LOCAL EFFECTS OF THE SPHINGOSINE 1-PHOSPHATE ON PROSTAGLANDIN F2ALPHA-INDUCED LUTEOLYSIS IN THE PREGNANT RAT

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Abstract

Since the regression of the corpus luteum (CL) occurs via a tightly controlled apoptotic process, studies were designed to determine if local administration of the antiapoptotic agent sphingosine-1-phosphate (S1P) effectively blocks the luteolytic action of PGF-2alpha (PGF-2α). On day 19 of pregnancy, 2 h before systemic PGF-2α administration, rats were injected intrabursa with either S1P or vehicle (control). The activity of four caspases, which contribute to the initial (caspase-2, -8 and -9) and final (caspase-3) events in apoptosis was measured in pooled CL from 4 individual ovaries at 0 h and 4 h after PGF-2α injection. The expression of the phosphorylated form of AKT (pAKT) and TNF-α was analyzed by ELISA. In addition, cell death was evaluated by electronic microscopy (EM) in CL 4 and 36 h after PGF-2α injection. The activity of caspase-2, -3 and -8 was significantly greater by 4h after PGF-2α but not caspase-9 activity. In contrast, expression of pAKT and TNF-α decreased significantly. Administration of S1P suppressed (p<0.05) these effects, decreasing caspase activities and increasing pAKT and TNF-α expression. The administration of S1P also significantly decreased the percentage of luteal apoptotic cells induced by PGF-2α. PGF-2α treatment increased the prevalence of luteal cells with advanced signs of apoptosis (i.e. multiple nuclear fragments, chromatin condensation or apoptotic bodies). S1P treatment suppressed these changes and increased the blood vessel density. These results suggest that S1P blocks the luteolytic effect of the PGF-2α by decreasing caspase-2, -3 and -8 activities and increasing AKT phosphorylation and TNF- α expression.

Keywords

Luteolysis; apoptosis; corpus luteum; PGF-2α; Sphingosine 1-P

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Introduction

The corpus luteum (CL) is a transient endocrine gland that forms from the remaining wall of the ovarian follicle after ovulation. The formation and regression of the CL requires rapid remodeling, growth, differentiation and death of its heterogeneous population of cells. This gland consists of not only steroidogenic cells (large and small), but also vascular endothelial cells, fibroblasts, smooth muscle cells and immune cells (Channing, 1969a; Channing, 1969b). After ovulation, vascular elements in the theca layer of the antral follicle invade the avascular granulosa layer to establish an extensive capillary network that nourishes the developing CL (Koos R.D., 1993; Hazzard and Stouffer, R. L., 2000). The CL's main function is to secrete the steroid hormone progesterone (P), which is essential for implantation of the blastocyst and maintenance of pregnancy (Bowen-Shauver and Gibori, G., 2004; Stocco et al., 2007; Stouffer, 2004). However, if pregnancy does not occurs, at the end of each ovarian cycle or when it is not required for maintenance of pregnancy, the CL ceases to produce progesterone and regresses in a process called luteolysis. This process occurs in two phases. The first one is known as functional luteolysis, which consists of the loss of its ability to produce P. The second and following phase termed structural luteolysis consists of the morphological regression/involution of the CL. Moreover, the integrity and function of the luteal vasculature declines during regression of the CL near the end of the cycle (Hazzard and Stouffer, R. L., 2000).

Prostaglandin F-2α (PGF-2α) has been established as the main luteolytic factor in several species, including rodents and domestic animals (Pharriss, 1971; Pharriss et al., 1972; Pharriss and Wyngarden, L. J., 1969). The actions of PGF-2α, include reduction of ovarian blood flow, uncoupling of the LH receptor from adenylate cyclase, reduction of steroidogenic enzyme activity and progesterone production, decrease of LH receptor concentration, changes in membrane fluidity, changes in luteal cell population, and increase in lysosomal enzyme activity (Niswender GD and Nett TM, 1994; Hansel and Blair, R. M., 1996; Milvae et al., 1996).

Apoptosis or programmed cell death has been related with luteal regression in many species, (Bacci et al., 1996; Bowen et al., 1996; Gaytan et al., 1998; Goodman et al., 1998; Guo et al., 1998; Hasumoto et al., 1997; Telleria et al., 2001; Yadav et al., 2005), but the precise cellular events associated with this mechanism are not well established. Signaling for apoptosis occurs through multiple independent pathways that are initiated either from triggering events within (intrinsic pathway) or from outside the cell (extrinsic pathway). All these pathways converge in a common machinery of cell destruction that usually involves the effector caspases, e.g., caspase-3. Members of the caspase family are cysteine aspartic acid-specific proteases that play a key role in the apoptosis, and their activation is a critical event in this process (Alnemri et al., 1996; Guo et al., 2002; Martin and Green, D. R., 1995; Nicholson, 1999). These proteases regulate the selective destruction of key proteins of the cell, such as PARP [poly (ADP-ribose) polymerase], lamin, fodrin (Chinnaiyan and Dixit, V. M., 1996; Cryns and Yuan, J., 1998; Thornberry and Lazebnik, Y., 1998). Several caspases have been identified within the CL in different species (Krajewska et al., 1997; Boone and Tsang, B. K., 1998; Rueda et al., 1999; Khan et al., 2000; Abdo et al., 2001; Peluffo et al., 2005; Peluffo et al., 2006; Yadav et al., 2005; Peluffo et al., 2007). Moreover,

there is evidence that PGF-2α plays an important role in regulating the activity of several members of the caspase family in the rat CL(Yadav et al., 2005; Peluffo et al., 2007).

The sphingolipid metabolites ceramide, sphingosine and the sphingolipid metabolite, sphingosine 1-phosphate (S1P) play and important role in the regulation of cellular fate. Ceramide and sphingosine usually inhibit proliferation and promote apoptosis, while the further metabolite S1P has been implicated in the protection from apoptosis and stimulation of angiogenesis (Cuvillier et al., 1996; Spiegel and Milstien, S., 2003a; Oyama et al., 2008). It has been described that ceramide and acid sphingomyelinase (the enzyme responsible for the conversion of sphingomyelin to ceramide) are potent inducers of apoptosis in luteinized granulosa cell (Pru et al., 2002). Concerning S1P, this is an important signaling molecule in ovarian cancer that regulates both growth and metastatic potentials of ovarian cancer cells (Xu et al., 1995; Xu et al., 1995; Xu et al., 1998; Hong et al., 1999; Xu et al., 2001; Schwartz et al., 2001). In rodents, intrabursa ovarian injection of S1P two hours before ionizing radiation prevents the massive follicular/oocyte destruction caused by irradiation, presumably by a S1P -mediated apoptosis inhibition (Morita et al., 2000; Hancke et al., 2007).

