

The role of intra-luteal factors in the control of the porcine corpus luteum

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In this paper we review three intra-luteal factors and their roles in the corpus luteum (CL). Insulin-like growth factor (IGF)-I, together with its receptor and IGF-binding proteins (IGFBPs), represent an important control system in the CL. IGF-I is a product of small luteal cells and has steroidogenic (i.e. luteotrophic) actions on large luteal cells via the type I receptor, while IGFBPs (e.g. BP-2 and 3; small cells) generally inhibit IGF-I's actions. IGF-I is particularly important in early CL development (up to day 7 of the oestrous cycle) in the pig. Tumour necrosis factor (TNF)- α is a product of luteal macrophages that infiltrate CLs in increasing numbers as the cycle progresses. TNF- α has been shown to play an important role in luteolysis, but we hypothesise that in the pig, this factor plays an additional role during the mid-luteal phase (days 7-13) in promoting the acquisition of luteal sensitivity to the luteolytic actions of prostaglandin (PG)F_{2 α} (= luteolytic sensitivity; LS). Endothelin (ET)-1 is a product of (luteal) endothelial cells, and along with its receptors (ET_A and ET_B) and endothelin-converting enzyme (ECE)-1, represent an intra-luteal system that also plays a role in luteolysis, in association with PGF_{2 α} . Since TNF- α induces endothelial cells to secrete ET-1, we hypothesise that ET-1 mediates the sensitising effects of TNF- α on the porcine CL during the mid-luteal phase (days 7-13). Finally, we hypothesise that TNF- α and/or ET-1 act to up-regulate luteal protein kinase C (e.g. isoforms β II and ϵ) activity and thereby sensitises luteal cells to PGF_{2 α} .

Introduction

Studies carried out almost 40 years ago clearly demonstrated that porcine corpora lutea (CLs) are capable of developing, functioning and regressing in the absence of pituitary gonadotrophins (Anderson *et al.*, 1967). Thus, our research focus has been on the role of intra-luteal factors in controlling these important processes. We first examined the insulin-like growth factor, IGF-I, as a luteotrophic factor in early luteal development and function. In more recent studies, we have investigated tumour necrosis factor (TNF)- α and endothelin (ET)-1, both of which are involved in luteolysis. However, we explored these factors for their possible roles in the acquisition of luteal sensitivity to PGF_{2 α} (luteolytic sensitivity = LS). In this chapter, the

literature documenting the roles of IGF-I, TNF- α and ET-1 in the CL is reviewed, and we present data and models to illustrate our hypotheses concerning the roles of these intra-luteal factors in the control of the porcine CL.

Insulin-like growth factor

IGF-I

IGF-I mRNA expression has been demonstrated in the CLs of several species including the pig (see Miller *et al.*, 2003 for refs). In the cow IGF-I mRNA levels were greatest on days 1-4 of the cycle and then declined (Einspanier *et al.*, 1990; Schams *et al.*, 2002). In ovine CLs, IGF-I mRNA expression increased from days 3-6 and remained unchanged for the rest of the cycle (Juengel *et al.*, 1997). IGF-I peptide was identified in both small and large bovine luteal cells and showed little variation during the oestrous cycle (Amselgruber *et al.*, 1994).

In our studies in the pig, we have shown that IGF-I mRNA expression was higher in small than in large, luteal cells (Gadsby *et al.*, 1996a; Fig. 1A), and was greater on days 4-6 than on days 10-16 of the oestrous cycle (Gadsby *et al.*, 1996a). Intra-luteal levels of IGF-I peptide were also significantly elevated on day 4 versus 7-16 of the oestrous cycle (Ge *et al.*, 2003).

IGF-IR

IGF-I exerts its biological effects via the type I IGF receptor (IGF-IR) (Valentinis and Baserga, 2001). IGF-IR has been detected in the CLs of the several species (see Miller *et al.*, 2003 for refs). In the bovine CL, IGF-IR mRNA expression was greatest on days 1-4 than on later stages of the oestrous cycle (Schams *et al.*, 2002).

In the pig, IGF-IR mRNA expression was significantly greater in large, compared with small, luteal cells, and was lowest in CLs during the early oestrous cycle, increasing to reach maximal levels on day 16. IGF-IR protein concentrations were also greater in large luteal cells (3-85 fold compared with small luteal cells), and were highest during the early (days 4-10) compared with later stages (day 12-16) of the cycle (Ge *et al.*, 2000).

IGF-I actions

IGF-I stimulated luteal progesterone secretion *in vitro* in several species including the pig (see Miller *et al.*, 2003 for refs; Ptak *et al.*, 2003). IGF-I also increased luteal progesterone secretion when administered *in vivo* in the cow (Sauerwein *et al.*, 1992). IGF-I acts to increase luteal steroidogenesis by elevating steroidogenic acute regulatory (StAR) protein concentrations (Balasubramanian *et al.*, 1997; Mamluk *et al.*, 1999).

We examined the steroidogenic response of CLs to IGF-I at different stages of the oestrous cycle *in vitro* and *in vivo* (Miller *et al.*, 2003). Porcine luteal cells were cultured with increasing doses of IGF-I or the IGF-I agonist, long-R₃-IGF-I, and the data obtained indicated that: 1) steroidogenesis was dose-dependently increased by IGF-I or long-R₃-IGF-I in large, but not small, luteal cells (confirming Yuan and Lucy, 1996); 2) luteal cells taken only from days 4 and 7 of the cycle, were sensitive to IGF-I and long-R₃-IGF-I (confirmed by Ptak *et al.*, 2003); and 3) the actions of IGF-I on the porcine CL were mediated via the PI-3 kinase pathway. Furthermore, IGF-I infused locally into the ovarian vasculature *in vivo* on days 6-7 of the oestrous cycle, acutely (1-2 hour) stimulated progesterone secretion.

