

UPR induces transient burst of apoptosis in islets of early lactating rats through reduced AKT phosphorylation via ATF4/CHOP stimulation of TRB3 expression

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Submitted 8 March 2010; accepted in final form 8 November 2010

Bromati CR, Lellis-Santos C, Yamanaka TS, Nogueira TC, Leonelli M, Caperuto LC, Gorjão R, Leite AR, Anhô GF, Bordin S. UPR induces transient burst of apoptosis in islets of early lactating rats through reduced AKT phosphorylation via ATF4/CHOP stimulation of TRB3 expression. *Am J Physiol Regul Integr Comp Physiol* 300: R92–R100, 2011. First published November 10, 2010; doi:10.1152/ajpregu.00169.2010.—Endocrine pancreas from pregnant rats undergoes several adaptations that comprise increase in β -cell number, mass and insulin secretion, and reduction of apoptosis. Lactogens are the main hormones that account for these changes. Maternal pancreas, however, returns to a nonpregnant state just after the delivery. The precise mechanism by which this reversal occurs is not settled but, in spite of high lactogen levels, a transient increase in apoptosis was already reported as early as the 3rd day of lactation (L3). Our results revealed that maternal islets displayed a transient increase in DNA fragmentation at L3, in parallel with decreased RAC-alpha serine/threonine-protein kinase (AKT) phosphorylation (pAKT), a known prosurvival kinase. Wortmannin completely abolished the prosurvival action of prolactin (PRL) in cultured islets. Decreased pAKT in L3-islets correlated with increased Tribble 3 (TRB3) expression, a pseudokinase inhibitor of AKT. PERK and eIF2 α phosphorylation transiently increased in islets from rats at the first day after delivery, followed by an increase in immunoglobulin heavy chain-binding protein (BiP), activating transcription factor 4 (ATF4), and C/EBP homologous protein (CHOP) in islets from L3 rats. Chromatin immunoprecipitation (ChIP) and Re-ChIP experiments further confirmed increased binding of the heterodimer ATF4/CHOP to the TRB3 promoter in L3 islets. Treatment with PBA, a chemical chaperone that inhibits UPR, restored pAKT levels and inhibited the increase in apoptosis found in L3. Moreover, PBA reduced CHOP and TRB3 levels in β -cell from L3 rats. Altogether, our study collects compelling evidence that UPR underlies the physiological and transient increase in β -cell apoptosis after delivery. The UPR is likely to counteract prosurvival actions of PRL by reducing pAKT through ATF4/CHOP-induced TRB3 expression.

endocrine pancreas; pregnancy; lactation; β -cell apoptosis; unfolded protein response

PREGNANCY IS HIGHLIGHTED AS a physiological state in which pancreatic β -cells from maternal pancreatic islets undergo a robust mass growth due to proliferation (39). Increase in pancreatic β -cell mass is an adaptive event that allows the

maternal organism to meet the insulin demand and compensate for peripheral insulin resistance (23). This morphofunctional adaptation is mainly attributed to lactogen action because in vitro treatment with this hormone induces β -cell proliferation (9). In addition, heterozygous null prolactin receptors (+/–) pregnant mice display reduced pancreatic β -cell mass compared with pregnant wild-type mothers (17).

RAC-alpha serine/threonine-protein kinase (AKT) is a protein kinase that governs general protein synthesis and cell survival by inhibiting apoptosis. In pancreatic β -cells, AKT activation protects from several proapoptotic insults (41, 10), while its inhibition leads to increased β -cell death (44, 15). It has been previously demonstrated that prolactin (PRL) activates pancreatic phosphatidylinositol 3 kinase (PI3K) upstream to AKT in vitro (2). Also, in vivo treatment with an antisense oligonucleotide targeted to the PRL receptor decreases AKT activation in pancreatic islets from pregnant rats (1). In parallel to AKT activation, PRL increases the expression of antiapoptotic genes in pancreatic islets (7).

A singular feature of pancreatic β -cell adaptation to pregnancy is that the increase in mass is time limited in such a way that the maternal endocrine pancreas returns to a nonpregnant state just after parturition. Scaglia et al. (34) have first demonstrated that maternal pancreatic islets undergo a transient burst of apoptosis as early as the 4th day postpartum, in spite of high levels of circulating PRL (20). As recently stressed, the intracellular mechanism underlying this reversal remains to be settled (32).

Endoplasmic reticulum (ER) stress is being currently considered a crucial event that drives pancreatic β -cell apoptosis in pathological conditions (14). ER stress occurs due to an imbalance of luminal ER homeostasis, e.g., increasing protein loading to the ER. Among several stimuli, accumulation of misfolded proteins within the ER of β -cells occurs as a consequence of increased proinsulin translation (35). ER stress bears unfolded protein response (UPR), which is an initially adaptive response that can ultimately lead to an execution phase, consisted of C/EBP homologous protein (CHOP)-mediated apoptosis (11). CHOP is a transcription factor that induces apoptosis by increasing Tribble 3 (TRB3) expression, a pseudokinase that inhibits AKT (27).

At the end of pregnancy, maternal islets display a twofold increase in general protein biosynthesis, including insulin (6). These data prompted us to investigate whether UPR is involved

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in the transient burst of apoptosis found in pancreatic islets from early lactating mothers. In the present study, we demonstrated an increase in hallmarks of UPR in pancreatic islets isolated from early lactating rats. We presently show that this response is associated with increased apoptosis and decreased AKT phosphorylation in pancreatic β -cell.

MATERIALS AND METHODS

Animals. Adult female Wistar rats at 8 wk of age (250–300 g) were kept at 24°C with 12:12-h light-dark cycle. Groups of two female rats were housed with one male for 5 days. The concomitant presence of spermatozoa and estrous cells in the vaginal lavage indicated the *day 0* of gestation, when the pregnant rats were isolated in a separate cage. On the day of the delivery, the number of pups was adjusted to 8 per lactating mother. The rats were used for experimental procedures at the 19th day of pregnancy (P19) and at the 1st (L1), 2nd (L2), 3rd (L3), 5th (L5), and 8th (L8) days postpartum. Virgin age-matched rats, submitted to male courtship, were used as the control group (CTL).

4-Phenyl butyric acid (PBA) (Sigma-Aldrich, St. Louis, MO) was diluted in vehicle [0.9% NaCl (wt/vol) and 0.15 mM NaHCO₃] to a final concentration of 100 mg/ml. L3 rats received PBA or vehicle intraperitoneally at the day of the delivery and at the first and the second days of lactation. PBA dosage was 250 mg·kg⁻¹·day⁻¹ divided into two injections of 125 mg/kg at 7:00 AM and 7:00 PM.

