

Progesterone induces apoptosis of insulin-secreting cells: insights into the molecular mechanism

V A Nunes^{1†}, E P Portioli-Sanches², M P Rosim², M S Araujo³, P Praxedes-Garcia³, MMR Valle², LP Roma², C Hahn⁴, E Gurgul-Convey⁴, S Lenzen⁴ and A K Azevedo-Martins¹

¹School of Arts, Sciences and Humanities and ²Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil

³Department of Biochemistry of Federal University of Sao Paulo, Sao Paulo, Brazil

⁴Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany

[†]V A Nunes is now at School of Arts, Sciences and Humanities, Avenida Arlindo Bettio, 1000, 03828-000 Sao Paulo, SP, Brazil

Correspondence should be addressed to V A Nunes

Email
vanunes@ib.usp.br

Abstract

Progesterone has been associated with the development of gestational diabetes (GD) due to the enhancement of insulin resistance. As β -cell apoptosis participates in type 1 and type 2 diabetes pathophysiology, we proposed the hypothesis that progesterone might contribute to the development of GD through a mechanism that also involves β -cell death. To address this question, RINm5F insulin-producing cells were incubated with progesterone (25–100 μ M), in the presence or absence of α -tocopherol (40 μ M). After 24 or 48 h, membrane integrity and DNA fragmentation were analyzed by flow cytometry. Caspase activity was used to identify the mode of cell death. The involvement of endoplasmic reticulum stress in the action of progesterone was investigated by western blotting. Oxidative stress was measured by 2',7'-dichlorofluorescein diacetate (DCFDA) oxidation. Isolated rat islets were used in similar experiments in order to confirm the effect of progesterone in primary β -cells. Incubation of RINm5F cells with progesterone increased the number of cells with loss of membrane integrity and DNA fragmentation. Progesterone induced generation of reactive species. Pre-incubation with α -tocopherol attenuated progesterone-induced apoptosis. Western blot analyses revealed increased expression of CREB2 and CHOP in progesterone-treated cells. Progesterone caused apoptotic death of rat islet cells and enhanced generation of reactive species. Our results show that progesterone can be toxic to pancreatic β -cells through an oxidative-stress-dependent mechanism that induces apoptosis. This effect may contribute to the development of GD during pregnancy, particularly under conditions that require administration of pharmacological doses of this hormone.

Key Words

- ▶ insulin-producing cells
- ▶ progesterone
- ▶ oestriol
- ▶ apoptosis
- ▶ oxidative stress
- ▶ gestational diabetes

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Introduction

The steroid hormones progesterone and the oestrogens have been shown to be involved in β -cell physiology, exerting direct effects on insulin secretion (for review see Lenzen & Bailey (1984) and Straub *et al.* (2001)) and islet

cell proliferation (Kawai & Kishi 1999, Nieuwenhuizen *et al.* 1999, Le May *et al.* 2006) through binding to their specific receptors (Pasanen *et al.* 1997, Le May *et al.* 2006). In addition, Picard *et al.* (2002) suggested that

the hormonal particularities of pregnancy, and more specifically progesterone, might contribute to the poor adaptation of insulin secretion (Straub *et al.* 2001) and action to the increased requirements during pregnancy.

In the pregnancy scenario, the diabetogenic effect of progesterone has been mostly explained by the enhancement of insulin resistance (Butte 2000), particularly in skeletal muscle and adipose tissue, through a reduction in glucose transporter 4 (GLUT4 (SLC2A4)) expression (Sugaya *et al.* 2000). However, considering that apoptosis is involved in the pathophysiology of type 1 and 2 diabetes and also contributes to the involution of the β -cell mass in the *postpartum* phase (Scaglia *et al.* 1995), we first proposed the hypothesis that progesterone might be involved in β -cell death, also contribute to the development of gestational diabetes (GD). Both progesterone's diabetogenic effect and its potential to induce β -cell death require attention with the increasing pharmacological use of progestagens throughout pregnancy for prevention of recurrent preterm delivery (Sanchez-Ramos *et al.* 2005, Dodd *et al.* 2008, Jayasooriva & Lamont 2009), which has been correlated to the enhanced incidence of GD (Rebarber *et al.* 2007).

Little is known about the possible mechanisms by which progesterone exerts its effects on β -cells; however, studies have shown that this hormone is able to modulate the generation of reactive oxygen species (ROS) and oxidative stress in different cell types (Nguyen & Syed 2011, Toyoda *et al.* 2011). Thus, as the pancreatic β -cells present very low activities of antioxidant enzymes (Tiedge *et al.* 1997) and it has been observed that the over-expression of these enzymes protects these cells against diabetogenic insults (Lortz *et al.* 2000, Azevedo-Martins *et al.* 2003, Gurgul *et al.* 2004), we considered that progesterone might act through an oxidative-stress-dependent mechanism that has been recognised as a stimulus for endoplasmic reticulum (ER) stress and for the unfolded protein response (UPR) (Cao & Kaufman 2012).

In contrast to the effects of progesterone, data from human and animal studies indicate that 17 β -estradiol (estradiol) prevents a decrease in insulin production in the diabetic state (Le May *et al.* 2006). Also, estradiol, at pharmacological concentrations, protects human pancreatic islets from apoptosis induced by proinflammatory cytokines *in vitro* (Contreras *et al.* 2002). This information led us to consider that an antidiabetic action of estradiol might be due, at the least in part, to prevention of β -cell apoptosis. Therefore we also evaluated the effect of oestriol, the major oestrogen produced during pregnancy, on progesterone-induced cell death.

We report herein that progesterone, at pharmacological concentrations, as used for preterm delivery prevention, induced apoptosis of RINm5F insulin-producing cells and rat islet cells, which could be almost abolished by pre-incubation with α -tocopherol. Cell death induced by progesterone was enhanced by oestriol, though this oestrogen *per se* had only little effect. In RINm5F insulin-producing cells, the ER stress-related proteins CCAAT/enhancer-binding protein homologous protein (CHOP) (DDIT3) and CREB2 (CRT2) were differentially expressed following exposure to progesterone, indicating that ER stress leading to an accumulation of unfolded/misfolded proteins may contribute to the observed effects.