We hypothesized that, if apoptosis plays a key role in PGF-2α-induced luteolysis in rodents, then S1P would prevent PGF-2α induced effects and rescue the CL from regression. Therefore, studies were designed to analyze the activity of three initiator caspases (caspases-2, -8 and –9) and the main effector caspase-3, as well as morphological and ultrastructural features of apoptosis, during PGF-2α-induced luteolysis and after local administration of the antiapoptotic agent S1P during PGF-2α treatment. We also examined the expression and phosphorylation of the kinase AKT, which is a key intracellular mediator of signals for cell survival, proliferation, and angiogenesis. Also, the expression of tumor necrosis factor- alpha (TNF-α), a key cytokine involved in both death and cell survival, was analyzed.

Results

Indices of luteal structure-function after PGF-2α and S1P/PGF-2α treatments: corpus luteum ultrastructural morphology, PECAM-1 expression and progesterone luteal levels

Ultrastructural analysis of the CL by EM (Figure 1), revealed that most of the luteal cells at day 19 of pregnancy exhibited a healthy nucleus as well as, many lipid drops; but very few indices of apoptotic and non-apoptotic cell death (Figure 1, Panel A, B). As expected, PGF-2α treatment increased luteal cells with signs of advanced apoptosis (i.e. multiple nuclear fragments, chromatin condensation or apoptotic bodies) by 36h post-injection (Figure 1, Panel C, D). Administration of S1P 2 hours prior PGF-2α suppressed these changes and most of the cells presented healthy nucleus (Figure 1, Panel E, F). And interestingly, an increase in blood vessel density was also observed in this group. None of these changes were observed after 4 h of treatments (data not shown).

To further examine blood vessels in the CL, luteal sections from all groups were stained for the vascular endothelial cell marker PECAM-1 (Figure 2) (DeLisser et al., 1993). Immunohistochemistry analysis revealed that PECAM-1 was expressed in the CL at day 19

of pregnancy (Figure 2, Panel B), and that treatment with PGF-2α appeared to decrease its expression (Figure 2, Panel C, D). Nonetheless, treatment with S1P prevented this decrease, and in fact, stimulated the PECAM-1 expression in the CL (Figure 2, Panel E, F). Progesterone content was measured in isolated CLs in all the groups (C0h, PG4h, PG+S4h, PG36h & PG+S36h) to examine whether S1P could revert the expected decrease in progesterone levels after PGF-2α treatment (Figure 3). Administration of PGF-2α to pregnant rats at day 19 of gestation rapidly (4h) decreased luteal progesterone content ($p<0.05$) and levels remained low at 36h. Notably, S1P failed to prevent this effect of the PGF-2α by 4h post-injection. But, by 36 hours after PGF-2α administration S1P treatment maintained the luteal progesterone content at levels observed in the control group (C0h). Accordingly, we found a significant increase in progesterone content in the PG+S36h group in comparison to the PGF-2α groups (PG4h & PG36h) as well as, to the PG+S4h group ($p<0.05$).

Local effects of S1P on caspase (-2, -8, -9 and -3) activity and luteal cell death during Prostaglandin F 2α-induced luteolysis in the rat

Following PGF-2α administration at day 19 of pregnancy, the activity of the initiator caspases (-2, and -8), as well as the main effector caspase-3, significantly increased by 4 hours ($p< 0.05$; Figure 4, Panel A, C, B, respectively). Intrabursa administration of the S1P 2 hours prior PGF-2α injection prevented ($p<0.05$) at least in part this increase. However, there were no significantly changes for the activity of the initiator caspase-9 between groups (Figure 4, Panel D). Although treatment with PGF-2α tended to increase caspase-9 activity, this effect was not statistically significant ($p>0.05$).

TUNEL analysis (Figure 5, Panel A) showed the presence of few cells with nuclear DNA fragmentation in the rat CL at day 19 of pregnancy. By 4 hours after PGF-2α administration, the number of cells showing advanced signs of apoptosis significantly increased ($p<0.05$). As expected, administration of the antiapoptotic agent S1P prevented this effect and significantly decreased ($p<0.05$) the percentage of apoptotic cells in the rat CL induced by PGF-2α administration (Figure 5, Panel B). Cells exhibiting TUNEL staining were expressed as the percentage of apoptotic cells in each group. Staining was not found in the negative control without the TdT enzyme (Panel A), nor in the surrounding stroma (not shown).

Stimulation of protein levels of TNF-α and the phosphorylated form of AKT by S1P on a Prostaglandin F2α-induced luteolysis model

Administration of PGF-2α almost abolished the luteal expression of TNF-α by 4 hours post injection (Figure 6, Panel A). In contrast, S1P treatment prevented ($p<0.05$) the effects of PGF-2α on TNF-α levels. At day 19 of pregnancy, PGF-2α treatment modestly but significantly decreased ($p<0.05$) the levels of the phosphorylated form of AKT (Figure 6, Panel B) in the CL. However, administration of S1P 2 hours prior PGF-2α injection prevented this decrease and increased levels to above those in controls ($p<0.05$, C4h).

Western blot of pAKT and AKT was used to determine whether protein expression of AKT was not modified by PGF-2α or S1P. Densitometric quantification of pAKT/AKT shows

similar results to those obtained using ELISA (data not shown), suggesting that the increase in the phosphorylated form of AKT is related with changes in the phosphorylation/ desphosphorylation rather than protein synthesis/turnover.