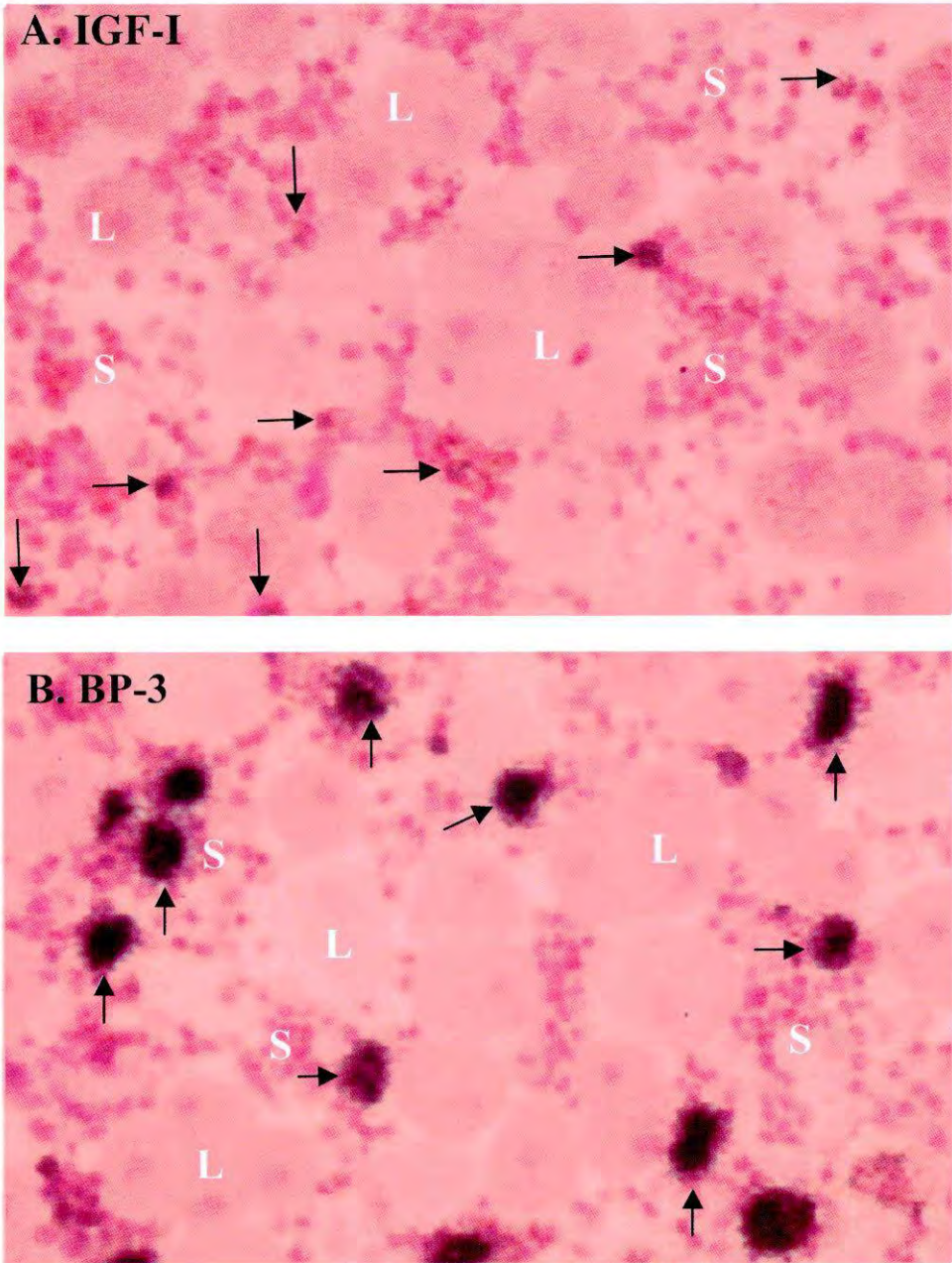


Fig. 1 Localisation of IGF-I and IGFBP-3 mRNAs in porcine luteal cells by *in situ* hybridisation. CLs were collected on day 7 of the oestrous cycle, enzyme-dissociated and centrifuged onto slides. **A.** IGF-I (40X magnification), **B.** IGFBP-3 (40X). Small cells are the primary sites of mRNA expression for IGF-I and BP-3. Arrows indicate positively labelled cells. L = large luteal cells (large pale staining cytoplasm with dark pink nuclei), S = small luteal cells (steroidogenic or non-steroidogenic).

IGFBPs

The IGFBPs are thought to modulate the biological actions of the IGFs (Baxter, 2000) and there are several reports of mRNA and protein expression in the CL of IGFBPs 1-5 (see Ge *et al.*, 2003 for refs). In the bovine CL, IGFBP-3 concentrations were highest during the early oestrous cycle (Brown and Braden, 2001; Schams *et al.*, 2002). IGFBP-3 specifically, was shown to localise to endothelial cells of the luteal microvasculature in rodent, primate and porcine CLs (see Ge *et al.*, 2003 for refs; Wandji *et al.*, 2000).

In our studies in the pig CL, we have demonstrated IGFBPs 2-5 mRNA (Gadsby *et al.*, 1996a; Fig. 1B) and protein (Nicholson *et al.*, 1999; Ge *et al.*, 2003) expression, predominantly in small luteal cells (Gadsby *et al.*, 1996a; Fig. 1B). While the concentrations (protein) of IGFBPs 2, 4, and 5 did not vary significantly during the oestrous cycle, IGFBP-3 concentrations were at their highest on days 4 and 7 (Ge *et al.*, 2003). We also demonstrated dose-dependent inhibitory effects of IGFBP-3 on IGF-I stimulated progesterone secretion (Ge *et al.*, 2003). Furthermore, luteal cells cultured with an IGFBP-3 antibody displayed dose-dependent stimulation of steroidogenesis, further supporting an inhibitory role for IGFBP-3 in the porcine CL (Ge *et al.*, 2003). IGFBP-2 and -3 show similar inhibitory effects in the bovine CL (Brown and Braden, 2001).