Materials and methods

Materials

RPMI-1640 medium, HEPES, penicillin and streptomycin were purchased from Invitrogen. Propidium iodide (PI) was purchased from ICN Biomedicals (Costa Mesa, CA, USA), sodium citrate from Merck and sodium bicarbonate from Labsynth Products (Diadema, SP, Brazil). The antibodies used were as follows: anti-CREB-2 and anti-CHOP (GADD 153) purchased from Santa Cruz Biotechnology, Inc. Thapsigargin was from Tocris (Bristol, UK). The ECL Western Blotting Analysis System kit was from Amersham Biosciences UK Limited. All cell culture plastic material was from Corning (Corning, NY, USA). Progesterone, oestriol and α -tocopherol as well as 2',7'-dichlorofluorescein diacetate (DCFDA) were from Sigma-Aldrich.

Cell culture conditions and islet isolation

RINm5F insulin-producing cells were cultured in RPMI-1640 medium, containing 11.1 mM glucose and 10% (v/v) FCS. The medium was supplemented with 2.0 mM glutamine, 20 mM HEPES, 100 μ g/ml streptomycin, 100 IU/ml penicillin and 24 mM sodium bicarbonate. The cells were maintained in an incubator (Forma, ThermoScientific, Asheville, NC, USA) in a humidified atmosphere at 37 °C and 5% CO₂.

Pancreatic islets were isolated from 3-month-old Wistar rats as described previously (Lenzen *et al.* 1983). The islets were washed and handpicked under a stereomicroscope. After an overnight culture, the islets were distributed into three batches and cultured at 37 °C in RPMI-1640 medium containing 10 mM glucose and 10% FCS with 0, 25 or 100 μ M progesterone for 48 h and were used in the subsequent experiments. The islets for caspase

3 activation and DCFDA oxidation determination were cultured on ECM-coated dishes for 10–14 days until formation of monolayer, and thereafter incubated with the test compounds then analysed.

Cell treatments

The cells were seeded the day before treatments into six-well plates for flow cytometric analyses (8×10^5 cells/well). Alternatively, pooled rat islets or rat islet-dispersed cells were used. Different concentrations of progesterone and/or oestriol were tested for 24 or 48 h. In order to evaluate the effect of α -tocopherol, the cells were pre-incubated for 2 h with this vitamin at a final concentration of 40 μ M, before addition of progesterone. The hormones, α -tocopherol and thapsigargin were dissolved in absolute ethanol. The final concentration of ethanol in the medium did not exceed 0.5%. Staurosporine was diluted in DMSO. Ethanol and DMSO, used as vehicles, did not affect the experimental results.

Cell proliferation assay

The effect of progesterone on cell proliferation was evaluated by using the BrdU *In situ* Detection kit (BD Bioscience, Heidelberg, Germany) according to the manufacturer's protocol. Briefly, the cells were seeded onto Nunc LabTek II CC2 chamber slides (Fisher Scientific, Schwerte, Germany), treated with test compounds and fixed overnight with fixation buffer. After fixation, the cells were washed three times with PBS for 5 min, followed by BrdU staining. Slides were thereafter mounted with Mowiol (Merck) plus 0.6% Dabco (St Louis, MO, USA). Images were captured and analysed using a Cell^R/Olympus BX61 inverted microscope system (Olympus, Hamburg, Germany). BrdU-negativity and BrdU-positivity were determined for 200–500 cells in each experiment. Four independent experiments were carried out. Data are expressed as the percentage of BrdU-positive cells for each treatment condition.

Cell membrane integrity assay

The cells were centrifuged at 400 *g* for 5 min at 4 °C and the pellet obtained was suspended in 500 μ l PBS. Thereafter, 50 μ l PI solution (50 μ g/ml in PBS) were added and the cells were analysed using a FACScalibur flow cytometer (Becton Dickinson, San Juan, CA, USA) (Nicoletti *et al.* 1991). Fluorescence was measured using the FL2 channel (orange-red fluorescence 585/42 nm). A total of 10 000

events were analysed per experiment. The cells with PI fluorescence were evaluated by using the Cell Quest software (Becton Dickinson).

For cell death evaluation of islets, after 48 h incubation with progesterone, rat pancreatic islets were disrupted by tryptic digestion and gentle pipetting. Living, apoptotic and dead cells were identified using the Guava ViaCount assay (Millipore, Billerica, MA, USA) based on the cytoplasmic and nuclear staining by two dyes. A membrane-permeant dye stained all nucleated cells, whereas a membrane-impermeant dye stained only damaged cells, thus indicating apoptotic and dying cells. Briefly, the cell suspensions were incubated with the ViaCount reagent for 5 min at room temperature. Data acquisition was carried out using a Guava EasyCyte flow cytometer (Millipore) and data analysis using the ViaCount software module.

DNA fragmentation assay

Apoptotic cells were evaluated by DNA fragmentation and loss of nuclear DNA content assays using the fluorochrome PI (Nicoletti *et al.* 1991). After trypsinisation, the cells were collected and incubated with a solution containing 0.1% Triton X-100. After incubation, the cells were centrifuged at 400 *g* for 5 min at 4 °C. The pellet was gently added to 300 μ l hypotonic solution, containing 50 μ g/ml PI, 0.1% sodium citrate and 0.1% Triton X-100. The cells were then incubated overnight at 4 °C and analysed by flow cytometry.

Islet cell apoptosis detection was carried out by using the Cell Death Detection ELISA PLUS kit (Roche Diagnostics), according to manufacturer's instructions, which is based on the measurement of the amount of histone-associated DNA fragments (mono- and oligonucleosomes). After cultivation of islets for 48 h in the presence of 0, 25 or 100 μ M progesterone, batches of 80 islets were lysed in 100 μ l lysis buffer, and the cytosolic and nuclear fractions were separated by centrifugation at 200 *g* for 10 min. The mean absorbance at 405 nm of each sample was normalised using the DNA content in the nuclear fraction, using a positive control available in the kit. Experiments were carried out three times in duplicates.