Pancreatic islets isolation and culture. Rats were euthanized by decapitation, and islets were isolated by collagenase digestion, as previously described (8). For the experiments with cultured islets, groups of ~400 islets were extensively and carefully washed with Krebs buffer containing antibiotics and cultured for 24 h in RPMI-1640 medium containing 11.1 mM glucose and 5% FBS. The culture was carried out with rat PRL (500 ng/ml) and/or wortmannin (100 nM) (Merck Biosciences, Darmstadt, Germany). DMSO, used to dilute wortmannin, was also added to CTL islets and PRL-treated islets. PRL was provided by the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases. All of the experiments involving animals were conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation. Our experiments were approved by the Ethics Committee on Animal Use at the Institute of Biomedical Sciences, University of Sao Paulo, Brazil.

DNA fragmentation. A group of 20 freshly isolated islets from each rat was dissociated after incubation with Ca²⁺-deprived Krebs buffer containing 0.5 mM EDTA at 37°C for 10 min. The same process was performed for cultured islets. Cells were pulled down with a brief spin and resuspended in 300 μ l of hypotonic solution containing 50 mg/ml propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100, and the solution was then incubated for 1 h at room temperature. Fluorescence was measured using the FL2 channel (orange-red fluorescence; 585/42 nm) using a FACSCalibur flow cytometer (Becton Dickinson, San Juan, CA). Ten thousand events were analyzed per experiment using the Cell Quest software (Becton Dickinson). DNA fragmentation was analyzed according to the method previously described (25).

Western blot analysis. Freshly isolated pancreatic islets were homogenized in 100 μ l of solubilization buffer and processed for protein extraction and Western blot, as previously described (4). Before incubation with the primary antibody, the membranes were blocked with blocking buffer (5% nonfat dried milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20) for 2 h at room temperature. The membranes were incubated for 4 h at room temperature with the primary antibody diluted in blocking buffer with 3% nonfat dried milk. Anti-CHOP, anti-TRB3, anti-ATF4, anti-AKT and anti-phospho-AKT (Ser473), anti-GADD34, anti-phospho-PERK, anti-PARP, and anti-caspase-3 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-BiP antibody was from Cell

Signaling Biotechnology (Beverly, MA), and anti- β actin was from Sigma (St. Louis, MO). Anti-phospho-eIF2 α antibody was from Abcam (Beverly, MA), and anti-PRL receptor (PRLR) was from Affinity Bioreagents (Golden, CO). Next, membranes were washed and incubated with peroxidase-conjugated secondary antibodies (GE Healthcare, Buckinghamshire, UK) for 1 h and processed for chemiluminescence detection. Quantitative analysis of the blots was performed by Scion Image software. When the primary antibody was a goat polyclonal, nonfat dried milk was replaced by 1% gelatin in all incubations.

RNA extraction and PCR. Total RNA was extracted from ~200 freshly isolated islets using TriZOL reagent (Invitrogen), and TRB3 expression was assessed by real-time PCR using SYBR Green master mix (Applied Biosystems, Foster City, CA), as previously described (25). The sequences of primers and amplification conditions were TRB3 sense 5'-GTTGGTGCTGGAGAACCTGGAG-3' and antisense 5'-AGAGTCCTGGAACGGGTATCGG-3', annealing temperature of 59°C; β -actin sense 5'-TGGCATTGTGATGGACTCCG-3' and antisense 5'-TTAATGTCACGCACGATTTCCC-3', annealing temperature of 58°C. Additionally, cDNAs were used for end-point RT-PCR analysis of PRLR, as previously described (7). The sequences of the primers were PRLR sense 5'-TGGACTCCCTGACTACAAGGC-3' and antisense 5'-GGAAGTCCTCCATCTGTC-CCAG-3', 56°C. In both analyses, β -actin was used as the internal control. RT-PCR was also performed for the analysis XBP-1 splicing, as previously described (21). Briefly, PCR products were incubated with the Pst-I restriction enzyme (Madison, WI) for 5 h at 37°C, followed by separation on a 2% ET-Br agarose gel. Primer sequences and amplification conditions were XBP-1 sense 5'-AAACAGAGTAGCAGCGCAGACTGC-3' and antisense 5'-GGATCTCTAAAAGTAGAGGCTTG-GTG-3', 600 bp, 52°C. All primers were synthesized by Integrated DNA Technologies (Coralville, IA). General reagents were from Invitrogen (Carlsbad, CA).

Chromatin immunoprecipitation assay. A group of 500 freshly isolated islets were processed using buffers and reagents from EZ ChIP chromatin immunoprecipitation kit (Upstate, Lake Placid, NY), according to a previously described protocol (3). Briefly, islets were fixed in Hanks buffer containing 1% formaldehyde and transferred to lysis buffer. DNA was sheared by sonication, and samples were diluted in dilution buffer and precleared with protein A-sepharose (50% slurry) (GE Healthcare, Buckinghamshire, UK) saturated with salmon sperm DNA. An aliquot of 10 μ l was collected as "input." The remaining supernatants were submitted to immunoprecipitation with protein A-sepharose and 2 μ g of anti-CHOP antibody. In parallel, one sample was incubated with protein A-Sepharose only, to generate the negative control (no-AB). DNA was eluted with elution buffer (SDS 1% and NaHCO₃ 0.1 M), submitted to cross-linking reversal and purified using phenol-chloroform. DNA samples were amplified for detection of TRB3 promoter. A 153-bp fragment corresponding to nucleotides -152 to +1 of the rat TRB3 gene was amplified by 40 cycles of PCR. The sequences of the primers were sense 5'-GTGC-CGCAGCACTTTAGCAG-3' and antisense 5'-TCTTGCTCTGAG-GTCTCAGAGCC-3', 60°C. The products were resolved in agarose gel containing ethidium bromide, and images were captured by digital photographs under UV illumination. CHOP binding was calculated after normalization by the input of each sample.

For Re-ChIP experiments, CHOP immunoprecipitates were eluted from the Sepharose beads using elution buffer added with DTT (10 mM). In this case, elution was carried out at 37°C. Next, eluted samples were diluted 40 \times with ChIP dilution buffer and incubated with 3 μ g of anti-ATF4 antibody. The following procedure was performed exactly as described for ChIP starting from the immunoprecipitation step.

Immunohistochemistry. The animals were anesthetized with ketamine/xylazine and perfused through the left cardiac ventricle initially with saline at 37°C and then with cold 0.1 M phosphate buffer (PB; pH 7.4) containing 2% paraformaldehyde. The pancreases were

dissected out and postfixed for 2 h. After this period, they were kept in a cryoprotective 30% buffered sucrose solution in PB for at least 4 h until sectioning. Transverse sectioning of the pancreatic tissue (12 μ m) was performed, embedding them in OCT compound, and cutting on a cryostat. All sections were blocked for 2 h in a PB solution containing 5% normal goat serum and 0.3% Triton X-100. Primary antibodies were incubated in 0.3% Triton X-100 in PB, diluted 1:200. Rabbit polyclonal antiserum against CHOP and TRB3 were the same used for Western blot, and guinea pig polyclonal antiserum against insulin (kindly provided by Dr. Angelo Carpinelli).

