Maximal expression of suppressors of cytokine signaling in the rat ovary occurs in late pregnancy

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Abstract

Maintenance of the rodent corpus luteum (CL) during pregnancy requires prolactin receptor (PRLR) signal transduction via STAT5. At the end of pregnancy, prostaglandin F_{2α} (PGF_{2α}) induces luteal regression through many mechanisms, including downregulation of PRLR signaling. We have previously shown that a PGF_{2α} analog upregulates suppressors of cytokine signaling (SOCS) proteins in the CL of day 19 pregnant rats leading to reduced STAT5 signaling. Here, we examined endogenous SOCS expression and STAT5 signaling in the rat ovary during normal pregnancy and luteolysis. The mRNA expression of *Socs1*, *Socs2*, and *Socs3* and related cytokine-inducible SH2-containing protein (*Cish*) was low in early pregnancy (day 7), but significantly increased at mid-pregnancy (days 10 and 13) associated with increased endogenous tyrosine phosphorylation (TyrP) of STAT5. In support of the notion that these changes are due to increasing placental lactogen levels at this time, we found that treatment with exogenous PRL on day 7 increased TyrP of STAT5 and induced SOCS mRNA expression, except *Socs3*. After mid-pregnancy, further significant increases in *Socs3* and *Cish* mRNA expression were observed. Such changes in mRNA expression correlated with protein levels, with protein levels of both SOCS3 and CISH being maximal in late pregnancy (days 19–21). In addition, a significant reduction in TyrP of STAT5 was first observed on day 20, with a further substantial decrease on day 21. Therefore, these results are consistent with the hypothesis that increased SOCS expression in the rat ovary during late pregnancy reduces STAT5 signaling, which may be important in PGF_{2α}-induced luteolysis.

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Introduction

In rodents, the secretion of progesterone by the corpus luteum (CL) is essential for normal pregnancy, with progesterone playing critical roles in embryo implantation, decidualization of the endometrial stroma, and maintenance of a quiescent myometrium before term. Luteotrophic support for the CL in early pregnancy is provided by pituitary prolactin (PRL; reviewed in Risk & Gibori (2000)). Twice daily surges of PRL induced by the mating stimulus enhance CL development and maintain progesterone secretion until mid-pregnancy, after which placental lactogens (PL) become the major luteotrophic support to term.

The luteotrophic effects of both PRL and PL on the CL are mediated via the PRL receptor (PRLR), a member of the cytokine class 1 receptor family (Clevenger & Kline 2001). In rodents, both the long (PRLRL) and short (PRLRS) PRLR variants are continuously expressed throughout gestation in the CL (Telleria *et al.* 1997). However, the PRLRL variant is more highly expressed at all stages of pregnancy and PRL signal transduction

occurs predominantly via JAK2 and STAT pathways. The importance of PRL and its receptor in maintaining the CL of pregnancy in rodents was demonstrated in studies with neutralizing antisera to PRL (Yoshinaga 1974) and more recently in both PRL and PRLR knockout mice (Horseman *et al.* 1997, Ormandy *et al.* 1997). Moreover, STAT5B appears to be the key signaling intermediate in PRLR signal transduction in the CL, as STAT5B-deficient mice abort mid-pregnancy due to reduced progesterone levels (Udy *et al.* 1997) while STAT5A/B double knockout mice fail to form or maintain a CL (Teglund *et al.* 1998).

The maintenance of CL function, in particular progesterone production, by PRL is thought to involve a wide range of complimentary effects (for review see Risk & Gibori 2000). One important element in the luteotrophic actions of PRL is thought to be the suppression of 20 α -hydroxysteroid dehydrogenase (20 α HSD), which metabolizes progesterone to inactive 20 α -hydroxyprogesterone. In rodents, little 20 α HSD activity is normally found in the CL during pregnancy, except immediately before parturition when 20 α HSD

expression rises rapidly, coincident with the fall in progesterone levels (Stocco *et al.* 2000). This appears to be important for normal parturition as 20 α HSD-deficient mice exhibit sustained progesterone levels and a delay in parturition (Piekorz *et al.* 2005), although such marked effects were not observed recently in another line of 20 α HSD-deficient mice (Ishida *et al.* 2008). Nevertheless, PRL activation of STAT5B appears important in suppressing 20 α HSD gene expression and maintaining progesterone levels, as early abortion in STAT5B-deficient mice can be partially corrected by 20 α HSD deficiency (Piekorz *et al.* 2005).