Determination of oxidative and nitrosative species generation

Cells (2×10^4) were seeded onto 96-well black plates and pre-incubated with 10 μ M dichlorofluorescein diacetate (DCF-DA) for 30 min at 37 °C. The medium containing the reagent was discarded, and fresh medium with different

concentrations of progesterone was added. The cells were cultured for 6, 12 and 24 h. After incubation, the plates were analysed at $\lambda_{\text{exc}}=480$ and $\lambda_{\text{em}}=520$ nm using the fluorescence reader Victor2 1420 Multilabel Counter (Perkin Elmer, Wiesbaden, Germany). Data were normalised to the number of viable cells and expressed as arbitrary units of DCF fluorescence.

For determination of oxidative stress by DCF fluorescence, rat islet cells were seeded onto ECM-coated dishes and pre-incubated with 10 μM DCFDDA for 60 min at 37 °C, followed by treatment with 25 or 100 μM progesterone for 48 h. Thereafter the cells were trypsinised and analysed using a CyFlow ML cytometer (Exc. 488 nm/Em. 520 nm) (Partec, Münster, Germany). A total of 10 000 events were counted. Data were analysed using FlowJo software (Tree Star, Ashland, OR, USA).

The superoxide content in islets was evaluated by flow cytometry (Guava EasyCyte, EMD Millipore Corporation, Billerica, MA, USA) through its reaction with hydroethidine (DHE), which generates the fluorescent product hydroxyethidium (Molecular Probes, Life Technologies, Carlsbad, CA, USA) (Zhao *et al.* 2005). Batches of 30 islets were preincubated for 30 min at 37 °C in Krebs–Henseleit solution (KH), pH 7.4 containing 10 mM glucose. After this period, DHE was added at a final concentration of 50 μM . The samples, protected from light, were then incubated for an additional 20 min at room temperature. The islets were disrupted by trypsin and gentle pipetting and resuspended in 200 μl RPMI-1640 medium. A set of 5000 events was evaluated per sample, and the yellow fluorescence was measured using an excitation wavelength of 488 nm. The emission signal was acquired with the yellow filter (583 ± 26 nm). The experiments were done in triplicates on three different days.

Caspase activity determination

Caspase activity in cell lysates was determined by fluorimetric assay using the substrate Abz-DEVDGVQ-EDDnp, as previously described by Nunes *et al.* (2005). Substrate (20 μM) hydrolysis was determined in 25 mM HEPES buffer, pH 7.4, containing 2.0 mM EDTA, 10 mM dithiothreitol, 10% sucrose, 0.1% 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate, 10 $\mu\text{g}/\text{ml}$ pepstatin A, 10 $\mu\text{g}/\text{ml}$ leupeptin and 100 μM phenylmethylsulphonyl fluoride (PMSF) (caspase buffer), at $\lambda_{\text{exc}}=320$ and $\lambda_{\text{em}}=420$ nm and for 3 h at 37 °C in a microtiter plate Synergy Biotek (BioTek Instruments, Inc Winooski, VT, USA). Enzyme activity was expressed as arbitrary units of fluorescence (AUF) per microgram of protein. All determinations were carried out in triplicates

in, at least, three individual experiments. Caspase activity was confirmed by incubating cell lysates with the caspase inhibitor Z-VAD-fmk (0.1 or 1 mM). Z-VAD-fmk efficiency was tested using purified recombinant caspase 3, as described by Nunes *et al.* (2003, 2005).

Discriminating specific caspase activity

Specific activities of caspases 3, 9 and 12 were determined after 24 h of incubation with various concentrations of progesterone, using red caspase 3 and green caspases 9 and 12 staining kits (PromoCell, Heidelberg, Germany) according to the instruction manual. After staining and washing, cell suspensions and dispersed islet cells were immediately subjected to analysis using a CyFlow ML cytometer (Partec). A total of 20 000 events were acquired. The number of cells with caspase activity was normalised to the number for untreated cells. Data were analysed by FlowJo software (Tree Star). A cytokine mixture consisting of 60 U/ml IL1 β , 185 U/ml TNF α and 14 U/ml IFN γ (PromoCell) was used as positive control.

Western blotting

For western blotting experiments, the cells were lysed with 25 mM HEPES, pH 7.4, containing 0.5% Triton X-100 and a cocktail of protease inhibitors (100 μM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ pepstatin, 5 mM EDTA). Homogenates were centrifuged at 12 000 g for 10 min and the supernatants were collected. The protein concentration was determined according to the method described by Bradford (1976), using BSA as the standard. Aliquots with 50 μg protein were freeze-dried, dissolved in 15 μl of sample buffer (300 mM Tris, pH 7.2, 2% SDS, 20% glycerol, 1.2% bromophenol blue and 3% β -mercaptoethanol) and boiled for 7 min. Extracted proteins were separated by 12% PAGE and electrotransferred onto nitrocellulose membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 2 h at 0.35 A at 4 °C. The membranes were blocked with 5% non-fat milk in 10 mM Tris buffer, pH 7.5, 150 mM NaCl and 0.1% Tween 20 (TBS-T) for 2 h and subsequently incubated with MABs anti-CREB-2 (1:200) and anti-CHOP (1:200). After an overnight incubation with the primary antibodies at 4 °C, the membranes were washed three times with TBS-T for 10 min. The HRP-conjugated anti-goat secondary antibody (anti IgG-HRP) was incubated at 1:1500 for 1 h at room temperature. β -tubulin was used as a loading and transfer control in all experiments. Bound proteins were visualised using a commercially available chemiluminescence kit (ECL, GE Healthcare) according to

the manufacturer's instructions. The membranes were then exposed to autoradiography films (Kodak), which were developed and scanned. Densitometric analyses were carried out using the freeware program CP Atlas 2.0 (lazarsoftware.com). Western blots representative of three independent experiments are shown.

Statistical analysis

Results are expressed as mean \pm S.E.M. Statistical analyses were carried out using one-way ANOVA followed by Tukey's test for multiple comparisons using the Prism analysis program (Graphpad, San Diego, CA, USA).

Results

Effects of progesterone on proliferation of insulin-producing cells

Incubation of RINm5F insulin-producing cells for 24 h with progesterone did not affect cell proliferation significantly (Fig. 1A). After a 48 h incubation, a significant decrease in proliferation of insulin-producing cells was observed in the case of a high concentration of 100 μ M, which is a concentration in the pharmacological range (Fig. 1A).