IGF-I Summary – Model of IGF-I action in the pig (Fig. 2)

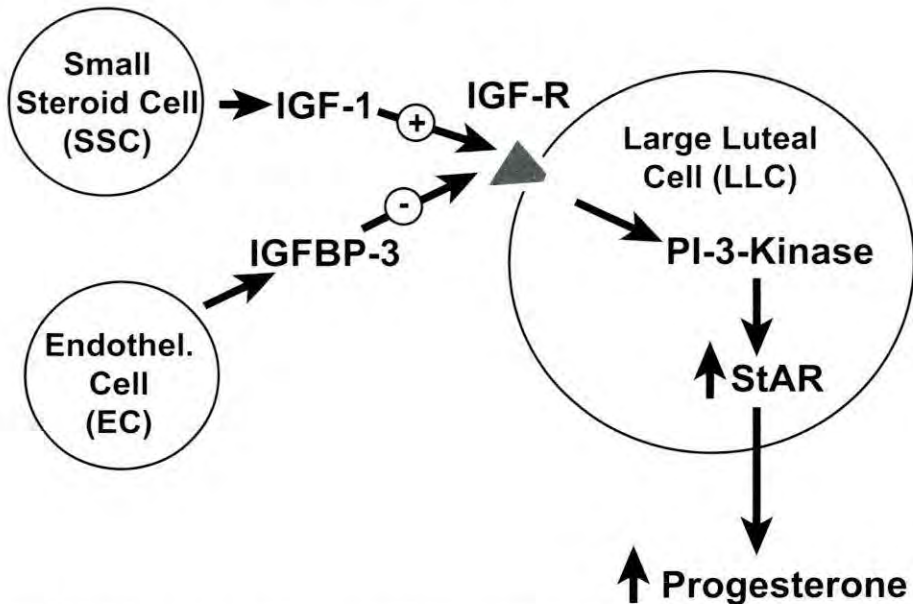


Fig. 2 Model illustrating the role of IGF-I in regulating the porcine CL during the “IGF-sensitive period” (~days 4-7). IGF-I originating from small luteal cells (probably steroidogenic; SSC) acts on IGF-R on large luteal cells (LLC) to activate the PI-3-kinase pathway and progesterone synthesis via increased StAR. IGFBP-3 secreted from endothelial cells (EC) inhibits IGF-I binding to its receptor.

Overall, these data strongly suggest that during the IGF-I sensitive period (up to day 7), IGF-I (probably from small steroidogenic cells; SSC) has paracrine actions on large luteal cells (LLC) via IGF-IR, PI-3-kinase and up-regulation of StAR, to stimulate steroidogenesis. This model also

illustrates how IGFBPs (e.g. BP-3) from endothelial cells (EC) act in a paracrine manner to inhibit the action of IGF-I on LLC. While our studies have focused on steroidogenesis, IGF-I also blocks apoptosis and stimulates cell proliferation in various cell types (Valentinis and Baserga, 2001) including ovarian steroidogenic cells (Chakravorty *et al.*, 1993; Westfall *et al.*, 2000; Ptak *et al.*, 2004).

Tumour necrosis factor- α

Tumour-necrosis factor (TNF)- α is a pro-inflammatory cytokine produced by resident tissue macrophages (MacEwan, 2002). Luteal macrophages accumulate in the CL towards the end of the oestrous cycle and thus the role of TNF- α in luteal regression has been studied extensively (Pate and Keyes, 2001).

TNF- α

TNF- α mRNA expression did not vary as the oestrous cycle progressed in the bovine CL (Petroff *et al.*, 1999; Sakumoto *et al.*, 2000), but was elevated in response to PGF_{2 α} treatment at mid-cycle (Neuvians *et al.*, 2004). TNF- α protein secretion increased in late luteal phase CLs (Sakumoto *et al.*, 2000), and in CLs whose progesterone secretory activity had begun to decline (Shaw and Britt, 1995). Recently, the number of luteal macrophages (or monocytes) were found to increase significantly between early and mid (days 6–12) cycle, raising the possibility that in the cow these cells (or their cytokines) may have effects on the CL before luteolysis (Townson *et al.*, 2002).

Likewise in the pig, the number of luteal macrophages secreting TNF- α increased most dramatically (~4-fold) between the early (days 4-6) and mid (days 8-12) luteal phase, but also increased an additional 1-2 fold in the late luteal phase (days 14-18; Zhao *et al.*, 1998), suggesting that TNF- α may have effects prior to luteolysis in the pig also.

TNF-receptors

TNF- α acts on target cells via TNF-receptors (TNFR) type 1 or 2. TNF- α can then activate multiple signalling pathways via the intracellular TNF-receptor associated factor (TRAF) proteins (MacEwan, 2002). Recent studies in the TNFR1 knockout mouse suggest that the luteolytic actions of TNF- α are mediated via TNFR1 (Roby *et al.*, 1999).

TNFR1 mRNA concentrations in the bovine CL were unchanged during most of the oestrous cycle, but increased ~3 fold in regressing and PGF_{2 α} -treated CLs (Friedman *et al.*, 2000). TNFR1 was found in all luteal cell sub-types but was most highly expressed in endothelial cells (Okuda *et al.*, 1999; Friedman *et al.*, 2000). In another study in the cow, the expression of TNFR1 mRNA and protein were greatest during the early and mid-luteal phase CLs (days 3-12) and decreased in the late luteal phase CLs (Sakumoto *et al.*, 2000).

TNF- α receptors have been detected on porcine small and large steroidogenic luteal cells as well as endothelial cells. Luteal TNF- α receptor levels remained unchanged between days 4 and 12 of the oestrous cycle, but increased on day 15 (Richards and Almond, 1994a; Miyamoto *et al.*, 2002).

