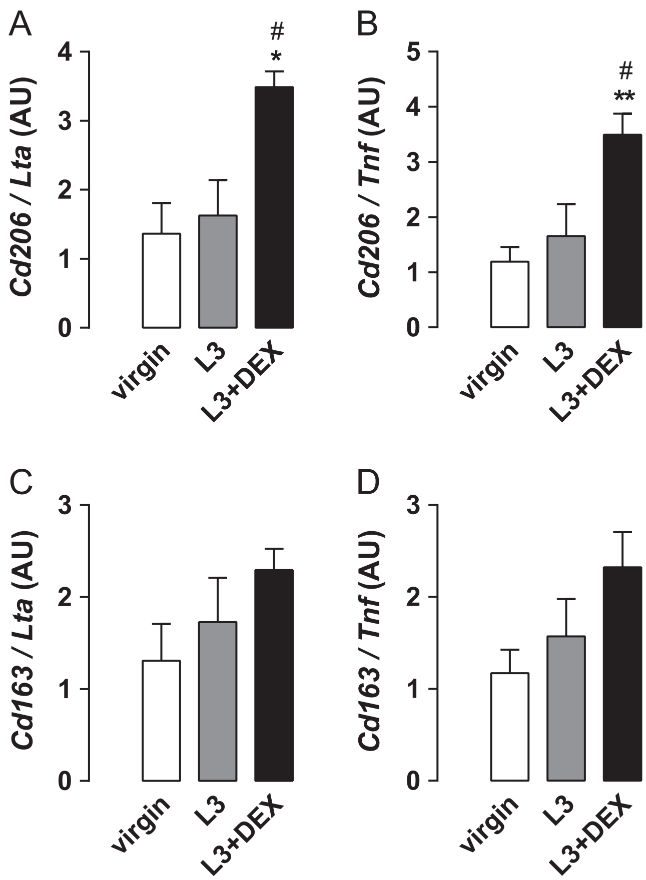

Figure 5
Rats that underwent undisturbed pregnancies (CTL) or received dexamethasone between the 14th and 19th gestational days (DEX) were killed on the 3rd day of lactation (L3). Virgin rats were also killed. Pancreatic islets were isolated and processed for qPCR detection of *cd206* relative to *lta* (A) or *tnf* (B) and *cd163* relative to *lta* (C) or *tnf* (D). Data are presented as the mean ± s.e.m. (N = 7–5). *P < 0.05 vs virgin; **P < 0.01 vs virgin; #P < 0.05 vs CTL at L3.

Discussion

The present data support the concept that rat pancreatic β-cell mass, which increases during undisturbed pregnancies, recovers to nonpregnant mass values 3 days after delivery with a coinciding increase in apoptosis and reduction of cellular proliferation. Notably, we revealed that morphological features of pancreatic islet neogenesis sequentially and transiently appear in the maternal pancreas 8 days after delivery. Additionally, we showed that the morphological adaptation of the endocrine pancreas to pregnancy as well as its recovery to the nonpregnant state during lactation was affected by the exposure of pregnant rats to DEX.

Neogenesis of pancreatic β-cells in rats during the neonatal period has been reported to occur immediately following birth and during the weaning period (21). More recently, the formation of new pancreatic islets derived from small extra-islet insulin-positive cell clusters (EICs) has been described to occur in adult rats (22, 23).

Our data demonstrated that an increase in pancreatic β-cell mass at P20 correlates not only with an increase in cellular proliferation (evidenced by the number of Ki-67 positive cells) but also with pancreatic islet neogenesis (evidenced by the percentage of EICs and ducts associated with islets). An increase in cellular proliferation in pancreatic islets on the 20th day of pregnancy has been previously described (15), but no studies have previously investigated morphological parameters related to pancreatic islet neogenesis in the pancreata of pregnant rats.

The contribution of pancreatic islet neogenesis to an increase in pancreatic islet mass during pregnancy seems to depend on the species. In accordance with our study in rats, it has been previously reported that an increase in endocrine pancreatic mass during human pregnancy relies on the neogenesis of small pancreatic islets (13). In mice, however, the contribution of neogenesis to an increase in pancreatic islet mass during pregnancy remains controversial (24, 25). In addition to pregnancy, experimental conditions that cause damage to the pancreas of an adult rat, such as a pancreatectomy, have also been reported to stimulate the neogenesis of insulin-positive cells from the ductal epithelium (26).