Effects of progesterone on death of insulin-producing and rat islets cells

Incubation of RINm5F cells with progesterone resulted also in a loss of cell membrane integrity at concentrations ranging from 50 to 100 μ M as shown in Fig. 1B. The effect was more pronounced after 48 h than after 24 h of incubation. About 60% of cells lost membrane integrity after 24 h and 90% after 48 h of incubation with 100 μ M progesterone. Staurosporine was used as a positive control for apoptotic cell death (Fig. 1B).

Progesterone also induced significant DNA fragmentation at a concentration range of 25–100 μ M in RINm5F cells (Fig. 1C). This effect was significantly stronger after 48 h comparing with after 24 h of incubation with the hormone (Fig. 1C).

In addition to our experiments using RINm5F cells as a model cell line for β -cells, we also carried out key experiments with islet cells. In general, rat islet cells presented a similar response to progesterone treatment. As shown in Table 1, 100 μ M progesterone induced apoptotic death of dispersed islet cells after 48 h incubation. This effect was specific because the rate of

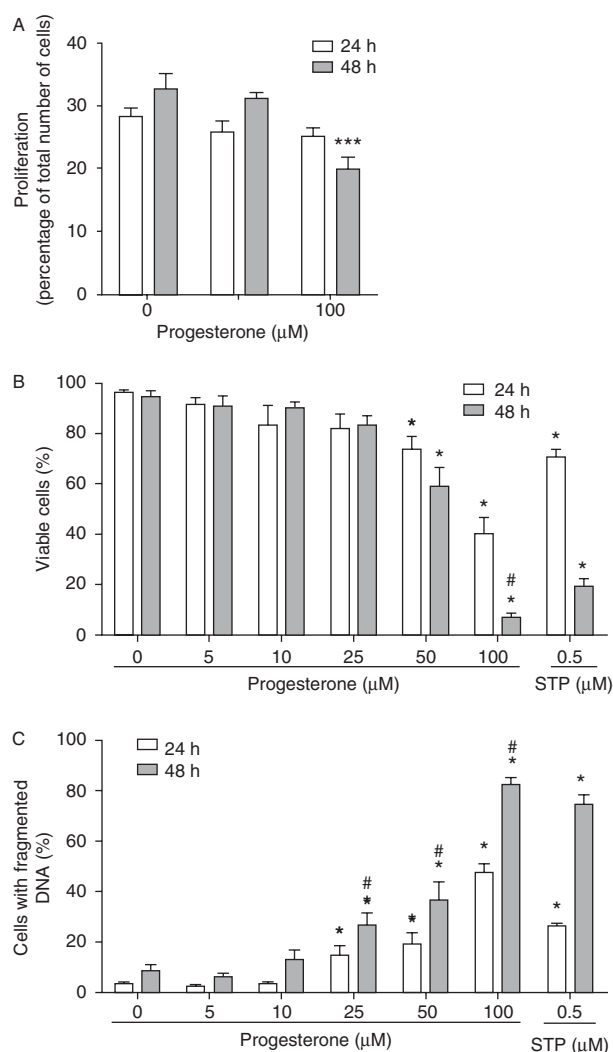


Figure 1 Concentration-dependent toxic effects of progesterone in RINm5F insulin-producing cells after 24 or 48 h of incubation. After incubation with progesterone, (A) cell proliferation; (B) cell membrane integrity and (C) DNA fragmentation were analysed. Data are presented as mean \pm S.E.M. of four to five experiments in triplicates. Differences were analysed for statistical significance by ANOVA followed by Tukey's test; * P < 0.05 compared with untreated cells, *** P < 0.01, P < 0.05 compared with the same treatment for 24 h. STP, staurosporine.

nonapoptotic cell death was not affected by progesterone treatment (Table 1). Progesterone at a final concentration of 25 μ M did not cause a significant induction of cell death. The apoptotic nature of cell death was confirmed by detection of DNA internucleosomal cleavage in islet cells incubated with 100 μ M progesterone for 48 h (Fig. 2A). The amount of mono- and oligonucleosomes was almost 20-fold higher in this group in comparison with the control group (Fig. 2A). Moreover, the measurement of caspase 3

Table 1 Effect of progesterone on rat islet cell death. $P < 0.05$.

Progesterone (μM)	Viable	Apoptotic	Dead (non-apoptotic)
0	92.2 ± 1.4	4.3 ± 1.0	3.5 ± 0.5
25	90.7 ± 1.2	4.6 ± 0.9	4.7 ± 0.5
100	$74.4 \pm 4.9^*$	$19.0 \pm 3.7^*$	6.6 ± 1.4

activity revealed a significant induction of this pathway by 100 μM progesterone after a 48 h incubation (Fig. 2B).

Effect of oestriol on viability of RINm5F β -cells

Oestriol was much less toxic to RINm5F cells than progesterone. After incubation for 24, as well as for 48 h, oestriol caused a significant reduction in cell viability (25%) only at the highest concentration (100 μM) (Fig. 3A). Incubation of cells with 25 μM oestriol and 100 μM progesterone strongly enhanced the loss of cell membrane integrity at both time points.

Oestriol (5–50 μM) did not cause significant DNA fragmentation after 24 or 48 h of incubation (Fig. 3B). Only the highest concentration of 100 μM caused a slight significant increase in the number of cells with fragmented DNA (Fig. 3B). The toxic effect of progesterone (100 μM) was significantly potentiated by oestriol (25 μM) (Fig. 3B).

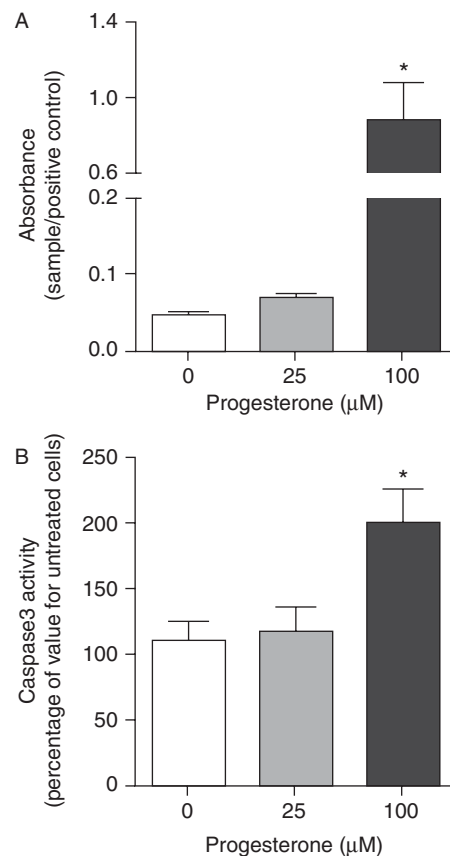
Looking for the mechanisms by which progesterone induced cell death

We evaluated the effect of progesterone in promoting caspase activation in RINm5F cells. Treatment of cells with progesterone resulted in a concentration-dependent increase in caspase activity as an indicator of apoptotic cell death (Fig. 4). Progesterone increased the activities of caspases 9, 12 and 3 (Fig. 5).