TNF actions

In general TNF- α 's effects on luteal steroidogenesis in the cow and pig are inhibitory (Pate and Keyes, 2001). However, while low doses of TNF- α induced luteolysis *in vivo*, high doses stimulated the CL and delayed luteolysis in cows (Skarzynski *et al.*, 2003a). In pigs, TNF- α inhibited LH or hCG-stimulated progesterone secretion, mainly in small steroidogenic luteal cells (Pitzel *et al.*,

1993; Richards and Almond, 1994b; Pate and Keyes, 2001), although basal progesterone secretion by large luteal cells was also inhibited (Pitzel *et al.*, 1993). In the rat, TNF- α inhibited luteal steroidogenesis via an inhibition of luteal LH receptor and StAR mRNA expression (Chen *et al.*, 1999). In the pig, TNF- α has luteolytic actions because it inhibits oestradiol, which is luteotrophic in this species (Pitzel *et al.*, 1993). TNF- α also stimulates prostaglandin (PGE₂, PGF_{2 α} and 6-keto-PGF_{1 α}) production by bovine (Okuda *et al.*, 1999; Sakumoto *et al.*, 2000; Pate and Keyes, 2001) and porcine (Richards and Almond, 1994b; Miyamoto *et al.*, 2002) luteal cells. It has been suggested that the luteolytic actions of TNF- α may be mediated by PGF_{2 α} in the pig (Richards and Almond, 1994b), but not in the cow (Pate and Keyes, 2001). In addition, TNF- α synergises with PGF_{2 α} to promote functional luteolysis in the pig both *in vitro* and *in vivo* (Pitzel *et al.*, 1993; Wuttke *et al.*, 1998). Finally, TNF- α induced apoptosis of bovine luteal endothelial cells (Pru *et al.*, 2003), an effect that could be prevented by treatment of cells with progesterone (Friedman *et al.*, 2000). However, TNF- α was not cytotoxic to steroidogenic luteal cells except in combination with interferon (IFN)- γ (Pate and Keyes, 2001).

TNF- α Summary – Models

A. Role of TNF- α in de-sensitising the porcine CL to IGF-I: As described above, porcine luteal cells lose their sensitivity to IGF-I on days 10-16 of the oestrous cycle, in spite of the elevated levels of IGF-IR on day 10 (Ge *et al.*, 2000; Miller *et al.*, 2003). Since the numbers and TNF- α secretory capacities of luteal macrophages increased \sim 4-fold during this period (Zhao *et al.*, 1998), we hypothesised that TNF- α may play a role in de-sensitising porcine luteal cells to IGF-I *in vivo*. In support of this hypothesis, TNF- α has been shown to block IGF-I signalling pathways in several different cell types (Urban *et al.*, 1996; Venters *et al.*, 2000; Shalita-Chesner *et al.*, 2001). In our studies (Fig. 3), TNF- α dose-dependently inhibited the steroidogenic actions of IGF-I on porcine luteal cells in culture.

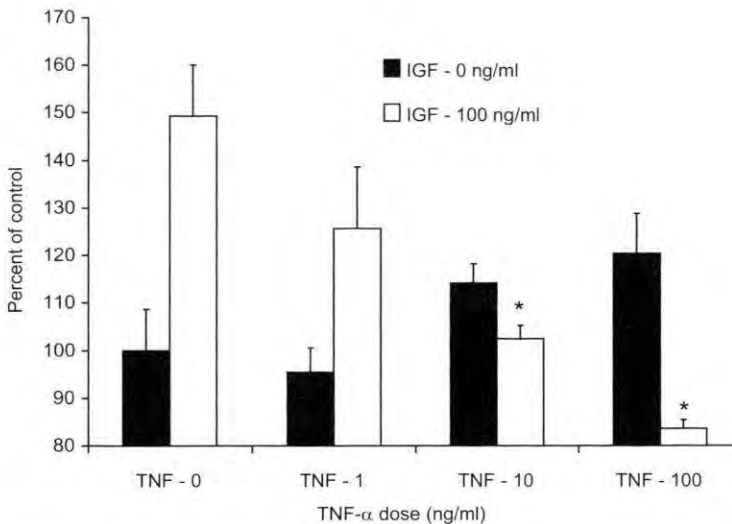


Fig. 3 Inhibitory actions of tumour necrosis factor (TNF)- α on IGF-I stimulated progesterone secretion by porcine luteal cells *in vitro*. Mixed porcine luteal cells (day 7 of cycle) were cultured with 0, 1, 10 or 100 ng/ml ($n=6$) TNF- α , in the presence (100 ng/ml) or absence (0 ng/ml) of IGF-I. Data shown are representative of 3 separate experiments and are presented as progesterone concentrations, expressed as a percent of the control (no TNF- α , no IGF-I) values. * Indicates significant difference ($p < 0.05$; ANOVA) at 10 and 100 ng/ml TNF- α versus controls (no TNF- α) in the presence of 100 ng/ml IGF-I.

Thus we propose a model (Fig. 4) in which macrophage-borne $\text{TNF-}\alpha$ decreases the sensitivity of porcine luteal cells to IGF-I, probably by blocking PI-3 kinase activation and up-regulation of StAR.

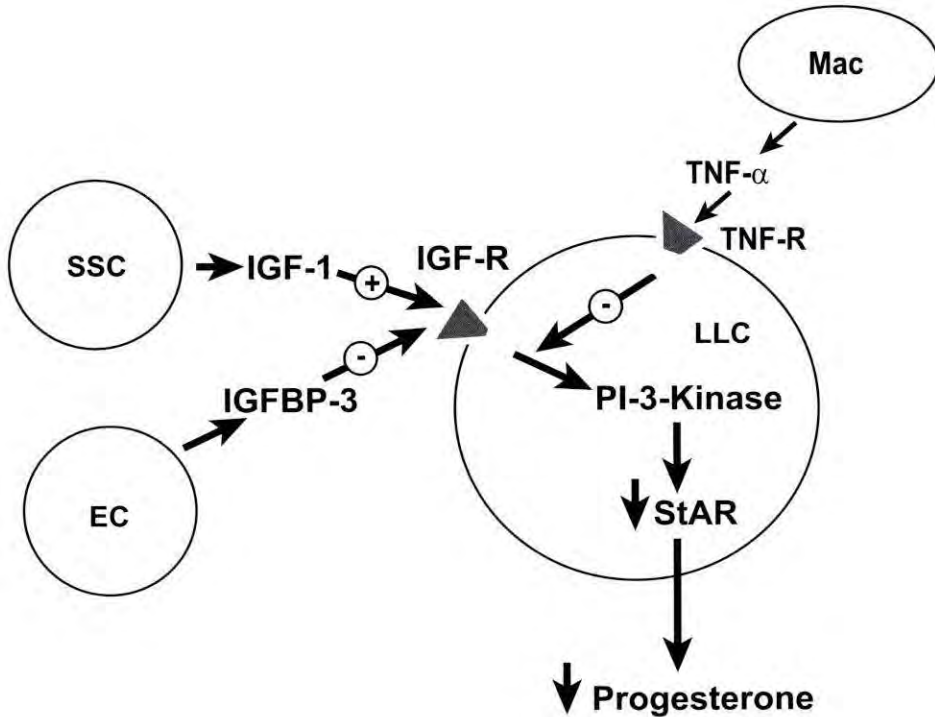


Fig. 4 Model illustrating the role of macrophage (Mac) - derived $\text{TNF-}\alpha$ in desensitising porcine luteal cells to IGF-I (between days 7-13). $\text{TNF-}\alpha$ acts via TNF-R on large luteal cells (LLC) to inhibit IGF-I actions at the post-receptor level (PI-3-kinase), leading to decreased StAR and progesterone secretion.