Morphological analysis of the pancreas throughout lactation allowed us to clarify how and when the changes that occur during pregnancy are reversed. In the present study, we observed an increase in cellular apoptosis (as assessed by DNA fragmentation) in pancreatic islets isolated from early lactating rats (L3). This result is

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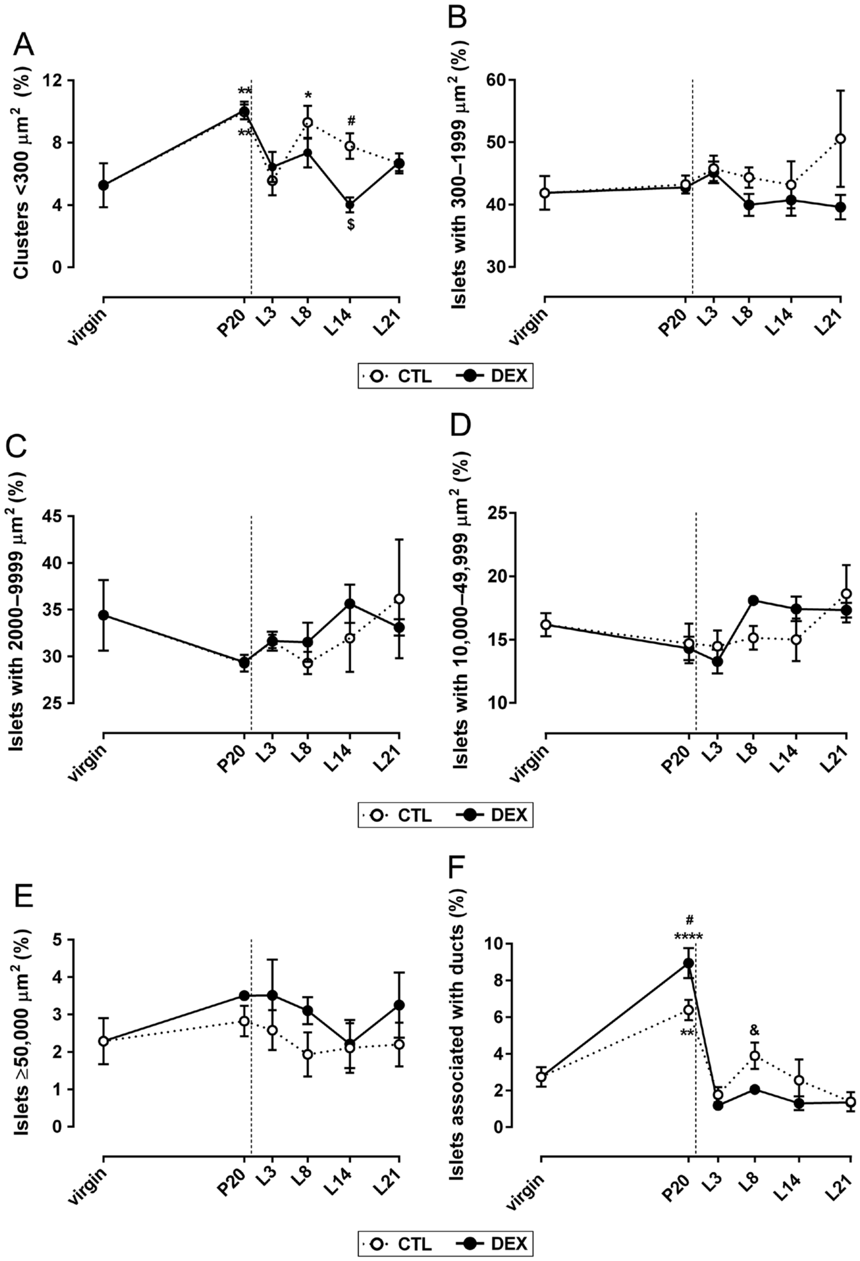

Figure 6
Rats that underwent undisturbed pregnancies (CTL) or received dexamethasone between the 14th and 19th gestational days (DEX) were killed on the 20th day of pregnancy (P20) or on the 3rd (L3), 8th (L8), 14th (L14) and 21st (L21) days of lactation. Virgin rats were also killed. Pancreata were removed for immunohistochemical detection of insulin. Sections were used for quantification of the percentage of extra-islet insulin-positive cell clusters (EICs) (A) or small (300–1999 μm²) (B), medium (2000–9999 μm²) (C), large (10,000–49,999 μm²) (D) or very large (>50,000 μm²) (E) pancreatic islets and islets associated with ducts (F). Data are presented as the mean ± s.e.m. (N = 4). *P < 0.05 vs virgin; **P < 0.01 vs virgin; ****P < 0.0001 vs virgin; #P < 0.05 vs DEX at the same time point; $P < 0.05 vs DEX at P20; &P < 0.05 vs CTL at L21.

consistent with data previously published by us and others and has already been reported to be transitory since it was no longer observed in islets isolated from L8 mothers (15, 16). In addition to an increase in apoptosis, we observed synchronized reductions in the number of Ki-67-positive cells and the mass of pancreatic β-cells at L3 in the present study.

Interestingly, these alterations were followed by a transitory increase in morphological parameters that indicated pancreatic neogenesis. This result is supported by the observation of a second occurrence of increased percentages of EICs and ducts associated with islets in the maternal pancreas at L8. Therefore, our data show that pancreatic islet neogenesis in adult rats occurs transiently not only during pregnancy but also during lactation. These findings also support the unprecedented concept that lactation serves as a postpregnancy window for maternal pancreatic islet renewal during which an increase in apoptosis is followed by transitory neogenesis.