Discussion

The caspase measurements in this study showed that the activity of caspase-2, -3 and -8 was significantly greater by 4h after PGF-2α administration. However caspase-9 activity was unchanged, indicating that caspase-9 may be not essential or even necessary for prostaglandin F2alpha-induced luteolysis in the pregnant rat. In fact, these results are in accordance with results obtained from Yadav et al (Yadav et al., 2005). Although caspase-9 activity was not influenced by PGF-2α in the rat CL, the intrinsic apoptotic pathway cannot be discarded, as caspase-2 can link both the extrinsic death receptor and the intrinsic mitochondrial pathways through the cleavage of a member of the Bcl-2 family, Bid (Guo et al., 2002). Besides, there it could also be due of time, dose or lack of pulsatile secretion of this hormone. Therefore, physiologic luteal regression may involve the cooperation of both extrinsic and intrinsic apoptotic pathways. Several studies demonstrate the presence of the extrinsic pathway in the luteal cells (Abdo et al., 2003; Carambula et al., 2003; Yadav et al., 2005; Kuranaga et al., 1999), although there are also data supporting the involvement of the intrinsic mitochondrial pathway in luteal cell death (Goyeneche et al., 2006; Papadopoulos et al., 1999).

Interestingly, local treatment with S1P 2 hours prior PGF-2α could revert this increase in caspase-2, -3 and -8 induced by PGF-2α at least in part, tending to protect the CL from the caspase cascade. In agreement with caspase activities, the percentage of apoptotic cells significantly increased by 4 hours after PGF-2α administration. And, the administration of S1P significantly decreased the percentage of apoptotic cells in the rat CL induced by PGF-2α administration. S1P is a bioactive sphingolipid metabolite formed by the phosphorylation of sphingosine by sphingosine kinase. It is implicated in many biological processes and it is well known as an antiapoptotic agent (Spiegel and Milstien, S., 2000; Payne et al., 2002; Alvarez et al., 2007). Besides, ceramide can be metabolized via ceramidase to S1P and in some cell types the dynamic balance of S1P and ceramide acts as a 'cellular rheostat' that determines the fate of the cell (Payne et al., 2002). S1P was shown to inhibit the activation of effector caspases (eg. caspase-3, -6, and –7) in human leukemia cells, by counteracting the release of mitochondrial proteins, cytochrome c and Smac/ DIABLO induced by a variety of different factors (e.g. Fas monoclonal antibody, serum deprivation, or exogenous ceramides) (Cuvillier and Levade, T., 2001). Intrabursa ovarian injection of S1P two hours before ionizing radiation prevents the massive follicular/oocyte destruction caused by irradiation, presumably by a S1P -mediated apoptosis inhibition (Morita et al., 2000). However, this is the first study that describes the effect of S1P on apoptosis and different caspase activities in the corpus luteum. Experiments are in progress in our laboratory using sphingosine kinase inhibitors to demonstrate if sphingosine plays a role on the physiological luteolytic process associated with late pregnancy.

Surprisingly, PGF-2α treatment at day 19 of pregnancy almost abolished the luteal expression of TNF-α by 4 hours post injection. S1P reversed its expression in the CL,

suggesting that S1P pre-treatment is able to prevent prostaglandin effect. These results differ from the results obtained from Henkes et al 2008, where PGF-2α treatment causes a significant increase in ovarian TNF-α during CL regression in pseudopregnant mice (Henkes et al., 2008). Differences may be due on the different experimental models or PGF-2α protocols, since there are reports that suggest TNF-α can initiate luteotropic or luteolytic effects depending on the stage of the cycle, species or local milieu in the CL (Davis and Rueda, B. R., 2002). It is important to consider that the binding of TNF-α to its receptor can trigger the survival of the cells through the activation of the nuclear factor-kappa B (NF-κB) (Wang et al., 1996; Beg and Baltimore, D., 1996; Wu et al., 1996). The activation of NF-κB by TNF-α and the expression of adhesion molecules requires Sphingosine Kinase (SphK) and these events are crucial for the protection of endothelial cells against the apoptotic effects of TNF-α (Xia et al., 1998; Xia et al., 1999). S1P can stimulate several transcription factors that have been implicated in cell growth regulation and protection against apoptosis, including NF-κB (May and Ghosh, S., 1999). Also, there may be a cross-talk between TNF-α and S1P receptors (Alvarez et al., 2007). Accordingly, in our model of PGF-2α-induced luteolysis in pregnant rats, S1P may be stimulating its own synthesis and survival of the cell by stimulation the expression of TNF-α, and possibly the activation of NF-κB. TNF-α is primarily expressed in the monocytes or macrophages, but it is also found in the endothelial cells of the CL (Zhao et al., 1998; Petroff et al., 1999). Interestingly, immune cells, as well as cytokines, play a role in regulating luteal function in rats, mice, cows and women (Brannstrom and Friden, B., 1997; Friedman et al., 2000; Terranova and Rice, V. M., 1997).

It is well known that PI3K/AKT is a pathway involved in cell survival and proliferation. PI3K/AKT signaling was shown to play a key role in regulating cell cycle progression by promoting G1-S phase transition. AKT can block cell-cycle inhibitors and stabilizes proteins that promote cell-cycle progression (Gao et al., 2003; Gao et al., 2004; Engelman et al., 2006; Jiang and Liu, L. Z., 2008). In this study, we investigated whether PGF-2α may exert its luteolytic role and promote apoptosis by regulating this pathway. Treatment with PGF-2α at day 19 of pregnancy significantly decreased the levels of the phosphorylated form of AKT at Ser 473. In addition, administration of S1P 2 hours prior PGF-2α injection reverted this effect. These results suggest that PI3K/AKT signaling pathway is inhibited by PGF-2α in CL from pregnant rats and the antiapoptotic agent S1P was able to reverted this effect. Accordingly, in other system it was demonstrated that exogenous administration of S1P was found to activate AKT and partially rescued the cells from the apoptosis (Osawa et al., 2001).

We further investigated whether ultrastructural CL parameters were altered by PGF-2α or PGF-2α /S1P treatments. Analysis of the CL by EM, revealed that most of the luteal cells at day 19 of pregnancy presented healthy nucleus as well as, many lipid drops; but very few indices of apoptotic cell death. As expected, PGF-2α increased luteal cells with advanced signs of apoptosis (i.e. containing multiple nuclear fragments, chromatin condensation or apoptotic bodies) by 36h post-injection. S1P treatment, whereas suppressed these changes and most of the cells presented healthy nucleus. Interestingly, an increase in blood vessel density was also observed in this group. It is important to note that in addition to its antiapoptotic effects, S1P is also an angiogenic factor comparable to basic fibroblast growth