B. Role of $\text{TNF-}\alpha$ in sensitising the porcine CL to $\text{PGF}_{2\alpha}$: In addition, since porcine CLs acquire luteolytic sensitivity (LS), during the IGF-insensitive period (days 10-16) we hypothesised that $\text{TNF-}\alpha$ may be involved in this process as well. To test this hypothesis, we examined whether priming of porcine luteal cells with $\text{TNF-}\alpha$ *in vitro* could increase their sensitivity to $\text{PGF}_{2\alpha}$. Porcine luteal cells were cultured with $\text{TNF-}\alpha$ for 2 days (days 2-4 of culture), and then were challenged with 1 ng/ml $\text{PGF}_{2\alpha}$ on days 4-6 of culture. The data (Fig. 5) indicated that $\text{TNF-}\alpha$ sensitised luteal cells to the luteolytic actions of $\text{PGF}_{2\alpha}$. Since $\text{TNF-}\alpha$ increased ET-1 secretion by luteal cells *in vitro* (Okuda *et al.*, 1999; L. Rose and J. Gadsby, unpublished data), we hypothesise that ET-1 mediates the sensitising actions of $\text{TNF-}\alpha$. As suggested below this sensitising action of $\text{TNF-}\alpha$ /ET-1 may involve up-regulation of protein kinase C (Fig. 6).

Endothelin

Endothelin-1

Endothelin (ET)-1 is a potent vasoconstrictive peptide originally described in the cardiovascular system, but which exerts important effects on the CL (Meidan and Levy, 2002). ET-1 is a 21

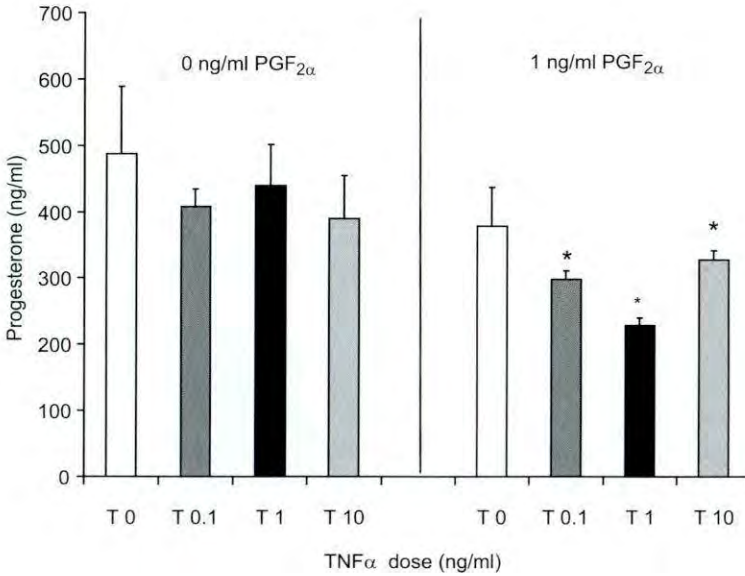


Fig. 5 TNF- α sensitises porcine luteal cells to PGF_{2 α} . Mixed porcine luteal cells (day 4 of cycle) were cultured for 2 days (days 2-4 of culture) with 0, 0.1, 1 or 10 ng/ml (n=6) TNF- α , and challenged with 0 or 1 ng/ml PGF_{2 α} for 2 days (days 4-6 of culture). Data shown are representative of 3 separate experiments and are presented as ng/ml progesterone (measured on day 6 of culture). * Indicates significant difference (p<0.05; ANOVA) at 0.1, 1 and 10 ng/ml TNF- α versus controls (no TNF- α) in the presence of 1 ng/ml PGF_{2 α} .

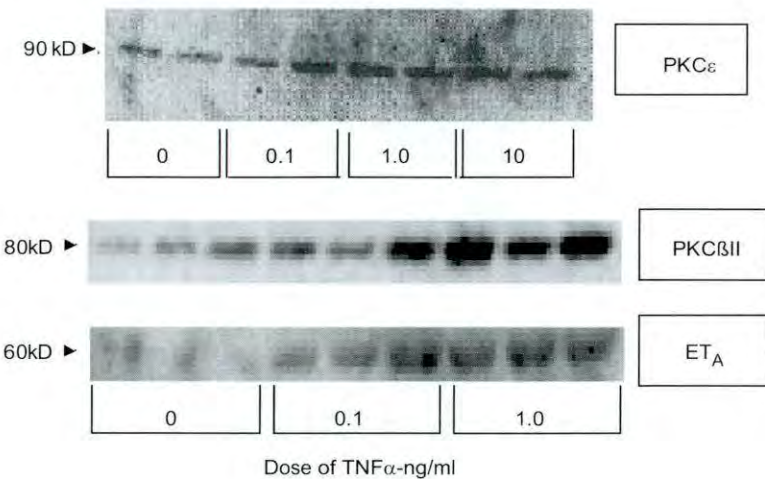


Fig. 6 TNF- α increases PKC ϵ , PKC β II and ET_A protein expression in porcine luteal cells in culture. Porcine luteal cells were cultured as described in Fig. 5, cellular protein extracted on day 4 of culture and examined by Western blot. Data show PKC ϵ , PKC β II and ET_A concentrations in response to TNF- α doses (0, 0.1, 1 or 10 ng/ml; 2-3 wells per treatment).

amino acid (aa) peptide that is synthesised as part of the precursor protein, pre-pro (pp)-ET-1 (212 aa), and which is proteolytically cleaved (via an endopeptidase) to generate the biologically inert Big-ET-1 (38 aa). Big-ET-1 is cleaved by endothelin-converting enzyme (ECE)-1 to produce ET-1 (Meidan and Levy, 2002).