After several washes in PB, pancreas sections were incubated with tetramethyl rhodamine isothiocyanate-conjugated donkey antiserum against guinea pig IgG and fluorescein isothiocyanate-conjugated donkey antiserum against goat or rabbit IgG (1:200; Jackson Laboratories, West Grove, PA), diluted in PB containing 0.3% Triton X-100, for 2 h at room temperature. Controls experiments consisted of the omission of primary antibodies, and no staining was observed in these cases. After washing, the tissue was mounted using VectaShield (Vector Laboratories, Burlingame, CA). Slides were analyzed on a Zeiss LSM 510 confocal microscope. Figures were mounted with Adobe Photoshop CS. Manipulation of the images was restricted to threshold and brightness adjustments of the whole image.

Statistical analysis. The results were expressed as means \pm SE. The results were compared using ANOVA followed by the Tukey-Kramer test or Student's unpaired *t*-test. *P* values <0.05 indicated a significant difference.

RESULTS

In vivo and in vitro relationship between apoptosis and AKT phosphorylation in pancreatic islets. AKT content increased in pancreatic islets from P19 rats and remained higher in L3 rats (1.51 \pm 0.06- and 1.59 \pm 0.06-fold of CTL, respectively), returning to CTL values in islets from L8 mothers (Fig. 1A). Absolute levels of AKT serine phosphorylation were increased

in pancreatic islets from P19 (1.39 \pm 0.16-fold of CTL) but reduced in L3 (0.56 \pm 0.04-fold compared with CTL; Fig. 1B). Representative blots of AKT, phosphorylated AKT, and the loading control β -actin, are shown in Fig. 1C. When relative AKT phosphorylation values were calculated (the ratio between phosphorylated AKT and AKT content), no changes were found in islets from P19 rats, but the relative AKT phosphorylation in islets from L3 rats was significantly reduced (0.52 \pm 0.35-fold of CTL). In parallel to the reduction in the absolute and the relative levels of AKT serine phosphorylation, islets from L3 rats displayed a transitory increase in DNA fragmentation. The percentage of cells with fragmented DNA rose from 16.92 \pm 1.18% in CTL islets to 26.00 \pm 1.19% in islets from L3 rats (Fig. 1D).

Pancreatic islets isolated from adult female rats cultured for 24 h displayed 15.06 \pm 1.07% of cells with fragmented DNA. PRL treatment decreased this value to 10.78 \pm 0.60%. The addition of wortmannin to the culture medium, a pharmacological inhibitor of the PI3K/AKT pathway, did not promote any effect by itself but blocked PRL-induced decrease in DNA fragmentation (Fig. 2A). PRL receptor mRNA expression displayed a biphasic pattern of up-regulation, peaking in islets of P19 and L8 rats (1.26 \pm 0.03-fold and 1.28 \pm 0.07-fold of CTL, respectively). PRLR mRNA levels in islets from L1, L2, L3, and L5 rats were similar to that found in CTL (Fig. 2B). Similar to its mRNA, PRLR protein content was increased in islets from P19 and L8 rats (1.86 \pm 0.17- and 1.87 \pm 0.09-fold of CTL, respectively) and was not altered in islets from L3 rats compared with CTL (Fig. 2C).

Increased UPR in pancreatic islets from early lactating rats. Figure 3A shows representative blots of several UPR-related proteins in pancreatic islets from pregnant and early lactating

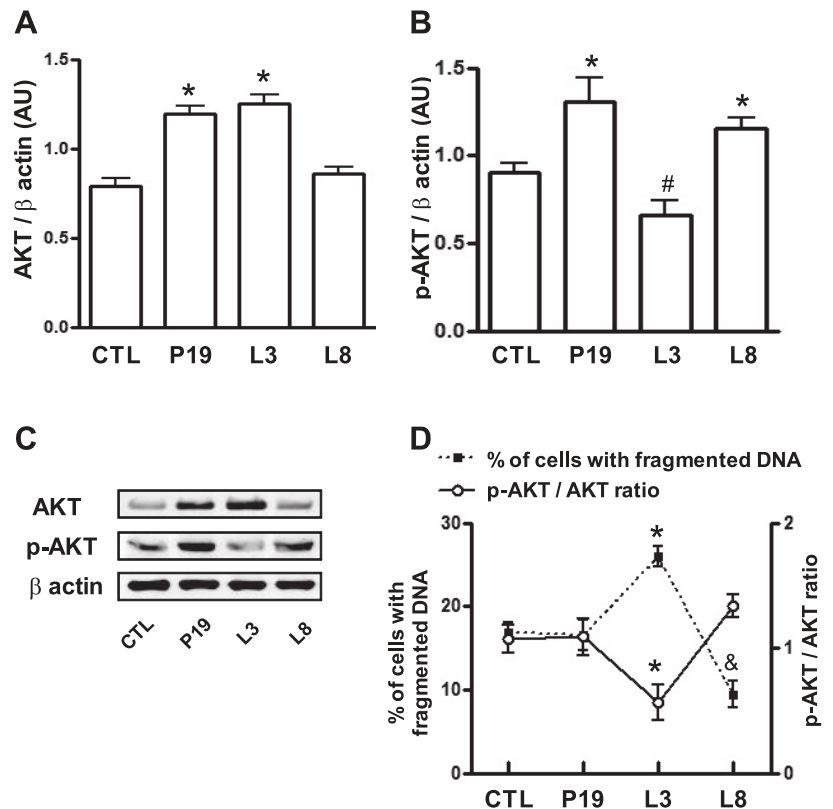


Fig. 1. Relationship between apoptosis and AKT phosphorylation in pancreatic islets. Pancreatic islets were isolated from rats at the 19th day of pregnancy (P19) and at the 3rd and the 8th day of lactation (L3 and L8, respectively). In parallel, islets were also isolated from virgin rats to be used as control (CTL). Isolated islets were processed for protein extraction and Western blot detection of AKT (A) and phosphorylated AKT (Ser 473) (B). C: representative blots are shown for membrane staining with anti AKT, anti phospho-AKT (Ser 473), and anti- β actin (used as loading control) (C). Isolated islets were also processed for DNA fragmentation analysis by flow cytometry after propidium iodide staining. D: results are shown as a percentage of cells with fragmented DNA relative to the total number of events in each condition (left y-axis). Phospho-AKT (Ser 473)/AKT ratio is shown in the same graph (right y-axis). Data are shown as means \pm SE. **P* < 0.05 vs. CTL; #*P* < 0.05 vs. P19; &*P* < 0.05 vs. L3 (*n* = 9 to 15 for DNA fragmentation and *n* = 4 to 12 for Western blot analysis).