factor (FGF-2) and VEGF in promoting vessel growth, and synergizes with these factors to promote development of vascular network in vivo (Igarashi et al., 2003; Spiegel and Milstien, S., 2003b; Balthasar et al., 2008; Ferrara et al., 2006). In previous studies (Abramovich et al., 2006; Parborell et al., 2008) we have demonstrated that angiogenic factors have an important role in the processes of follicular selection, dominance and atresia mediated by apoptosis. The present results show that PECAM-1, a specific protein for microvascular endothelial cells, was expressed in the CL at day 19 of pregnancy, and that treatment with PGF-2α vastly decreased its expression. Nonetheless, treatment with S1P reverted this decrease, and in fact, stimulated the PECAM-1 expression in the CL, suggesting that endothelial cell survival may be involved in the antiapoptotic effect of S1P. Furthermore, S1P receptors are expressed in vascular endothelial cells (Chae et al., 2004; Sanchez et al., 2007; Sanchez and Hla, T., 2004; Maines et al., 2006). Therefore, the blockage of luteolysis by S1P may be due to the stimulation of angiogenesis, an inhibition of luteal apoptosis, or a combination of both. Further information is needed to elucidate the relevance of the changes observed in the vasculature after S1P treatment.

Luteal progesterone content measurements reflected the ultrastructural changes observed by EM, since administration of PGF-2α to pregnant rats rapidly decreased progesterone content and by 36 hours after PGF-2α administration S1P treatment could recover the luteal progesterone content. Unexpectedly, S1P failed to prevent this effect of the PGF-2α by 4h post-injection. It was suggested that the reduced ability of luteal cells to produce progesterone can induce cell death (Liszewska et al., 2005). However, in our experimental model one could hypothesize that functional luteolysis occurs by 4 hours after PGF-2α administration involving changes in the activity of steroidogenic enzymes able to be reverted after a prolonged exposure to S1P.

In summary, the results reported here represent the first demonstration that in vivo intrabursal administration of the antiapoptotic agent S1P itself effectively blocks the luteolytic action of PGF-2α in pregnant rats by decreasing caspase-2, -3 & -8 activities, increasing the phosphorylation of AKT and TNF-α and PECAM-1 expression.

Materials and methods

Animals and Protocols

Adult female Sprague-Dawley rats received food and water ad libitum and were housed at room temperature in a range of 21–23°C in a 12L:12D cycle. Animals were sacrificed by CO2 asphyxiation. The ovaries were removed and cleaned prior to subsequent assays. All experimental protocols were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the OHSU (Oregon Health and Science University) IACUC (Institutional Animal Care and Use Committee) and the IBYME (Instituto de Biología y Medicina Experimental) ethical committees. Stages of the estrous cycle were determined by daily examination of vaginal cytology, and rats were mated overnight at proestrus.

Day 1 of gestation was designated as the morning on which spermatozoa were found in a vaginal smear. Pregnant animals were divided in three main groups (n=4 rats per group).

Control untreated group (C0h): Rats were untreated at day 19 of pregnancy. Control PGF-2α group (PG4h & PG36h): Anesthetized rats on day 19 of pregnancy were injected with the S1P vehicle PET (5% PEG, 2.5% ETOH, 0.8% Tween 80) into the bursa of both ovaries (10 μl/ovary) and 2 hours later they were injected IP with one dose of PGF-2α (400 μg/rat, Biogenesis, Bs As, Argentina). PGF-2α +S1P Group (PG+S4h & PG+S36h): Rats on day 19 of pregnancy were injected with S1P into the bursa of both ovaries (10 μg/ovary, Sigma Chemical Co., St. Louis, MO), and 2 hours later they were also injected IP with one dose of PGF-2α.. Ovaries were collected at specific times (0, 4, and/or 36 hours) after PGF-2α administration in both Control PGF-2α and PGF-2α +S1P Group. Day 19 of pregnancy is selected for PGF-2α treatment since this is the time when the CL becomes more sensitive to PGF-2α action (Bussmann, 1989).

Corpora lutea were dissected from ovaries under a stereoscopic microscope as previously described in our laboratory (Andreu et al., 1998). Pools of CL (n=5–6 CL for each rat), were frozen in liquid nitrogen and stored at −80 °C for protein isolation and steroid extraction. 0h and 4h timepoints for protein isolation and 0h, 4h and 36h for steroid measurement. For ultrastructural morphology analysis by electronic microscopy (EM), CLs (n=3–4 per group from different rats) were fixed in glutaraldehyde buffer. Also, half of one ovary was fixed in formalin to evaluate PECAM-1 expression, as well as, apoptosis. Samples at 0h, 4h and 36h post PGF-2α administration were used for ultrastructural analysis of the CL morphology and to analyze PECAM-1 expression and only 0/4h for apoptosis (TUNEL assay).

Electronic Microscopy

Isolated CLs were fixed for two hours by immersion in 2.5 % (w/v) glutaraldehyde prepared in 0.1 M phosphate buffer, pH 7.4. Then, samples were washed with 0.1 M phosphate buffer, pH 7.4; and distilled water. After rinsing, samples were post-fixed with 1.5 % osmium tetroxide for 90 minutes, washed with distilled water, and dehydrated with 50 % ethanol for 10 minutes. Prior dehydration, the samples were pre-stained overnight with 1 % uranyl acetate in 70 % ethanol. The samples were then dehydrated with graded ethanol solutions followed by infiltration in propylene oxide and embedding in Epoxy Resin (Durcupan ACM, SPI # 02809, Structure Probe, Inc./SPI Supplies, PA, USA). Ultrathin sections (700 nm thickness) were cut from the blocks using a Reichert-Jung ultramicrotome, collected on 300 mesh copper grids and post stained with Reynolds lead citrate solution (Reynolds, 1963). Ultrathin sections were then photographed with a Carl Zeiss 10C electron microscope at 80 kV using a 35 mm Kodak 5302 film.