In the cow, luteal ET-1 concentrations increased from the early to mid-luteal phase; this increase was thought to result from increased ECE-1 activity, since Big ET-1 concentrations declined at this time (Meidan and Levy, 2002). Expression of ppET-1 mRNA increased in the late luteal phase perhaps explaining increased ET-1 secretion during luteolysis (Meidan and Levy, 2002). In another study, ET-1 mRNA and peptide expression were high during the early luteal phase, declined in the mid and late luteal phase, and increased in regressing CLs (Berisha *et al.*, 2002). It is not clear why these studies differed, but both showed an increase in ET-1 during luteal regression, pointing to its potential role in this process.

Endothelial cells are the primary source of ET-1 in the CL, as indicated by expression of ppET-1 mRNA (Meidan and Levy, 2002; Davis *et al.*, 2003; Klipper *et al.*, 2004). Other luteal cell types are capable of cleaving ET-1 from Big-ET-1 via membrane associated ECE-1 (Meidan and Levy, 2002; Davis *et al.*, 2003). The major regulators of ET-1 production are PGF_{2 α} and TNF- α . PGF_{2 α} has been shown to increase ET-1 mRNA and peptide expression in ruminant CLs *in vivo*, and by luteal cells *in vitro* (Hinckley and Milvae, 2001; Wright *et al.*, 2001; Meidan and Levy, 2002; Schams *et al.*, 2003; Choudhary *et al.*, 2004), although PGF_{2 α} treatment was only effective *in vivo* in the mid-late luteal phase (cow - Meidan and Levy, 2002). Furthermore, PGF_{2 α} infused by microdialysis into the ovine or bovine CL, increased the release of ET-1 (Ohtani *et al.*, 1998; 2004). TNF- α stimulates ET-1 secretion from bovine luteal endothelial cells (Okuda *et al.*, 1999) and porcine luteal cells (L. Rose and J. Gadsby, unpublished data) *in vitro*.

Endothelin-converting enzyme (ECE)-1

There are four isoforms of ECE-1 (a-d) that differ in their amino terminal sequences, which probably accounts for their different sub-cellular localisations and thus different intracellular sites for ET-1 synthesis (from Big ET-1; Klipper *et al.*, 2004). For example ECE-1a resides intracellularly, whereas ECE-1b is found on the cell membrane (Meidan and Levy, 2002). Steroidogenic luteal cell types express exclusively the ECE-1b isoform enabling these cells to generate bioactive ET-1 at the cell surface close to the ET-receptor (Meidan and Levy, 2002; Levy *et al.*, 2003). Endothelial cells possess all ECE-1 isoforms suggesting that these cells can convert Big-ET-1 to ET-1 both intra- and extra-cellularly (Meidan and Levy, 2002; Levy *et al.*, 2003; Klipper *et al.*, 2004).

In one study in the bovine CL, ECE-1 (mRNA and protein) increased between the early and mid-luteal phases (Meidan and Levy, 2002; Levy *et al.*, 2003). This increase in ECE-1 was hypothesised to account for the increase in ET-1 production between early and mid-luteal phases, and thus for the acquisition of LS in the bovine CL (Meidan and Levy, 2002). In other studies, ECE-1 mRNA expression levels were either reported to be relatively constant (Berisha *et al.*, 2002), or to increase between the early and mid-luteal phases (Choudhary *et al.*, 2004). In addition, while ECE-1 protein (Western blot) concentrations decreased, ECE-1 enzyme activity failed to show any significant changes during this period (Choudhary *et al.*, 2004), casting some doubt on the proposed critical role of ECE-1 described above (Meidan and Levy, 2002; Levy *et al.*, 2003). Little is known of the regulation of ECE-1 expression in the CL, although there is a report suggesting that PGF_{2 α} can transiently increase ECE-1 mRNA levels in the bovine CL (Schams *et al.*, 2003). Other studies in the cow suggest that LH inhibits, and IGF-1 or insulin stimulates, ECE-1 in granulosa-lutein cells, and ET-1 inhibits ECE-1 mRNA expression in luteal cells, *in vitro* (Levy *et al.*, 2003).

Endothelin receptors and actions

There are two ET-receptor sub-types, ET_A and ET_B, originally named after the location of their discovery (ET_A for aorta and ET_B for bronchus; Meidan and Levy, 2002). These receptors belong to the superfamily of seven-transmembrane G protein coupled receptors.

Based on ligand binding studies bovine CLs were shown to express ET-receptors, mainly the ET_A subtype (Meidan and Levy, 2002). All bovine luteal cell subtypes expressed ET_A mRNA, although the highest concentrations were seen in endothelial cells (Meidan and Levy, 2002); luteal endothelial cells were also found to express ET_B (Klipper et al., 2004). ET_A mRNA concentrations in bovine CLs remained relatively constant from early to late stages of the oestrous cycle, but increased ~2-5-fold in regressing CLs (Meidan and Levy, 2002). The elevated expression of ET_A in late stage CLs is consistent with an involvement of ET-1 in PGF_{2α} - induced luteolysis (Hinckley and Milvae, 2001; Meidan and Levy, 2002). Another study in the cow showed no changes in ET_A mRNA concentrations throughout the oestrous cycle but an increase in ET_B during CL regression was seen (Berisha et al., 2002). In an additional report, ET_A increased between early and mid-cycle, with a slight decline in the late cycle, while ET_B increased in the early stages and thereafter remained constant throughout the cycle (Wright et al., 2001; Choudhary et al., 2004). There is no definitive explanation for the differences observed between these studies. PGF_{2α} up-regulates the expression of ET_A mRNA in the bovine CL, but only when administered in the mid-late luteal phase (Meidan and Levy, 2002; Schams et al., 2003; Choudhary et al., 2004). The effects of PGF_{2α} on ET_B mRNA are more controversial, with one study showing an increase (Schams et al., 2003) and another study showing no effect (Choudhary et al., 2004). ET_A mRNA expression in luteal steroidogenic cells was negatively regulated during luteinisation; LH or Forskolin inhibited ET_A by small (theca derived), whereas insulin or IGF-I inhibited ET_A by large (granulosa derived) luteal cells in the cow (Meidan and Levy, 2002).