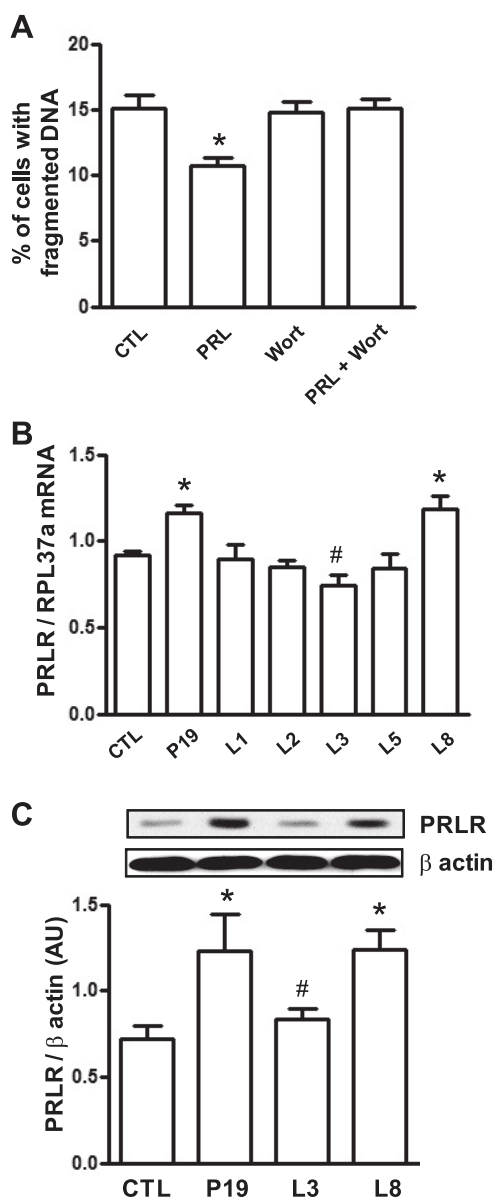


Fig. 2. DNA fragmentation in islets cultured with prolactin (PRL) and/or wortmannin and PRL receptor (PRLR) expression in islets from pregnant and lactating rats. *A*: islets were isolated from virgin female rats and plated in culture dishes (around 400 islets/plate). Islets were cultured for 24 h with PRL (500 ng/ml) and/or wortmannin (100 nM) processed for DNA fragmentation analysis by flow cytometry after propidium iodide staining. *B*: pancreatic islets were isolated from rats at the 19th day of pregnancy (P19) and at the 1st, 2nd, 3rd, 5th, and the 8th day of lactation (L1, L2, L3, L5, and L8, respectively). In parallel, islets were also isolated from virgin rats to be used as CTL. Isolated islets were processed for total RNA extraction and RT-PCR analysis of PRL receptor mRNA. *C*: an additional set of islets was processed for protein extraction and Western blot detection of PRL receptor. β -actin was used as a loading control. Data are shown as means \pm SE. * $P < 0.05$ vs. CTL; # $P < 0.05$ vs. P19 ($n = 10$ for DNA fragmentation, $n = 4$ for Western blot and $n = 4$ to 7 for RT-PCR).

rats. Immunoglobulin heavy chain-binding protein (BiP) and activating transcription factor 4 (ATF4) contents were not altered in pancreatic islets from P19 rats but were increased in pancreatic islets from L3 and L8 rats (1.65-fold of P19 for BiP content, Fig. 3C; and 1.56-fold of CTL and P19 levels, Fig. 3D). CHOP expression was decreased in P19 (0.54 ± 0.23 -fold

of CTL) and increased in L3 (1.45 ± 0.14 -fold of CTL). These alterations were completely reverted in islets from L8 rats (Fig. 3E). X-box binding protein 1 (XBP-1) splicing (Fig. 3B), and activating transcription factor 6 (ATF6) expression (not shown) were not modulated in islets from pregnant and lactating rats.

Figure 3F shows the detailed temporal variation in the phosphorylation of both double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) and eukaryotic initiation factor 2 alpha (eIF2 α), and the expression of growth arrest and DNA damage-inducible protein 34 (GADD34). PERK phosphorylation transiently increases in islets from L1 rats (2.05 ± 0.15 -fold of CTL), reaching levels lower than CTL at L3 (0.58 ± 0.08 -fold of CTL). Similarly to PERK, eIF2 α phosphorylation transiently peaks in L1 (1.35 ± 0.07 -fold of CTL) and falls to minimal values in islets from L3 rats (0.73 ± 0.05 -fold of CTL). Both PERK and eIF2 α phosphorylation returned to CTL levels in L8. GADD34 expression is upregulated in L3 (1.4 ± 0.04 -fold of CTL) and further increased in L8 (1.92 ± 0.02 -fold of CTL).

Increase in TRB3 expression and ATF4/CHOP binding to TRB3 promoter in pancreatic islets from early lactating rats. CHOP and ATF4 have been described to stimulate TRB3 expression and, by extension, mediate UPR-induced down-regulation of AKT serine phosphorylation. In Fig. 4, *A* and *B*, we show that TRB3 expression is upregulated in L3 islets (2.88 ± 0.30 -fold and 1.95 ± 0.27 -fold of CTL values, respectively for mRNA and protein contents). Using chromatin immunoprecipitation (ChIP) assay, we presently demonstrate that binding of CHOP to TRB3 promoter is increased in pancreatic islets from L3 (1.97 ± 0.10 -fold of CTL; Fig. 4C). In addition, Re-ChIP experiments further suggested increased formation of ATF4-CHOP heterodimer in islets from early lactating rats, since sequential immunoprecipitation of ATF4 from the DNA pulled down with anti-CHOP antibody has also yielded increased binding to the TRB3 promoter in L3 islets (2.45 ± 0.20 -fold of CTL; Fig. 4D). No binding was observed in no-AB immunoprecipitation.

Chemical chaperone PBA restores AKT phosphorylation levels and abrogates CHOP- and TRB3-dependent apoptosis in pancreatic islets from L3 rats. PBA treatment was carried out for 3 days by means of intraperitoneal injections. In accordance with the data exhibited in Fig. 1, the percentage of cells with fragmented DNA rose from $17.65 \pm 0.94\%$ in islets from vehicle-treated CTL rats to $31.11 \pm 3.07\%$ in vehicle-treated L3 rats (Fig. 5A). PBA treatment of L3 rats reduced the percentage of islet cells with fragmented DNA to values similar to that found in CTL ($19.22 \pm 1.32\%$). Other classic apoptotic markers, e.g., cleaved poly(ADP-ribose) polymerase (PARP) and caspase-3, were increased in L3 islets and reduced by PBA treatment (Fig. 5B), further suggesting an apoptotic process dependent on ER stress. Moreover, AKT serine phosphorylation was reduced in islets from L3 rats treated with vehicle (0.49 ± 0.16 -fold vehicle-treated CTL values). PBA treatment of L3 rats restored AKT serine phosphorylation to values similar to that found in islets from vehicle-treated CTL rats (Fig. 5C).