PECAM-1 Immunohistochemistry (IHC)

Ovaries from the different groups (C0h, PG4h, PG+S4h, PG36h & PG+S36h) were fixed in 10% neutral buffered formalin for 1 week. Then tissues were dehydrated in a series of ethanol solutions (50, 70 and 100%) and paraffin-embedded. Four μm sections were deparaffinized and hydrated through xylene and a graded series of ethanol. Endogenous peroxidases were then quenched with 25-min incubation in 3% H_2O_2. Sections were incubated in phosphate-buffered saline (PBS) prior to antigen retrieval (0.1% Triton X-100 in PBS for 30 min plus a 30 min incubation in Proteinase K, 20 μg/ml). Sections were placed in a blocking buffer (2% BSA in PBS) for 20 minutes, and then incubated with the primary

antibody anti-PECAM-1 (cat # 550300, CD 31 purified mouse anti rat cd31 pecam-1 monoclonal, BD Pharmingen, CA, USA) in a concentration of 1:100 in PBS buffer for 1 h at room temperature and overnight at 4°C. Primary antibody was detected using a biotinylated anti-mouse IgG secondary antibody (1:400; Vector Laboratories, Burlingame, CA, USA) and the Vector ABC-Elite Kit, visualized with DAB (diaminobenzidine) with a metal enhanced substrate as described by the manufacturer (1718096, Roche, Penzberg, Germany), and counterstained with hematoxylin. A negative control lacking primary antibody were processed on adjacent tissue sections.

Luteal Progesterone Levels

Steroids were extracted as previously described in our laboratory (Irusta et al., 2003). Progesterone content in CL was measured in rats from each group (n=3–4 rats per group; C0h, PG4h, PG+S4h, PG36h & PG+S36h). A pool of 3–4 CLs was used to represent luteal progesterone content in each rat. CLs were homogenized in acetone with Ultra-Turrax (IKA Werk, Breisgau, Germany). Known quantities of labeled progesterone (3H-progesterone) in acetone (approximately 3000 cpm), were added to each sample as internal standard. An aliquot was taken from each homogenate for protein measurement. After homogenization, the samples were centrifuged (1,600 x g for 10 min) and the resultant supernatant was evaporated to dryness. Following the addition of distilled water and vortexing, the samples were twice extracted with diethyl ether and the upper ether phase was transferred to conical tubes and again evaporated to dryness. The remaining residue was dissolved in methanol and, after adding distilled water, the samples were submitted to a solvent partition with n-hexane and dichloromethane and the upper layer was discarded and the lower phase evaporated. Finally, samples were stored in distilled water for later analysis by radioimmunoassay (Irusta et al., 2003) and each sample was tested in duplicate.

Caspase-2, -3, -8, and -9 Activity Assays

From each rat (n=3–4) a pool of CL per ovary (C0h, PG4h & PG+S4h) was used to evaluate enzyme activity (n=3–4 ovaries/per group). A fluorometric assay kit (630225, Clontech, CA, USA), which contains fluorogenic substrates specific for different caspases (-2, -3, -8 and -9), immobilized in separate wells, was used following the procedure previously described by our laboratories (Peluffo et al., 2005; Peluffo et al., 2006). Twenty μg (caspase-2 and -3) or 40 μg (caspase-8 and -9) of the extracted protein from each pool of CL was added in homogenization buffer to the wells and each concentration was tested in duplicate. The plate was incubated in the fluorescence plate reader at 37° C for 3 hours and fluorescence was read every 10 minutes. The activity was determined by fluorometric detection (Ex: 380nm, Em: 460nm) and the negative control (blank, without sample) was subtracted from all the samples. The two-hour incubation point was selected for analyses because it was located on the linear section of the activity curve. Baseline values of negative controls and samples with specific caspase inhibitors did not increase during the 2 hr interval.

Apoptotic Cell Analysis (TUNEL)

Serial 4 μm sections of paraffin-embedded fixed ovaries (n=3–4) from the different groups (C0h, PG4h & PG+S4h) were used. Nuclear DNA fragmentation in luteal cells was detected using the DeadEnd Colorimetric TUNEL System (G7130, Promega, Madison, WI, USA),

following the manufacturer's instructions with minor modifications as previously described (Peluffo et al., 2005; Peluffo et al., 2006). A microscope with a 100X objective was used and three randomly selected fields were analyzed from each CL section (3 CLs per section/ovary, 3–4 ovaries per stage). Results were expressed as the percentage of apoptotic cells.

TNF-α ELISA

Concentrations of TNF-α in homogenized CL from the different treatment groups (C0h, PG4h & PG+S4h) were quantitated by enzyme-linked immunosorbent assay (Quantikine Rat TNF-alpha/TNFSF1A immunoassay, Cat # RTA00, R&D Systems Inc., MN, USA). Based on the manufacturer's instructions, 120 μg of the extracted proteins from the CL were added into the plate (n=3–4 ovaries/per group), where a monoclonal antibody specific for rat TNF-α was pre- coated onto each well, and incubated for 2 hours at room temperature. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat TNF-α was added to the wells and the plate was incubated for 2 hours at room temperature. Then, following a wash, a substrate solution was added to the wells and the plate was incubated in the dark 30 minutes at room temperature, and the reaction ended by adding a stop solution. The intensity of the color measured was in proportion to the amount of rat TNF-α bound in the initial step. The TNF-α protein levels were determined by absorbance, at 450 nm, against a standard curve. Each sample was tested in duplicate and negative controls (blanks, without sample) were subtracted.

pAKT ELISA

An ELISA kit was used to detect and quantify the level of Akt protein that is phosphorylated at serine residue 473 (CBA005, Calbiochem) in CL from the different groups (C0h, PG4h & PG+S4h) was used following the manufacturer's procedure. Similar to the caspase activity assay, a pool of CL from each ovary was used to evaluate the levels of the phosphorylated form of AKT (n=3–4 ovaries/per group). Thirty μg of the extracted protein from each pool of CL diluted in stardand diluent was added into the wells, that were coated with a monoclonal antibody specific for Akt1 (regardless of phosphorylation state), and the plate was incubated at room temperature for 2 hours. After washing, a rabbit antibody anti-Akt (pSer473) was added to the wells and the plate was incubated for 1 hour at room temperature. After removal of excess detection antibody, a horseradish peroxidase-labeled anti-rabbit IgG (anti-rabbit IgG-HRP) was added and the plate was incubated for 30 minutes at room temperature. Then, after rinsing a stabilized chromogen was added, and the plate was incubated in the dark for 30 min at room temperature, and the reaction ended by adding a stop solution. The AKT (pSer473) levels were determined by absorbance, at 450 nm, against a standard curve. Each sample was tested in duplicate and the plate was blanked against a reaction tube containing only the stabilized chromogen and the stop solution.