ET-1 decreased luteal progesterone secretion *in vitro* (Hinckley and Milvae, 2001; Meidan and Levy, 2002; Choudhary et al., 2005) and *in vivo* (Hinckley and Milvae, 2001; Ohtani et al., 2004), an effect which appears to be mediated via the ET_A receptors (Hinckley and Milvae, 2001; Meidan and Levy, 2002). ET-1's vasoconstrictive actions are also probably mediated via ET_A receptors on vascular smooth muscle cells of ovarian arterioles, and presumably play an important role during luteolysis by reducing blood flow to the CL (Klipper et al., 2004; Ohtani et al., 2004). In contrast, the ET_B receptor subtype (Klipper et al., 2004) appears to mediate ET-1's action on nitric oxide production, which has vasodilatory effects (Klipper et al., 2004). How the balance of these opposing actions of ET-1 is achieved in the regressing CL, is not currently known.

ET-1's actions via ET_A are similar to those of PGF_{2α} via its receptor; activation of phospholipase C (PLC), release of intracellular calcium and activation of protein kinase C (PKC; Schmitz-Spanke and Schipke, 2000). As discussed above, studies using the ET_A selective antagonists, have shown that the ET_A receptor subtype mediates the luteolytic actions of ET-1 and PGF_{2α} in the CL (Hinckley and Milvae, 2001; Meidan and Levy, 2002). In addition, since PGF_{2α} also induces ET-1 production (see above), it is clear that interactions between these factors are critical to the luteolytic process *in vivo*. ET-1's actions via ET_B on luteal endothelial cells appear to involve the stimulation of the inducible form of nitric oxide synthase (iNOS) leading to the generation of Nitric Oxide (NO; Klipper et al., 2004). Since NO also has been shown to be involved in PGF_{2α}'s luteolytic actions (Skarzynski et al., 2003b), it is possible that ET-1 acting via ET_B may also be involved in CL regression.

In ruminants, sub-luteolytic doses of PGF_{2α} given in the presence of ET-1 *in vivo* will cause complete luteal regression, showing that ET-1 and PGF_{2α} act synergistically (Hinckley and Milvae, 2001). A similar synergistic response was demonstrated for TNF-α and PGF_{2α} in the pig

in vitro (Pitzel *et al.*, 1993) and *in vivo* using CL microdialysis (Wuttke *et al.*, 1998). In our preliminary studies (Fig. 5), we have demonstrated that TNF- α "sensitised" porcine luteal cells *in vitro* to the luteolytic effects of PGF_{2 α} . In view of the fact that TNF- α increases ET-1 secretion by endothelial cells (Okuda *et al.*, 1999) and porcine luteal cells (L. Rose and J. Gadsby, unpublished data), and that TNF- α dose-dependently increased ET_A expression on porcine luteal cells (Fig. 6), we hypothesise that ET-1 mediates TNF- α 's role in inducing LS in the porcine CL during the oestrous cycle (see below).

PGF_{2 α} luteolytic sensitivity (LS) and luteolysis

In order to understand the roles of TNF- α and/or ET-1 in the regulation of LS in the pig, it is critical to review the topic of LS and the role of PGF_{2 α} in the process of luteolysis.

In ruminants, the early CL displays a period of insensitivity to PGF_{2 α} (i.e. lacks LS) that lasts ~ 5-6 days (see Estill *et al.*, 1995 for refs). After this period, CLs display LS and thus PGF_{2 α} or its analogues will cause premature luteal regression and shortening of the oestrous cycle (see Estill *et al.*, 1995 for refs; Meidan and Levy, 2002). Several theories have been proposed for the mechanism of LS in ruminants: 1) only CLs displaying LS respond to PGF_{2 α} by up-regulating the luteal cyclooxygenase (COX)-2 enzyme, leading to increased intra-luteal PGF_{2 α} production, which appears necessary for luteolysis (Diaz *et al.*, 2002); 2) only CLs displaying LS (mid-cycle) have elevated ET-1 levels (discussed above - Meidan and Levy, 2002); 3) CLs lacking LS (early) showed elevated levels of PGF_{2 α} metabolism compared with those at mid-cycle (Silva *et al.*, 2000); and 4) protein kinase (PK) C ϵ specifically, is up-regulated in bovine CLs that display LS and it is this PKC isoform that mediates PGF_{2 α} luteolytic signalling *in vitro* (Sen *et al.*, 2004; 2005).

The pig is unusual since CLs are insensitive to the luteolytic actions of PGF_{2 α} (i.e. lack LS) until after day 12 of the oestrous cycle (see Estill *et al.*, 1993 for refs). Consequently, PGF_{2 α} analogues cannot be used to shorten the oestrous cycle (Estill *et al.*, 1993). Our original studies designed to address this problem measured the luteal PGF_{2 α} (FP) receptor, and we showed a ~ 3 fold increase on days 13-14 (vs. days 4-12; Gadsby *et al.*, 1990; 1993), coinciding with the onset of LS in this species (Estill *et al.*, 1993; 1995). Recently we found that PGF_{2 α} -receptor mRNA levels were significantly elevated on day 10, reaching a maximum on day 13 of the oestrous cycle (Boonyaparakob *et al.*, 2003). However, important functional differences between CLs lacking and those possessing LS, probably occur at the post-FP receptor level, as suggested by the following studies. Previously it was shown that repeated administration of PGF_{2 α} analogues (Lutalyse and Cloprostenol) on days 5-10 of the oestrous cycle, induced premature luteolysis and shortened cycle length by up to 7 days (Estill *et al.*, 1993; 1995; Gadsby *et al.*, 1996b). These studies indicated that LS can be advanced in the pig, although this occurred without an increase in FP receptors (Estill *et al.*, 1995). In another study Cloprostenol administration induced COX-2 (mRNA and protein) in both CLs lacking (day 9 of oestrous cycle), and displaying (day 17 of pseudopregnancy), LS (Diaz *et al.*, 2000). However, Cloprostenol did not increase PGF_{2 α} or decrease progesterone secretion on day 9, whereas it did significantly increase PGF_{2 α} and decreased luteal progesterone secretion on day 17 (Diaz *et al.*, 2000). Furthermore, the PKC-activator (PDD) was found to be equally capable of increasing luteal PGF_{2 α} synthesis on days 9 and 17, but was less effective at decreasing progesterone secretion on day 9 compared with day 17 (Diaz *et al.*, 2000). These data suggested that: 1) protein kinase C levels may be rate-limiting to the luteolytic actions of PGF_{2 α} in CLs which lack LS; and 2) PGF_{2 α} and progesterone synthetic pathways may be regulated by different PKC-isoforms (Diaz *et al.*, 2000).