TRB3 expression was also increased in pancreatic islets of vehicle-treated L3 rats (3.18 ± 0.54 -fold vehicle-treated CTL values). In this case, PBA treatment reduced the mRNA of TRB3 to values lower than that found in vehicle-treated CTL

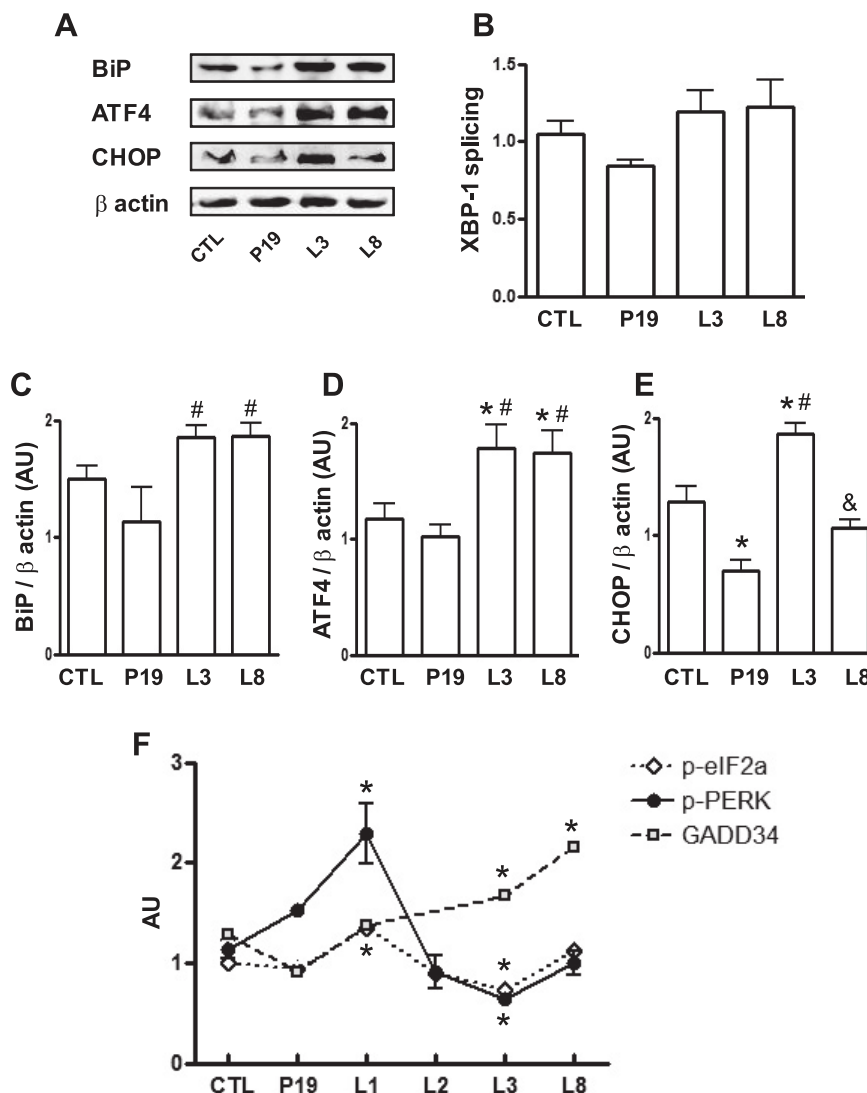


Fig. 3. UPR markers in pancreatic islets from pregnant and lactating rats. *A*: pancreatic islets were isolated from rats at the 19th day of pregnancy (P19) and at the 1st, 2nd, 3rd, and the 8th day of lactation (L1, L2, L3, and L8, respectively). In parallel, islets were also isolated from virgin rats to be used as CTL. Isolated islets were processed for protein extraction and Western blot detection of immunoglobulin heavy chain-binding protein (BiP), activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP), and β -actin. *B*: an additional set of islets were processed for total RNA extraction, reverse transcription, and amplification of a fragment of the X-box binding protein 1 (XBP-1) transcript. XBP-1 splicing was assessed after resolving the products of XBP-1 fragment digestion with *Pst*-I in EtBr agarose gel. Data were obtained by the densitometric analysis of the X-ray films exposed to membranes probed with BiP (*C*), ATF4 (*D*), and CHOP (*E*) antibodies. *F*: The results of Western blot analysis of phospho-PERK, phospho-eIF2 α , and GADD34 are shown in the same graph. In the latter graph, significance symbols are placed at the top for GADD34 and p-PERK, and at the bottom for p-eIF2 α . Data are shown as means \pm SE. * P < 0.05 vs. CTL; # P < 0.05 vs. P19; & P < 0.05 vs. L3; n = 7 for ATF4, XBP-1 splicing, CHOP, BiP, and phospho-eIF2 α ; n = 3 to 5 for phospho-PERK and GADD34.

rat islets (0.14 ± 0.60 -fold of vehicle-treated CTL values) (Fig. 5D). CHOP binding to TRB3 promoter was increased in vehicle-treated L3 rats (2.08 ± 0.20 -fold vehicle-treated CTL values). PBA treatment of L3 rats reduced CHOP-TRB3 promoter association to vehicle-treated CTL values (Fig. 5E).

Immunohistochemical analysis showed that CHOP expression is mostly located in insulin-producing cells, as demonstrated by insulin/CHOP merging. Although not quantitative, representative images revealed an increase β -cell CHOP staining, which was reversed by PBA treatment (Fig. 6A). Western blot approach showed that CHOP content was increased in islets of vehicle-treated L3 rats (1.56 ± 0.18 -fold of vehicle-treated CTL islets). PBA treatment reduced CHOP levels in L3 to 0.60 ± 0.24 -fold of CTL (Fig. 6B).

We also found that TRB3 is coexpressed with insulin in CTL islet cells. In L3 islets, TRB3 has a spread pattern of expression, being also detected in peripheral islet cells and exocrine pancreas. Similar to CHOP, TRB3 staining in L3+PBA is less apparent than in untreated L3 β -cells (Fig. 6C). Indeed, TRB3 protein content was increased in islets from L3 rats ($2.17 \pm$

0.24 -fold of CTL), and PBA treatment restored TRB3 islet content (Fig. 6D).

DISCUSSION

In the present study, we demonstrate that reduced levels of AKT serine phosphorylation are involved in the burst of apoptosis that occurs in the maternal endocrine pancreas just after delivery. This response correlates with the activation of UPR and is linked to ATF4/CHOP-dependent stimulation of TRB3 expression. In addition, we further demonstrate that reduced AKT phosphorylation and increased apoptosis/UPR are suppressed by PBA, an inhibitor of ER stress.