It noteworthy that dynamic changes in the endocrine pancreas may therefore play a role in the metabolic resetting of maternal metabolism that occurs during lactation. This metabolic reset hypothesis supports the theory that complete metabolic resetting to a nonpregnant state occurs during lactation and has long-term benefits to maternal health (27). For example, observational studies

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have shown that women who breastfeed for longer periods of time have a reduced risk for type 2 diabetes (28, 29). Similarly, in a previous study, rats that were not permitted to lactate after delivery become glucose intolerant later in life (30). Taking into account the present data, we hypothesize that absent or insufficient lactation may allow incomplete renewal of the maternal endocrine pancreas, thus increasing the long-term risk for glucose intolerance.

The data presented in this study also demonstrated that exposing rats to DEX during pregnancy affected the morphology of the endocrine pancreas at three critical stages: late pregnancy (P20), early lactation (L3) and peak lactation (L8).

At P20, we observed that pancreatic β-cell mass, the number of Ki-67-positive cells, and the percentage of EICs and ducts associated with islets were further increased by treatment with DEX. Pancreatic islets of DEX rats at P20 also demonstrated an increase in mass. These findings suggest that treatment of pregnant rats with DEX exacerbates the pregnancy-associated increase in the pancreatic β-cell mass of rats by stimulating cellular proliferation and β-cell neogenesis. Interestingly, the treatment of rats with DEX has been previously reported to stimulate an increase in pancreatic β-cell mass and proliferation in male rats but not in nonpregnant female rats (17, 31). We thus conclude that DEX interacts with components of the internal *milieu* associated with pregnancy to exert morphological effects that are described in the present study. In agreement with our data, DEX has also been reported to enhance pancreatic islet neogenesis by promoting an additional increase in the number of small β-cell clusters located close to the ductal complex in 90% pancreatectomized rats (32).

Pancreatic islets of mothers treated with DEX also did not exhibit an increase in apoptosis during the early lactation stage. Instead, the islets of rats treated with DEX demonstrated increases in pancreatic islet and β-cell mass at L3. In addition, we demonstrated that islets from DEX rats had increased levels of M2-polarized macrophages relative to either T lymphocyte or M1-polarized macrophage markers at L3. Lymphotoxin-α (LTα), produced by T lymphocytes and tumor necrosis factor-α (TNF-α), produced by M1-polarized macrophages and NK cells, are known to potentiate IFN-γ-induced pancreatic β-cell apoptosis during type 1 diabetes mellitus insulitis (33, 34). In contrast, M2-polarized macrophages, characterized by CD206 and CD163 expression (35), can infiltrate pancreatic islets and delay the development of autoimmune diabetes in NOD mice by promoting pancreatic β-cell survival (36). In accordance to our data, M2 polarization, which is characterized by low levels of inflammatory cytokines such as IL-1, TNF-α and IL-6, has been previously demonstrated to be induced by glucocorticoids (37). In the context of pancreatic islets, M2 infiltration promotes cell proliferation, inhibits cell apoptosis and delays the development of insulinopenic diabetes in NOD mice (36, 38, 39).

Therefore, we conclude that changes in cellular apoptosis and pancreatic β-cell mass observed in islets from early lactating DEX rats is associated with an altered profile of infiltrating immune cells. It was also previously reported that pharmacological glucocorticoid receptor activation in rats treated with streptozotocin changes the infiltration of macrophages into pancreatic islets from M1- toward M2-polarized macrophages and protects pancreatic cells (40).

Similarly, in the present study, we demonstrated that an increase in M2 relative to M1 markers in pancreatic islets of DEX rats at L3 is associated with a reduction in the expression of TRIB3 and restoration of AKT phosphorylation compared to CTL at L3. TRIB3 has been previously shown to act as a mediator of pancreatic β-cell apoptosis induced by pro-inflammatory cytokines such as IL-1β, IFN-γ and TNF-α (41) through a mechanism that relies on the inhibition of AKT phosphorylation (42, 43).

Interestingly, although morphological changes associated with pancreatic islet neogenesis were exacerbated during the late stage of pregnancy, the treatment of pregnant rats with DEX caused a late-term effect characterized by the suppression of pancreatic islet neogenesis at L8. Pancreatic islets from DEX rats also exhibited a singular increase in non-β-cell mass at this stage of lactation.

One limitation of our study is that we cannot assure that changes in M2-polarized macrophages infiltration actually occur in islets of rats treated with DEX during pregnancy. Such limitation is due to the low efficiency and availability of antibodies designed for flow cytometry and immunohistochemistry that recognize these rat antigens.

Altogether, our data demonstrate that the renewal of the endocrine pancreas that occurs during lactation was impaired by DEX treatment during pregnancy. Although the relative small size of the effect, we believe that the presently described alteration induced by antenatal DEX treatment may yield a long-term effect. This interpretation is based on our previous results showing that exposure to DEX during pregnancy results in glucose intolerance, impaired insulin secretion and increased expression of senescence markers in maternal pancreatic islets 12 months after delivery (44). Considering the data

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described herein, we conclude that the improper renewal of the endocrine pancreas after delivery may exert long-term impacts on maternal metabolism.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/EC-18-0505.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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