PGF_{2 α} acts on the CL to stimulate phospholipase-C leading to the generation of inositol triphosphate (IP-3) and diacylglycerol (DAG); IP-3 in turn increases intracellular Ca²⁺ levels and DAG activates several isoforms of protein kinase C, specifically PKC- α , β I, β II, ϵ and θ in inducing

functional luteolysis (Davis *et al.*, 1996; Chen *et al.*, 2001; Diaz *et al.*, 2002; Sen *et al.*, 2005). The PKC isoforms have been classified based on their mode of activation: 1) PKC- α , β I, β II and γ , which depend on calcium, DAG, and phosphatidylserine (PS) for activation ("classical or conventional PKCs"); 2) PKC-isoforms δ , ϵ , η , μ and θ , that are independent of calcium, but which require DAG and PS ("novel PKCs"); 3) PKC-isoforms ζ and ι/λ , which only require PS ("atypical PKCs"); and 4) a new structurally distinct type of PKC isoform called "PKC-related kinases", that also require only PS (DaRocha *et al.*, 2002).

As discussed above in the porcine CL, PKC levels appear to be limiting to $\text{PGF}_{2\alpha}$'s actions leading us to hypothesise that the activities of PKC (e.g. α , β I, β II, ϵ or θ) will increase during the acquisition of LS, and will be elevated in response to $\text{TNF-}\alpha$ (via ET-1). In preliminary studies we showed that $\text{TNF-}\alpha$ up-regulated PKC- β II and PKC- ϵ (Fig. 6) in cultured porcine luteal cells (*in vitro*) implicating these isoforms at least, in the $\text{PGF}_{2\alpha}$ sensitisation process, as suggested for the bovine CL (Sen *et al.*, 2004; 2005).

Overall model for the acquisition of LS

Fig. 7 shows a model illustrating our hypotheses concerning the roles of $\text{TNF-}\alpha$ and ET-1 in regulating LS in the pig. $\text{TNF-}\alpha$ originating from macrophages, stimulates endothelial cells to secrete ET-1 (Okuda *et al.*, 1999; L. Rose and J. Gadsby, unpublished data). ET-1 acts via ET_A on large luteal cells to increase PKC expression PKC- β II and - ϵ (Fig. 6), to mediate the increased LS of luteal cells to $\text{PGF}_{2\alpha}$. This model represents fundamentally new thinking about the roles of $\text{TNF-}\alpha$ and ET-1 as factors which sensitise luteal cells to $\text{PGF}_{2\alpha}$ and is under current examination in our efforts to understand the control of LS in the pig.

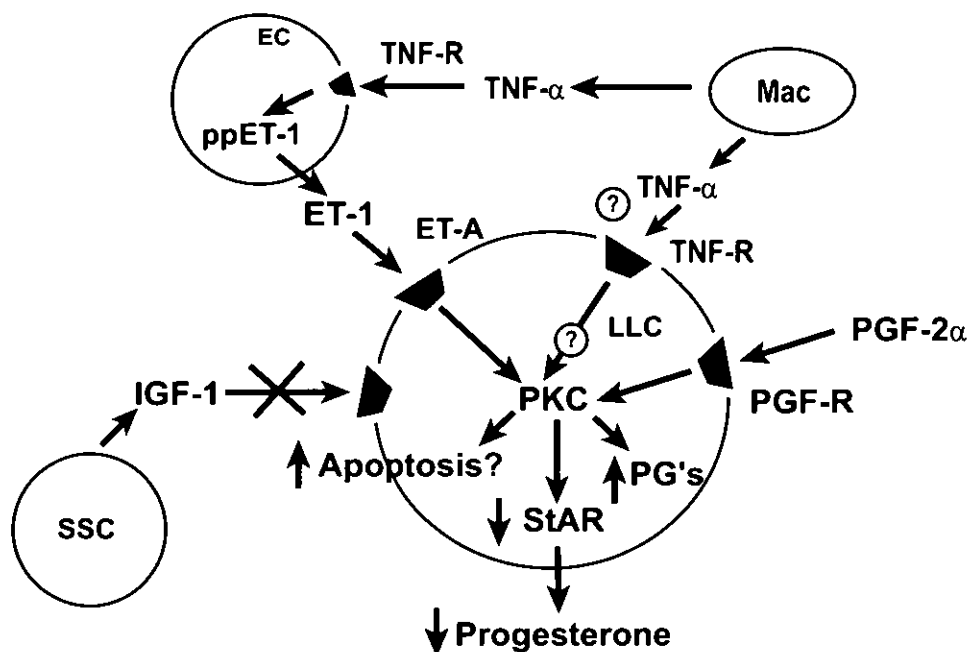


Fig. 7 Model illustrating the role of macrophage (Mac) - derived $\text{TNF-}\alpha$ in sensitising porcine luteal cells to $\text{PGF}_{2\alpha}$ (between days 7-13). $\text{TNF-}\alpha$ acts via TNF-R on endothelial cells (EC) to increase ET-1 production. ET-1 acts via ET_A receptors on LLC to increase PKC isoform expression (e.g. PKC β II or PKC ϵ), which increases LLC sensitivity to $\text{PGF}_{2\alpha}$ at a post-receptor (FP) level, leading to decreased STAR, decreased progesterone and increased prostaglandin (PG) production, and possibly increased apoptosis.

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