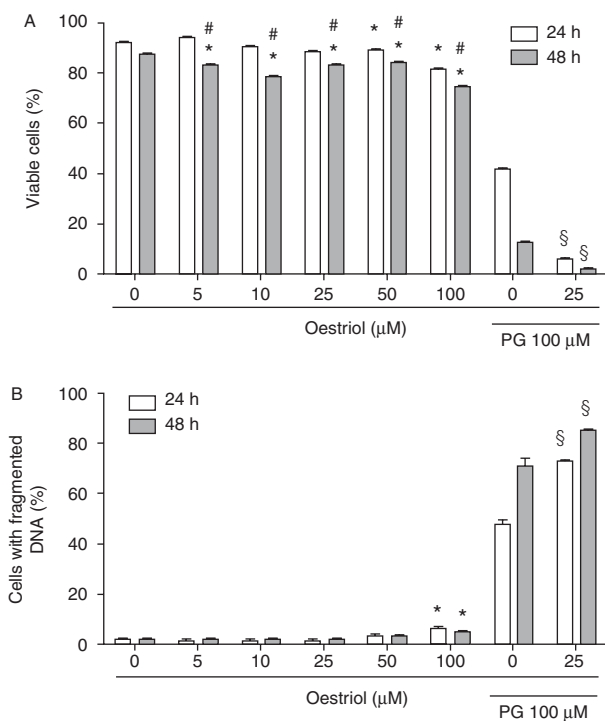
In order to investigate the effect of progesterone on generation of ROS, we used a fluorescence assay based on DCFDA oxidation. Significant production of ROS was observed after 12 h of incubation with 100 μM progesterone and after 24 h with 50 μM (Fig. 6). Similar effects were observed in dispersed rat islet cells, showing a significant induction of ROS formation after a 48 h incubation with 100 μM progesterone (Fig. 7A). We also evaluated the effect of progesterone on formation of superoxide radicals in disrupted islets cells of rat. Progesterone at a final concentration of 100 μM caused a significant enhancement of superoxide radical production, which was not evident with 25 μM progesterone (Fig. 7B).

In the following experiments, we investigated the potential protective effect of the dietary antioxidant α -tocopherol (40 μM) against progesterone toxicity. This treatment reversed cell membrane integrity loss (Fig. 8A) and DNA fragmentation (Fig. 8B) of RINm5F cells. At concentrations of progesterone up to 50 μM , the toxic effect was abolished and, even at the highest progesterone concentration of 100 μM , α -tocopherol (40 μM) strongly counteracted the toxic effect of progesterone after both 24 and 48 h of incubation.

To investigate the participation of proteins and transcription factors involved in the UPR on progesterone-induced cell death, we evaluated the expression,

**Figure 2**

Effects of progesterone on islet cell apoptosis. After 48 h of treatment with progesterone, batches of 80 rat islets were (A) lysed and the presence of mono- and oligonucleosomes in the cytosolic fraction was evaluated by using the Cell Death Detection ELISA PLUS kit; (B) trypsinised, and incubated with a staining kit and caspase 3 activation was measured by flow cytometry. (A) The mean absorbance of each sample was normalised to that of the positive control provided in the kit and corrected for differences in islet DNA content of the nuclear fraction. (B) Data were normalised to the value for untreated cells. Data are presented as mean \pm s.e.m. of four experiments made in duplicate. Differences were analysed for statistical significance by ANOVA followed by Tukey's test; * $P < 0.05$ vs untreated.

**Figure 3**

Concentration-dependent toxic effects of oestriol and progesterone (PG) in RINm5F insulin-producing cells after 24 or 48 h of incubation. After incubation with test compounds, (A) cell membrane integrity and (B) DNA fragmentation were analysed. Cells were incubated with progesterone and/or oestriol at different concentrations for 24 or 48 h. Data are presented as mean \pm s.e.m. of three to four experiments in triplicates. Differences were analysed for statistical significance by ANOVA followed by Tukey's test; * $P < 0.05$ compared with untreated cells, # $P < 0.05$ compared with the same treatment for 24 h, § $P < 0.05$ compared with treatment with progesterone alone at the same time point.

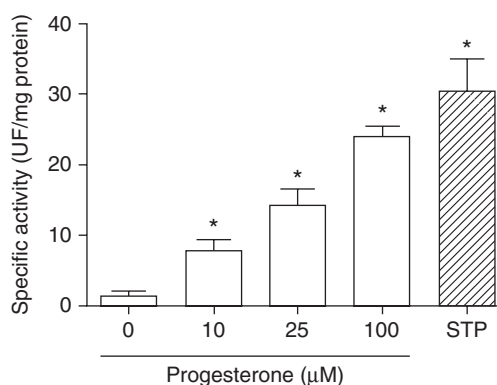
by western blotting, of CHOP and cAMP-response element-binding protein 2 (CREB2, also called ATF4). Thapsigargin, an inhibitor of sarcoplasmic/ER Ca^{2+} ATPases (SERCAs), which pumps Ca^{2+} into the ER (Nakagawa *et al.* 2000), was used as a positive control of ER stress, at concentrations that were able to induce cell death as assessed by flow cytometry (data not shown).

Our findings showed a concentration-dependent increase in the expression of CHOP and CREB2 after incubation of cells with progesterone (Fig. 9). Densitometric analyses revealed that CHOP expression (Fig. 9A) was sixfold and tenfold higher after incubation with 25 and 100 μ M progesterone, respectively, in comparison with untreated cells, when normalised to β -tubulin expression (Fig. 9A). These effects were observed at both time points (24 and 48 h) and expression was increased even more after the longer incubation period.