Western blotting

After boiling for 5 min, extracted proteins from the different CL groups (40 μg protein, C0h, PG4h & PG+S4h) were separated by 10% SDS PAGE and transferred to a nitrocellulose membrane using an electroblotting apparatus as previously described (Peluffo et al., 2007; Abramovich et al., 2009). Non-specific binding sites were blocked overnight (ON) in TBS (4 mM Tris-Cl, pH 7.5, 100 mM NaCl) containing low-fat powdered milk (5%) and Tween

20 (0.5%) at 4°C. The membranes were then incubated overnight with the appropriate anti-rabbit primary antibodies (AKT: Cat # 9272 Cell Signaling, Boston, MA or phospho AKT, phosphorylated at serine residue 473: Cat # 7985 Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized by incubating the blots for 1 hour with peroxidase-conjugated secondary anti-rabbit IgG (1:1000, Cat # A4914 Sigma Chemical Co., St. Louis, MO). Finally, the membranes were incubated with Western Lightning. Chemiluminescence Reagent Plus (cat # NEL104, PerkinElmer Life Sciences, Inc. Boston, MA) for 1 min and exposed to X-ray film in a dark room. Protein expression was quantified by densitometric analysis using Scion Image Software for Windows (Scion Corporation, Worman's Mill, CT). Consistency of protein loading was evaluated by staining the membranes with Ponceau-S and the density in each specific band was normalized to the density of an internal control, GAPDH (1:10000, Cat # 2118 Cell Signaling Boston, MA).

Statistical Analysis

Statistical analyses were performed using the ANOVA Test for comparison between timepoints followed by Student-Newman-Keuls method using the Sigma Stat software package (SPSS, Chicago, IL). Differences were considered significant at $p < 0.05$. Percentage data were analyzed using the ANOVA Test (non parametric, Kruskal-Wallis statistic) for comparison between timepoints followed by Dunn's Multiple Comparison method.

Acknowledgments

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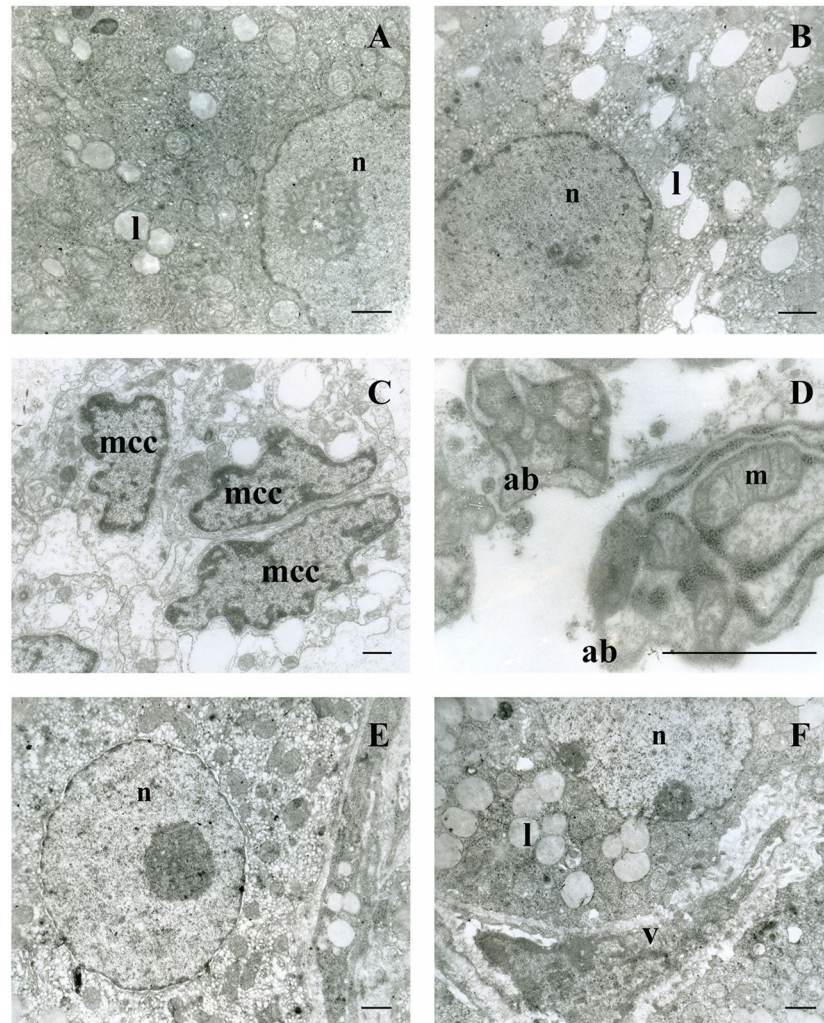
Figure 1.
Ultrastructural features of the rat CL after 36 h of PGF-2α (PG) administration. Rats were intrabursa treated with S1P (S) (PG+S36h) or the vehicle PET (PG36h), two hours prior PGF-2α injection. C0: Rats were untreated at day 19 of pregnancy. Photomicrographs showing ultrastructural aspects of the corpus luteum obtained from the different groups: C0h (A–B), PG36h (C–D) and PG+S36h (E–F). Typical morphological features of apoptosis evident after PGF-2α treatment: the presence of multiple nucleus with marginated and condensed chromatin, as well as the presence of apoptotic bodies; whereas not in the control group or the S1P group. For all: n =nucleus; m =mitochondrion; ab=apoptotic body; l = lipid droplets; mcc=marginated and condensed chromatin; v=blood vessel. Each bar represents 1µm in all the panels.
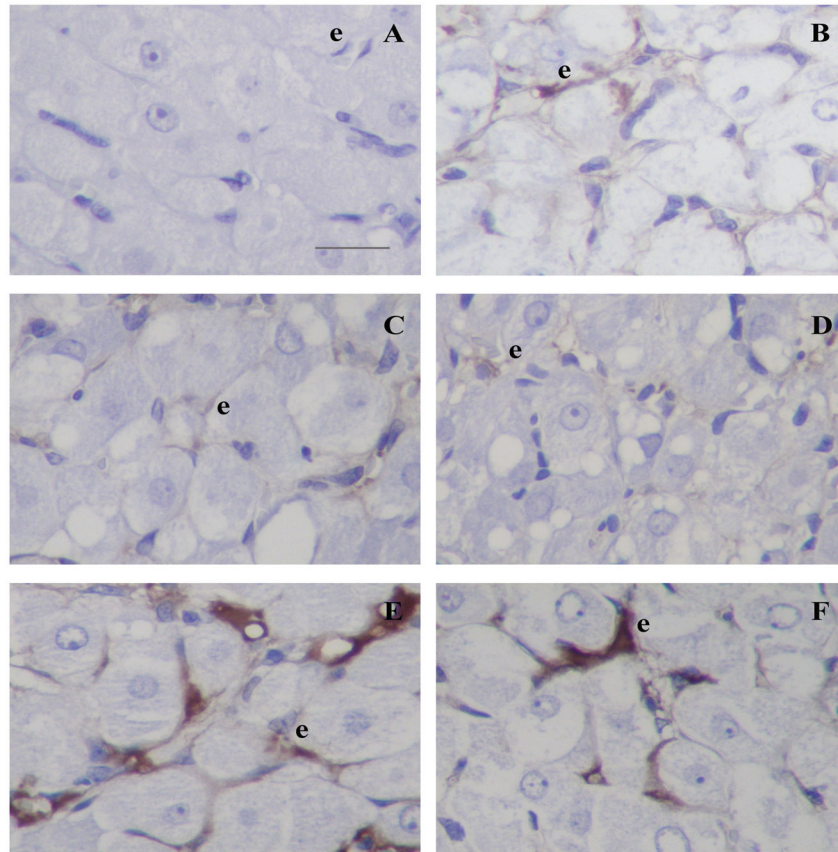
Figure 2.
Immunohistochemistry for PECAM-1 in the rat CL after 4h or 36 h of PGF-2α (PG) administration. Rats were intrabursa treated with S1P (S) (PG+S4h or S36h) or the vehicle PET (PG4h or PG36h), two hours prior PGF-2α injection. C0h: Rats were untreated at day 19 of pregnancy.