Our first aim was to investigate whether alterations in AKT activation were associated or not with the previously described transient increase in apoptotic rate of maternal islets after the delivery (34). We found a reduced AKT serine phosphorylation matching with an increased DNA fragmentation in L3 islets, thus suggesting the significance of AKT in the physiological burst of apoptosis in this period. In addition, we demonstrated that in vitro

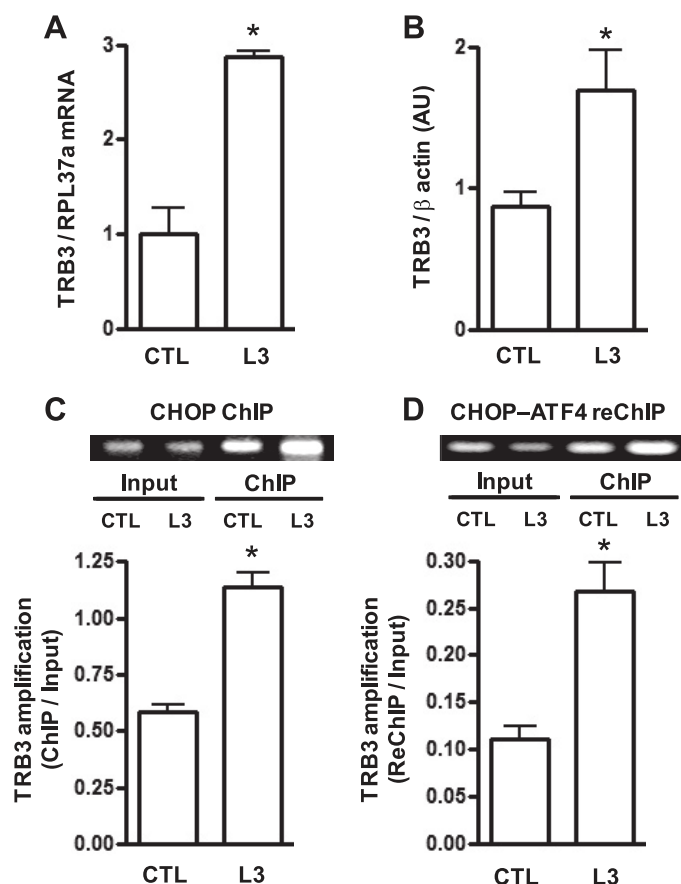


Fig. 4. Tribble 3 (TRB3) expression and CHOP/ATF4 binding to TRB3 promoter in pancreatic islets of lactating rats. Pancreatic islets were isolated from virgin rats (CTL) and rats at the 3rd day of lactation (L3) and processed for total RNA and protein extraction. **A**: Approximately 2 μ g of RNA from each sample were used for cDNA synthesis and real-time PCR analysis of TRB3 mRNA. **B**: total protein from each sample was resolved by SDS-PAGE and processed for Western blot detection of TRB3 protein content. **C**: TRB3 promoter was amplified from DNA extracted from the Input, and ChIP originated from CHOP-immunoprecipitated samples. **D**: CHOP immunoprecipitated DNA complexes were eluted and reimmunoprecipitated with ATF4 antibody (Re-ChIP). PCR products were resolved in agarose gels and densitometric values of ChIP and Re-ChIP from each sample were normalized by the respective Input. Data are shown as means \pm SE. * P < 0.05 vs. CTL (n = 4 for Western blot and RT-PCR and n = 3 for ChIP and Re-ChIP).

antiapoptotic action of PRL in islets isolated from adult female virgin rats was suppressed by wortmannin, a PI3K/AKT inhibitor. This result particularly shows that AKT activation by PRL mediates the antiapoptotic effect of this hormone.

We next found that PRL receptor (PRLR) content was decreased in L3 to values similar to that found in CTL, compared with P19 islets. Thus, the reduction in PRLR by itself does not satisfactorily explain the reduction of AKT phosphorylation to levels below that found in CTL. We then hypothesized that an intracellular mechanism, other than downregulation of PRLR, would be inhibiting AKT activation and inducing apoptosis in pancreatic islets from L3 rats.

ER stress-mediated UPR activation and pancreatic β -cell death are currently considered key events in the pathogenesis of diabetes mellitus (22). Many insults are able to trigger ER stress-mediated UPR in pancreatic β -cells. For instance, depletion of ER Ca^{2+} stores, increased insulin biosynthesis, and

palmitate-induced protein overload were described to stimulate UPR-mediated apoptosis (14, 35, 19, 30). Thus, both changes in Ca^{2+} handling due to the modulation of sarcoendoplasmic reticulum Ca^{2+} -ATPase (SERCA2) expression in islets from early lactating rats (4, 18), and the increased insulin biosynthesis by maternal islets at the end of pregnancy (6), point to UPR as a mediator of the apoptotic burst postpregnancy.