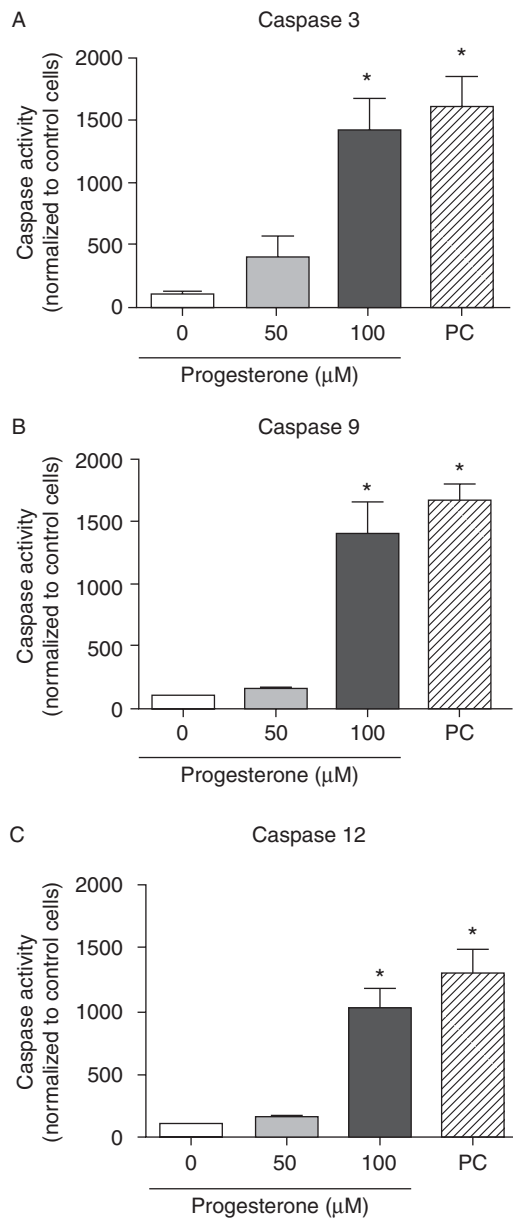
The CREB2 protein level in cells incubated with 25 or 100 μ M progesterone was 2.7 times higher than that for untreated cells (Fig. 9B). After 48 h of incubation with the same progesterone concentrations, an increase in CREB2 expression of 1.8- and 1.2-fold was observed, in comparison with treatment for 24 h. Oestriol alone did not affect CREB2 expression. However, in combination with 100 μ M progesterone for 48 h, the amount of this protein was increased compared with cells treated with progesterone alone (Fig. 9B).

Discussion

Although the diabetogenic potency of progesterone is well-known (for review, see Lenzen & Bailey (1984) and Butte (2000)), the present study provides new insights into the mechanisms of its direct action on insulin-producing cells under conditions that require pharmacological use of this hormone, such as for prevention of recurrent preterm delivery. Accordingly, it seems especially relevant to evaluate the potential effects of progesterone-based medications on mother and foetus. Considering the diabetogenic effect of progesterone, Rebarber *et al.* (2007) reported that the use of 17 α -hydroxyprogesterone caproate (17P), in the micromolar range, by women having a history of preterm delivery, increased the risk of development of GD. Another study involving women of hispanic descent with no previous history of diabetes showed that the use of an injectable progestagen-based contraceptive for long periods of time was associated with an increased

**Figure 4**

Concentration-dependent effects of progesterone on caspase activities of RINm5F insulin-producing cells after 24 h of incubation. Results were expressed as arbitrary units of fluorescence (UF) per minute per milligram of cytosolic protein. Data are presented as mean \pm s.e.m. of triplicates of three separate experiments. Differences were analysed for statistical significance by ANOVA followed by Tukey's test; * $P < 0.05$ different from untreated cells. STP, staurosporine.

**Figure 5**

Concentration-dependent effects of progesterone on caspases 3, 9 and 12 activities in RINm5F insulin-producing cells after 24 h of incubation. Data were normalised to values for control cells and expressed as mean \pm S.E.M. of triplicates of three separate experiments. Differences were analysed for statistical significance by ANOVA followed by Tukey's test; * $P < 0.05$ vs untreated. PC, positive control (cytokine mixture).

risk of developing GD (Xiang *et al.* 2006). Though another group did not find a correlation between progesterone use and an increasing risk of GD (Gyamfi *et al.* 2009), we have demonstrated in this study a direct effect of this hormone on the pancreatic β -cell line, RINm5F, and on rat islet cells causing apoptotic cell death, at concentrations ranging from 50 to 100 μ M which correspond to the

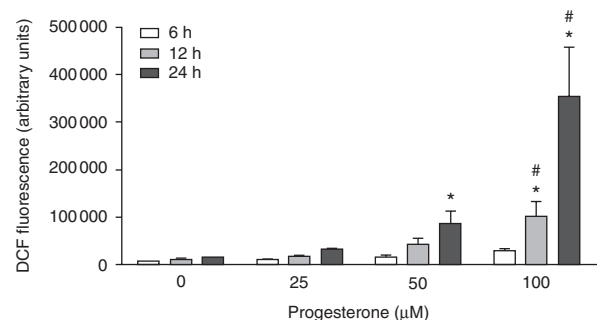
pharmacological concentrations currently used for the prevention of preterm delivery (Dodd *et al.* 2008).

It is well-known that during development of diabetes β -cells undergo apoptosis, causing impairment of insulin production and secretion. In this study, we showed that exposure of both insulin-producing cells and rat islets to progesterone resulted in caspase activation and DNA fragmentation, confirming the occurrence of apoptosis. Moreover, we could show that progesterone at high micromolar concentrations was able also to decrease the proliferation rate of insulin-producing cells.