Microscope images from C0h (B), PG4h (C), PG36h (D), PG+S4h (E) and PG+S36h (F). Compared to the negative control incubated without the primary antibody (A). Staining was evident in the endothelial cells (e), but not in the luteal cells (small and large), nor in the stroma. Scale bar = 20 μm. PECAM-1was highly expressed in the CL of the C0h, PG+S4h and PG+S36h samples. While, little or no expression of PECAM-1 was detected in the CL after PGF-2α administration (PG4h and PG36h).
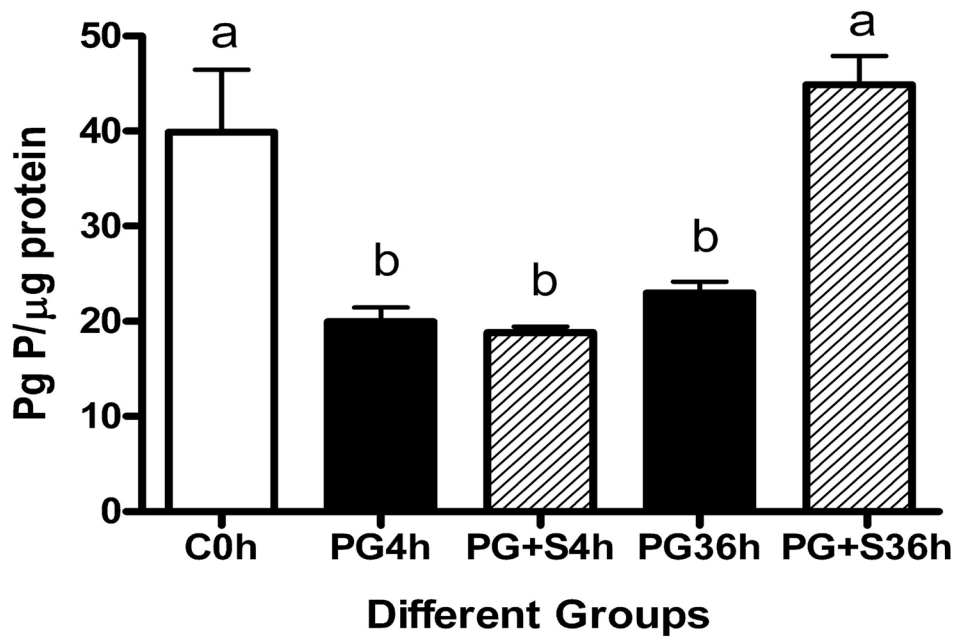
Figure 3.
Luteal progesterone (P) levels (mean ± SEM) at different times (4h or 36h) after PGF-2α (PG) administration. Rats were intrabursa treated with S1P (S) (PG+S4h or S36h) or with the vehicle PET (PG4h or 36h), two hours prior PGF-2α injection. C0h: Rats were untreated at day 19 of pregnancy. Different letters (a, b, c) over standard error bars indicate significant differences (p<0.05) between groups (n=3–4 per group).
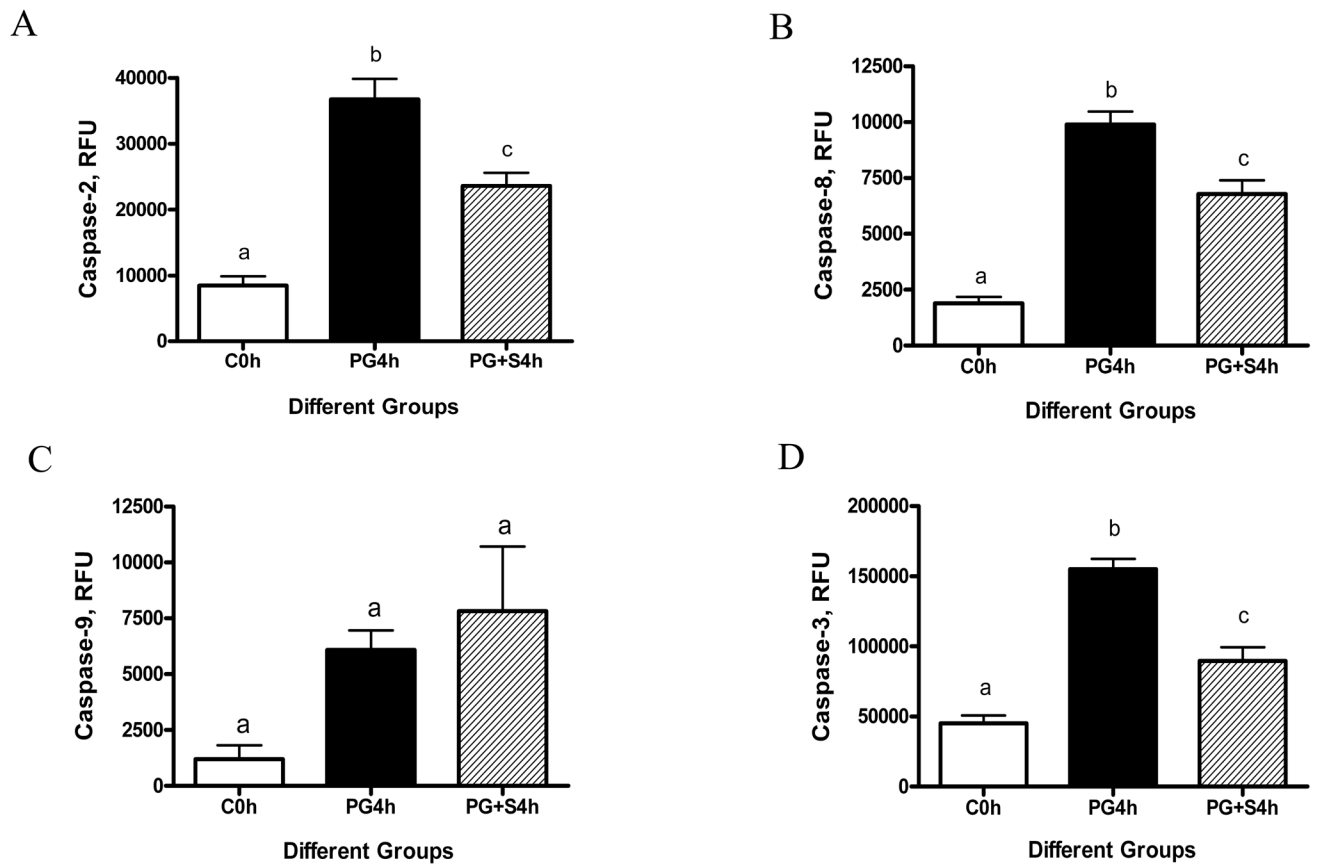
Figure 4.
Enzyme activity levels (mean ± SEM) for the initiator caspases -2 (A), -8 (B), -9 (C) and the effector caspase-3 (D) in the rat CL after 4 h of PGF-2α (PG) administration. Rats were treated with S1P (S) (PG+S4h) or the vehicle PET (PG4h), two hours