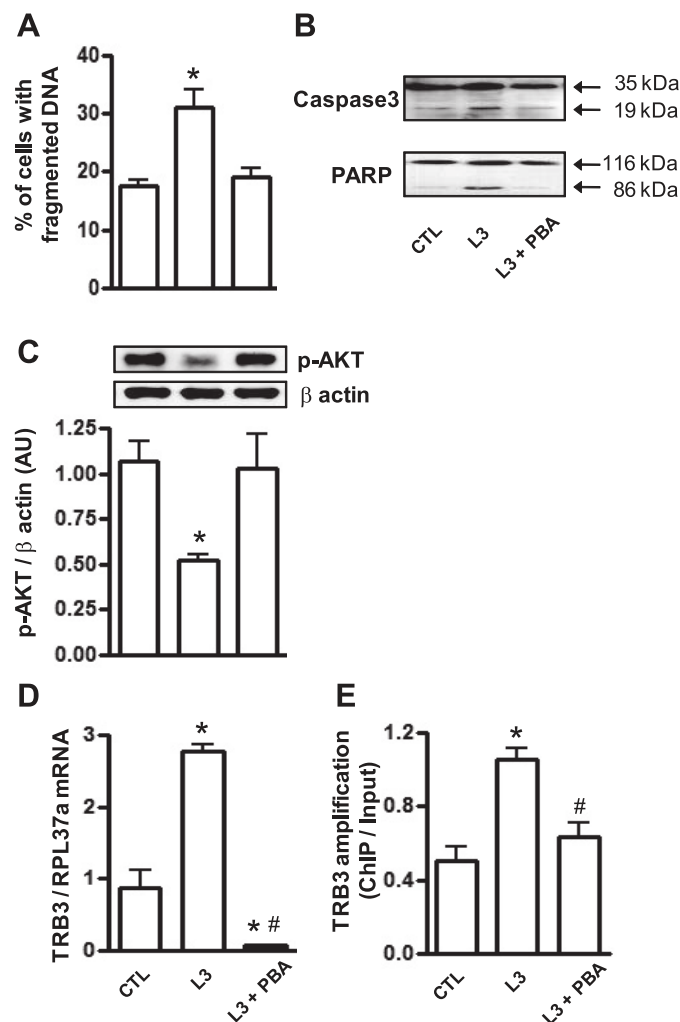
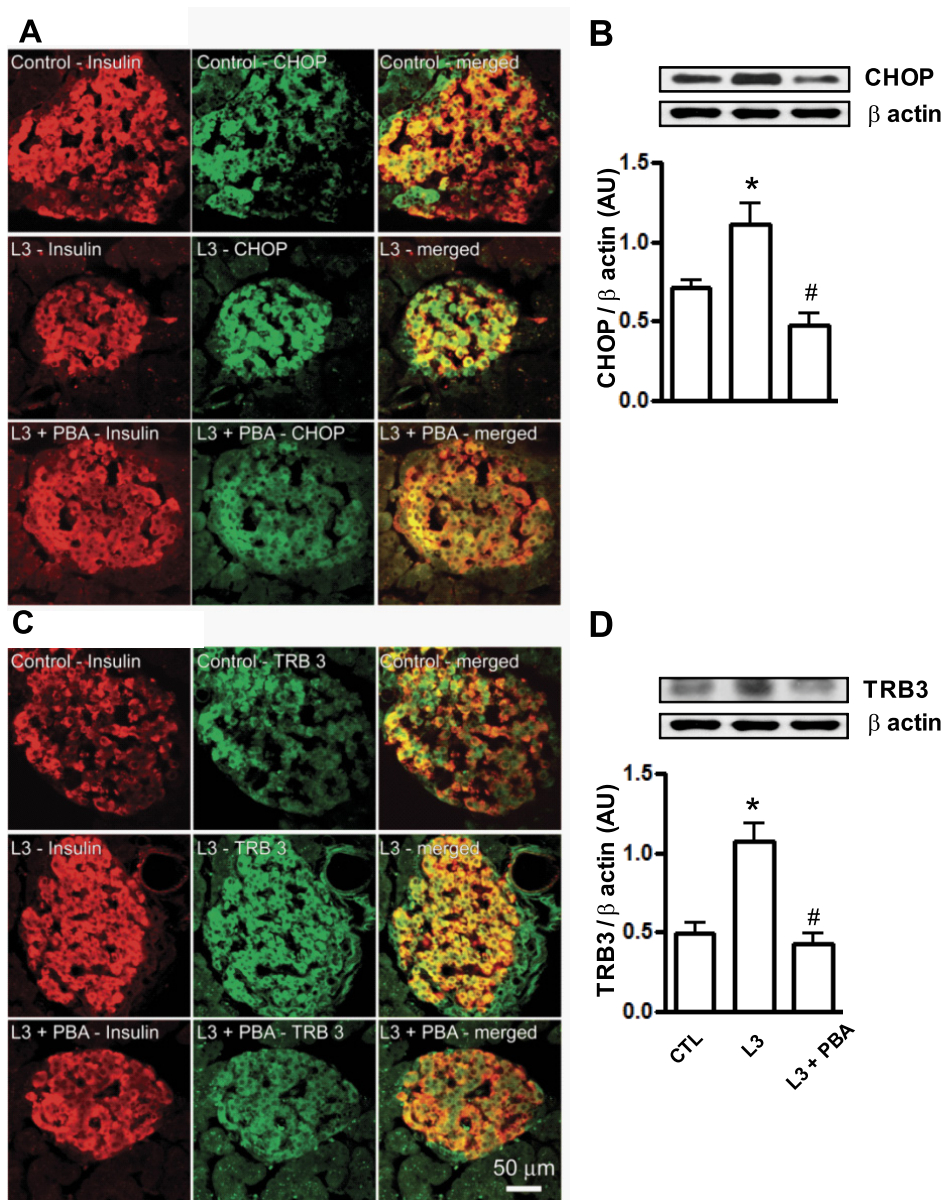


Fig. 5. Apoptosis, AKT phosphorylation, TRB3 expression, and CHOP binding to TRB3 promoter in pancreatic islets of lactating rats treated with 4-phenyl butyric acid (PBA). Lactating rats were treated with PBA (250 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) at the day of the delivery and at the first and the second day of lactation, and euthanized at the 3rd day of lactation (L3+PBA). Another group of lactating rats was treated with the vehicle at the same schedule and also euthanized on L3. Virgin rats were treated for 3 days with the vehicle and used as CTL. **A**: pancreatic islets were isolated and processed for DNA fragmentation analysis by flow cytometry after propidium iodide staining. Another set of islets was processed for protein extraction and Western blot detection of cleaved caspase-3 and PARP (**B**) and phospho-AKT and β -actin (**C**). **D**: These data were obtained by the densitometric analysis of the X-ray films exposed to membranes. Islets isolated from CTL, L3, and L3+PBA rats were processed for total RNA extraction and real-time PCR analysis of TRB3 mRNA. Also, TRB3 promoter was amplified from DNA extracted from the Input and ChIP originated from CHOP immunoprecipitated samples (CTL, L3, L3+PBA). **E**: PCR products were resolved in agarose gel, and densitometric values of ChIP from each sample were normalized by the respective Input. Data are shown as means \pm SE. * P < 0.05 vs. CTL; # P < 0.05 vs. L3 (n = 10 for DNA fragmentation, n = 3 for ChIP assay, PARP, and caspase-3 detection, and n = 7 for phospho-AKT detection).

Fig. 6. CHOP and TRB3 expression and distribution in pancreatic islets of lactating rats treated with PBA. Lactating rats were treated with PBA (250 mg·kg⁻¹·day⁻¹) at the day of the delivery and at the first and the second day of lactation; then, they were euthanized at the 3rd day of lactation (L3+PBA). Another group of lactating rats was treated with the vehicle at the same schedule and euthanized on L3. Virgin rats were treated for 3 days with the vehicle and used as CTL. **A**: anesthetized animals were perfused through the left cardiac ventricle with proper solutions, and the pancreas was dissected out and processed for immunohistochemical staining of insulin (using a secondary antibody conjugated to TRITC-red images) and CHOP (using a secondary antibody conjugated to FITC-green images). **C**: pancreas sections were also stained with anti-insulin and anti-TRB3 antibody (using a secondary antibody conjugated to FITC-green images). Another set of rats was euthanized, and islets were processed for protein extraction and Western blot detection of CHOP and β actin (**B**) and TRB3 and β -actin (**D**). Data were obtained by the densitometric analysis of the X-ray films exposed to membranes. Data are shown as means \pm SE. * $P < 0.05$ vs. CTL; # $P < 0.05$ vs. L3 ($n = 3$ for immunohistochemistry and $n = 8$ for Western blot).