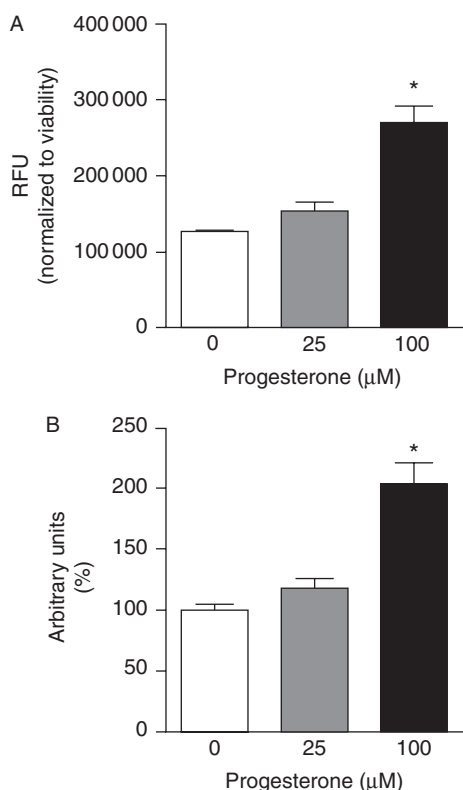
Studies investigating progesterone as a death inducer using other cell types are controversial. Candolfi *et al.* (2005) showed that progesterone is able to antagonise the apoptosis induced by TNF α in somatotrophs and lactotrophs. On the other hand, Cheng *et al.* (2006) obtained the opposite results using hepatoma HuH-7 cells, indicating that the beneficial or deleterious effects of this hormone may be tissue-specific. Though the mechanism of progesterone cytotoxicity is not completely clarified, the ability of progesterone to induce oxidative stress has been investigated (Verma & Rana 2008).

In fact, it has been shown that ROS and redox-dependent mechanisms can affect the process of apoptosis (Sen 1998). A relationship between ROS and apoptosis is supported by many experimental findings (Jabs 1999, Wei *et al.* 2000), and studies have reported that antioxidants can suppress or delay apoptosis by acting as scavengers of ROS in different systems (Zamzami *et al.* 1995).

We have shown that progesterone caused an augmentation of oxidative species generation in both RINm5F insulin-producing cells and rat islets, which may be related

**Figure 6**

Concentration-dependent effects of progesterone on reactive oxygen species (ROS) generation in RINm5F insulin-producing cells after 6, 12 or 24 h of incubation as measured by the increase in DCF fluorescence. Data are presented as mean \pm S.E.M. of three experiments in triplicates. Differences were analysed for statistical significance by ANOVA followed by Tukey's test; * $P < 0.05$ compared with untreated cells, # $P < 0.05$ compared with the 50 μ M dose at the same time point.

**Figure 7**

Effects of progesterone on oxidative stress and superoxide radical formation in rat islets. For measurement of overall oxidative stress (A) dispersed rat islet cells were preincubated with DCF-DA (10 μM) for 60 min, followed by incubation with progesterone for 48 h. For superoxide radical detection (B) cells were first treated for 48 h with progesterone followed by incubation with DHE (50 μM) for 20 min, and then dispersed using trypsin and gentle pipetting, and resuspended in 200 μl RPMI-1640 medium. A set of 5000 (DHE) to 10 000 (DCF) events were evaluated per sample by flow cytometry. Data are presented as mean \pm s.e.m. of three to four experiments in triplicates. Differences were analysed for statistical significance by ANOVA followed by Tukey's test; * $P < 0.05$ vs untreated.

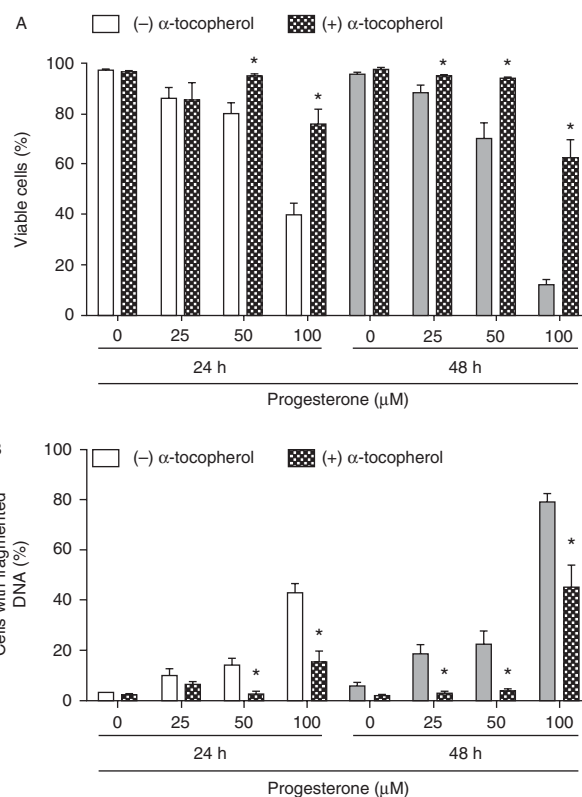
to the mechanisms by which progesterone exerts its effects on pancreatic β -cells.

Supporting the idea that antioxidants can suppress apoptosis induced by progesterone and based on the fact that pancreatic β -cells are very sensitive to redox changes (Lenzen 2008), which correlated with their low antioxidant enzyme activity (Tiedge *et al.* 1997), we found that α -tocopherol, a natural antioxidant, reduced or even abolished progesterone toxicity in insulin-producing cells, resulting in both reduced rates of cell death and small percentages of cells with fragmented DNA. However, we could show that oestriol, the main oestrogen in pregnancy (Reyes *et al.* 2006), was not able to protect β -cells against progesterone-induced cell death, as originally proposed. Rather, this hormone alone was able