prior PGF-2α injection. C0h: Rats were untreated at day 19 of pregnancy. Bars with different letters (a, b, c) represent a significant difference (p<0.05) in the activity between groups (n=3–4 per group). The activity is expressed in RFU (Relative Fluorescence Units).
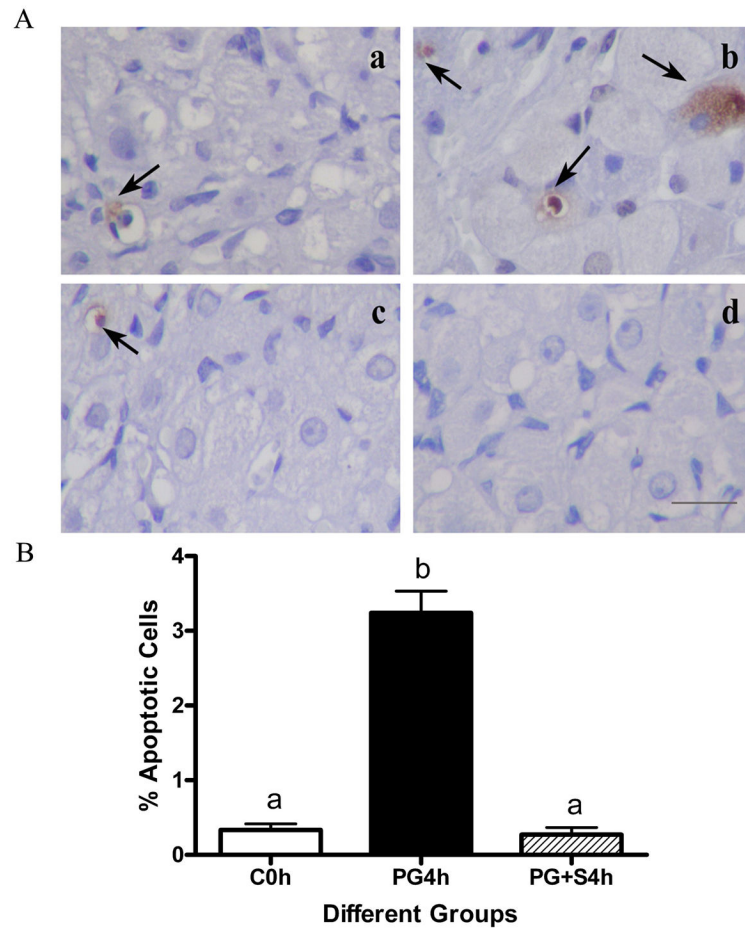
Figure 5.
In situ 3′ end labeling (TUNEL) of rat CL obtained from different treatment groups (panel A). C0h (a), PG4h (b) and PG+S4h (c); Cells showing advanced signs of apoptosis were positively stained in the TUNEL assay (arrows). Negative control without the TdT enzyme, showed no staining (d). Bar = 20 μm. Panel B: TUNEL analysis expressed as the percent of apoptotic cells in the CL. Different letters (a, b, c) represent significant differences (p<0.05) between the different groups. Six randomly selected fields were analyzed from each CL section (3–4 CL per section/ovary, 4 ovaries per group).
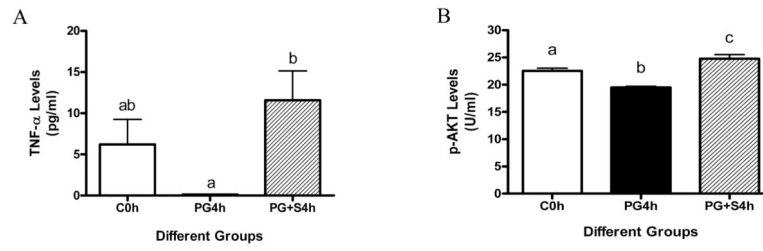
Figure 6.
Levels of TNF-α protein (Panel A) and phosphorylated-Akt (Panel B) as measured by immunoassay in the rat CL in the different groups (C0h, PG4h and PG+S4h). Different letters (a, b, c) over the error bars represent significant differences (p<0.05) between treatments (n=3–4 per group).