Activation of UPR is triggered by the dissociation of the chaperone BiP from the transducer proteins PERK, ATF6, and inositol-requiring enzyme 1 (IRE1). These three proteins constitute the initial steps of independent branches that direct the adjustment of ER folding rate. IRE1 activation stimulates the processing of XBP-1 mRNA, and ATF6 pathway is redundant to that of IRE1/XBP-1 but also potentiates the IRE1 pathway by increasing the transcription of XBP-1 mRNA (46, 47). Our initial UPR screening revealed that the PERK pathway, but not ATF6 expression and XBP-1 splicing, was altered in pancreatic islets during the transition from pregnancy to lactation. These data suggest that UPR is partially activated during the physiological adaptation of pancreatic islets along the peripartum.

PERK activation is described to trigger eIF2 α phosphorylation on serine 51, which contributes to the attenuation of mRNA translation due to eIF2 α inhibition. The generation of

mice with constitutive active eIF2 α generates severe diabetic phenotype (5, 36). Our results show a transient increase in PERK phosphorylation concomitant with the stimulation of eIF2 α phosphorylation in L1 islets, which precedes the burst of DNA fragmentation associated with the reduction in PERK and eIF2 α phosphorylation. Although apparently controversial, the immediate increase in PERK/eIF2 α phosphorylation just after delivery can have a putative physiological role. Translation of most of the mRNAs is inhibited by eIF2 α serine 51 phosphorylation, but paradoxically, it increases the translation of the mRNA that encodes the transcription factor ATF4 (24). ATF4 has a transcriptional activity over several genes, including GADD34, a protein phosphatase that dephosphorylates eIF2 α , therefore, exerting a negative feedback on the PERK/eIF2 α /ATF4 pathway (26). We found that GADD34 progressively increases in maternal islets after parturition, reaching significant higher values at L3 that temporally coincides with the

peak of ATF4 protein content. Thus, an upregulation in GADD34 expression, possibly mediated by ATF4, would explain the decreased levels in eIF2 α phosphorylation observed in L3. In addition, our data indicate that activation of PERK/eIF2 α pathway as early as in the first day after delivery is likely to increase ATF4 content later at L3, thus triggering these sequential events that culminate with β -cell death.

Phosphorylation of eIF2 α is a critical convergence point of the integrated stress response that leads to global protein translational arrest and stimulation of specific transcription factors (32a). In addition to PERK, there are at least three other different mammalian eIF2 α kinases that may be activated by different stressors. These kinases are known as protein kinase RNA-activated, general control nonderepressible 2, and heme-regulated eIF2 α kinase. Therefore, we do not discard that kinases other than PERK could also be collectively contributing to apoptosis by mediating the increase in ATF4 and CHOP expression.

Besides, it cannot be ruled out that the increase in pancreatic β -cell death of L3 islets would also result from an oxidative stress. We have no data to sustain this suggestion, but the absence of eIF2 α phosphorylation has been described to cause an amplified and unregulated proinsulin translation, leading to an oxidative stress response (5). This could be due to increased production of oxidative species secondary to the formation of hydrogen peroxide (40) and the consumption of glutathione (12). Together, with a putative oxidative stress, the induction of CHOP by ATF4 might also mediate pancreatic β -cell death (29, 33, 38). Accordingly, we have found that the increase in ATF4 and DNA fragmentation in L3 islets correlates with an increase in CHOP expression.

The mechanism by which CHOP targets apoptosis is not completely understood, but one important mechanism is the formation of CHOP-ATF4 heterodimers that, in turn, induces TRB3 expression (27). Here, we demonstrate that the increase in TRB3 mRNA expression in islets from L3 rats parallels the reduction in AKT phosphorylation. In addition to the participation of TRB3 in a physiological burst of apoptosis, which is being presently proposed, TRB3 has already been suggested to mediate the pathological apoptosis of pancreatic β -cells from diabetic rodents (31, 16), and in INS1 cells treated with palmitate (31). The participation of the ATF4-CHOP heterodimers in the upregulation of TRB3 expression was reinforced by the present demonstration that the binding of CHOP and the heterodimer CHOP-ATF4 to the TRB3 promoter was increased in islets from L3 rats. The fragment that we amplified from the DNA pulled down with both CHOP and CHOP-ATF4 ranged between the -152 and the +1 nucleotide of the rat TRB3 gene, which encompass the binding site for ATF4-CHOP (27).

To further demonstrate the relevance of ER stress for the downregulation of AKT phosphorylation and increase in apoptosis, we next treated lactating rats with PBA, a short-chain fatty acid described as a chemical chaperone because of its ability to stabilize protein conformation, to improve ER folding capacity, and to facilitate the trafficking of mutant proteins (42). PBA was already demonstrated to inhibit ER stress and improve glucose intolerance by enhancing AKT phosphorylation in liver (28). In agreement with these, we found that 3-day treatment with PBA restored AKT phosphorylation and decreased DNA fragmentation in islets from L3 rats. Inhibition of

β -cell apoptosis by PBA was further supported by the reduction of PARP and caspase-3 processing that was found in islets of PBA-treated L3 rats. PARP degradation by caspase into 85- to 90-kDa fragments is considered a biochemical marker for apoptosis (43), already detected in pancreatic β -cell undergoing programmed cell death (13). Cleavage of caspase-3, in turn, is proportional to its proteolytic activity and apoptosis in pancreatic β -cells (45). The reversal of these apoptotic parameters by PBA was accompanied by a reduction in CHOP expression and binding to the TRB3 promoter and, consequently, downregulation of TRB3 expression in islets of PBA-treated L3 rats. Importantly, we also demonstrated that the increase of CHOP and TRB3 in islets of L3 rats were detected in insulin-positive cells and inhibited by PBA.

Perspectives and Significance

To our knowledge, the present study is the first to describe the UPR as a mechanism for a physiological and transitory burst of apoptosis in pancreatic β -cell. The apoptosis triggered by ER stress and the activation of UPR (increased ATF4 and CHOP expression) in pancreatic islets from early lactating mothers have a transitory feature and was already detected as early as the 3rd day after delivery. Moreover, we show that the mechanism by which UPR triggers physiological apoptosis in pancreatic islets is probably a result of CHOP/ATF4-induced TRB3 expression and a consequent downregulation of AKT activity. These data provide mechanistic information to explain the transitory burst of apoptosis that contributes to maternal pancreatic β -cell renewing postpregnancy.

ACKNOWLEDGMENTS

The authors thank José L. dos Santos and Luciene Ribeiro for technical assistance.

GRANTS

This study was supported by the Brazilian foundations Fundação de Amparo a Pesquisa do Estado de São Paulo, Conselho Nacional de Desenvolvimento e Tecnológico, and Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior.

DISCLOSURES

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

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