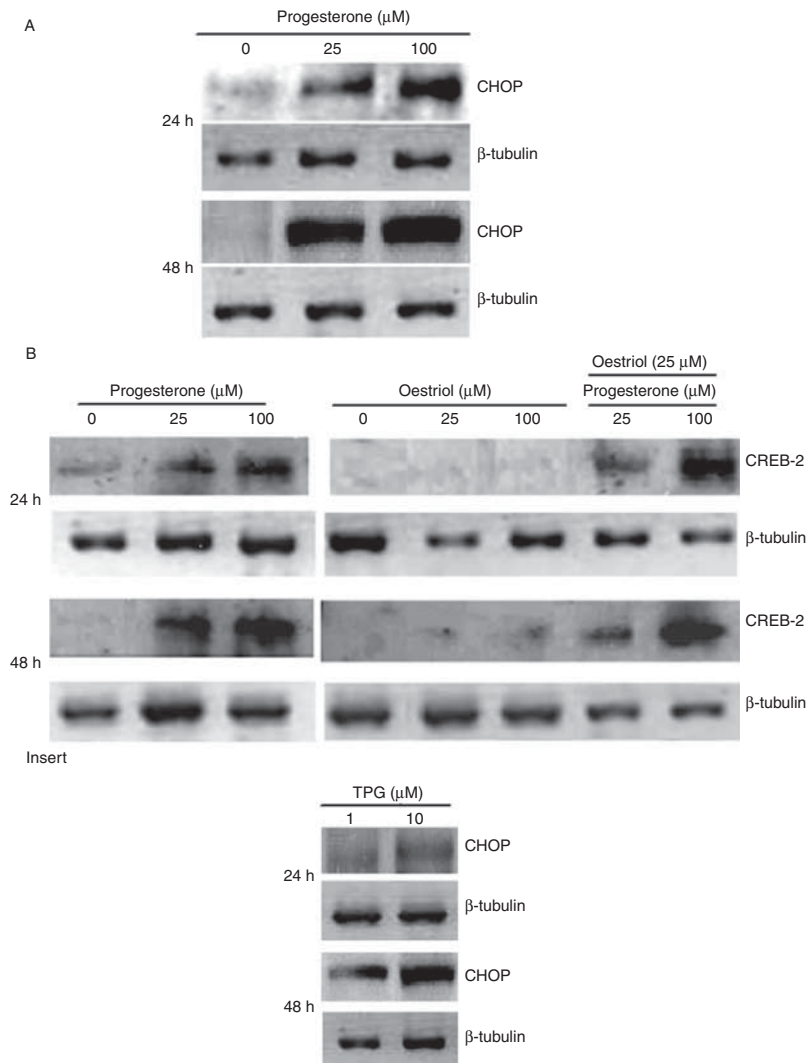
to induce cell death, when used at concentrations up to 25 μM, although it was much less toxic than progesterone. Furthermore, our results showed that oestriol is able to slightly enhance the effect of progesterone on RINm5F cell death.

The oxidative imbalance has been also recognised as a stimulus for ER stress, which affects the ability of the cells to properly fold proteins, and plays an important role in pancreatic β -cell death (Oyadomari & Mori 2004, Wu & Kaufman 2006, Eizirik & Cnop 2010).

A number of mechanisms have been proposed for connecting ER stress to apoptotic cell death, including the direct activation of proteases (Nakagawa *et al.* 2000, Momoi 2004), kinases (Ito *et al.* 2001), transcription factors, Bcl-2-family proteins and their modulators (Wei *et al.* 2001, Scorrano *et al.* 2003). Therefore, with certain stimuli, the activation of different signal transduction cascades takes

**Figure 8**

Protective effect of α -tocopherol (40 μM) against progesterone-induced membrane integrity loss and DNA fragmentation in RINm5F insulin-producing cells after 24 or 48 h of incubation. After incubation, (A) cell membrane integrity; (B) DNA fragmentation were analysed. Cells were preincubated for 2 h with α -tocopherol before incubation with progesterone for 24 or 48 h at different concentrations. Data are presented as mean \pm s.e.m. of four experiments in triplicates. Differences were analysed for statistical significance by ANOVA followed by Tukey's test; * $P < 0.05$ compared with PG treatment alone.

**Figure 9**

Immunodetection of proteins involved in UPR in progesterone and/or oestriol-treated RINm5F insulin-producing cells after 24 or 48 h of incubation. Cells were incubated with progesterone and/or oestriol with thapsigargin (TPG) at different concentrations for 24 or 48 h. Western blots representative of three independent experiments for (A) CHOP and (B)

CREB-2 are depicted. For both proteins, cells were treated for 24 and 48 h. This figure is composed of images of different gels, where samples of progesterone and oestriol-treated cells were run in the same experiment at each time point. β -tubulin expression was used as a loading control. Thapsigargin was used as a positive control for ER stress.

place in the ER lumen, collectively called the UPR. The UPR, which initially acts to counteract toxicity, can eventually trigger cell death if ER dysfunction is severe or prolonged (Rao *et al.* 2001). In this perspective, proteins downstream of all UPR pathways have been identified as having proapoptotic functions. An example is the proapoptotic protein CHOP, which is upregulated by the transcription factor activating transcription factor 4 (ATF4 or CREB2), and exclusively responsive to ER stress (Welihinda & Kaufman 1996). CHOP causes downregulation of the anti-apoptotic mitochondrial protein *BCL2* (Yoshida *et al.* 1998), favouring a proapoptotic pathway involving proteins that promote mitochondrial

damage, cytochrome *c* release and caspase 3 activation (Matsumoto *et al.* 1996, Allagnat *et al.* 2012).

In this scenario, we proposed the hypothesis that progesterone might trigger ER stress and UPR, thus contributing to pancreatic β -cell death. Accordingly, we studied the expression of CHOP and CREB2 in RINm5F cells in response to progesterone and/or oestriol exposure. Progesterone-induced CHOP protein expression increased in parallel to the hormone concentration up to ten times. Progesterone also increased the expression of CREB2 protein. Oestriol alone did not affect CREB2 expression; however, it slightly enhanced the upregulatory action of progesterone on RINm5F cells.

Activation of caspases observed after incubation of RINm5F cells with progesterone indicates that the mechanism of progesterone-induced cell death is by apoptosis. In this study we showed activation of different caspases, but particularly of the executioner caspase 3 as well as of the mitochondrial caspase 9, and of the ER-stress-related caspase 12.

In summary, the present data indicate that progesterone induces apoptosis of insulin-producing cells in tissue culture and primary β -cells in rat pancreatic islets. As progesterone potently induced ROS formation and the toxic effect was antagonised by the antioxidant α -tocopherol, it can be assumed that progesterone acts through a mechanism dependent on ROS generation, which may trigger ER stress and ultimately apoptotic cell death. Thus the results of this study provide new insights into the mechanisms underlying progesterone-induced death of β -cells, indicating that this hormone could contribute to development of GD when used under conditions where its use at pharmacological doses is recommended.

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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