At the end of pregnancy, luteolysis occurs in response to increasing secretion of uterine prostaglandin F_{2 α} (PGF_{2 α}). A key component in luteal regression is the decline in progesterone levels (functional luteolysis), which is not observed in the PGF_{2 α} receptor knockout mouse. The mechanisms by which PGF_{2 α} induces functional luteolysis have been intensively investigated (for review see (Stocco *et al.* 2007)), but the initiating molecular events that result in a decline in progesterone secretion by the CL remain somewhat elusive. One early event involves upregulation of 20 α HSD by the transcription factor NUR77, which is absent in the PGF_{2 α} receptor knockout mouse (Stocco *et al.* 2000). However, there is also a reduction in luteotrophic signaling to the CL via the PRLR. Recent gene array data have shown opposite effects of PGF_{2 α} and PRL on many genes in the rodent CL suggesting a functional antagonism between these two hormones (Stocco *et al.* 2001). Indeed, as noted above, the expression of both PRLR variants in the CL decreases abruptly in late pregnancy (Telleria *et al.* 1997, Russell & Richards 1999), an effect that has been shown to be PGF_{2 α} dependent (Stocco *et al.* 2003). Furthermore, besides a reduction in PRLR expression, downstream signaling via STAT5 appears curtailed. We have shown (Curlewis *et al.* 2002) that administration of the PGF_{2 α} receptor analog, cloprostenol, to day 19 pregnant rats induces expression of members of the suppressors of cytokine signaling (SOCS) protein family, which are well known to inhibit PRLR signal transduction via JAK2/STAT pathways. Specifically, we have shown increased expression of SOCS3 and to a lesser extent SOCS1 in response to cloprostenol, and this is coincident with decreased tyrosine phosphorylation (TyrP) and nuclear translocation of STAT5, many hours before a reduction in PRLR levels. Therefore, we hypothesize that the induction of SOCS proteins by PGF_{2 α} and the consequent inhibition of PRLR signal transduction via STAT5 are critical early events in functional luteolysis. In the present study, we aimed to characterize the expression of endogenous SOCS proteins and examine STAT5 signal transduction in the rat ovary during normal pregnancy.

Results

SOCS expression in the ovary during pregnancy

The mRNA expression of all SOCS examined (*Socs1*, *Socs2*, *Socs3*, and cytokine-inducible SH2-containing protein (*Cish*)) was the lowest on day 7 of pregnancy (Fig. 1). In general, SOCS mRNA expression significantly increased from early (day 7) to mid-pregnancy (day 10–13), and thereafter either remained at mid-pregnancy levels (*Socs1* and *Socs2*) or increased further during late pregnancy (*Socs3* and *Cish*). Nevertheless, mRNA expression of all SOCS examined was high in late

Figure 1 Ovarian SOCS mRNA expression increases during pregnancy. Total RNA was extracted from the ovaries of pregnant rats, reversed transcribed, and SOCS expression determined by real-time qPCR as described in Materials and Methods. SOCS results were normalized relative to β -actin and are expressed as the fold induction relative to day 7 of pregnancy. Samples were collected and processed as two separate sets (days 7–19 and days 19–21). Samples were obtained in the morning (1100–1200 h) on all days, with additional samples obtained at night (2300–2400 h) in late pregnancy (denoted P). Bars represent means $\pm$ S.E.M. ($n=5$ rats per day). Different superscripts denote a significant difference ($P<0.05$).

pregnancy (days 19–21). Overall, the induction of *Socs1*, *Socs2*, and *Cish* across pregnancy was more marked (20- to 30-fold) than the induction of *Socs2* (~4-fold).

For *Socs3*, a progressive increase in mRNA expression was observed across pregnancy, with significant ($P<0.05$) increases from day 7 to 13 and day 16–19 (Fig. 1). In late pregnancy (days 19–21), there was a trend (ANOVA, $P=0.06$) for increasing *Socs3* mRNA expression. Levels increased from the morning of day 19 into the evening of the same day, and remained high on day 20 before declining on the morning of day 21. However, there was marked variation in *Socs3* mRNA expression between animals in late pregnancy.

For *Cish*, like *Socs3*, significant increases in mRNA expression were observed during pregnancy, although an unusual pattern was observed in the second half of pregnancy. Increased *Cish* mRNA expression was observed from day 7 to 13, before a slight decrease on day 16, followed by a return to higher expression on the morning of day 19. Thereafter, *Cish* mRNA expression significantly ($P<0.05$) increased reaching maximal levels on the morning of day 20 and remained high through to day 21 (Fig. 1).

To confirm that the increase in *Socs3* and *Cish* mRNA expression during pregnancy results in increased protein expression, western blots were conducted on whole-cell extracts (Fig. 2). In general, induction of SOCS3 and CISH protein levels during pregnancy correlated with changes in mRNA expression described above. SOCS3 protein levels were very low on day 7 of pregnancy, significantly increased on day 13, further increased on day 19, and maximal on day 20 (Fig. 2A). Similarly CISH protein levels significantly increased during pregnancy, from low levels on day 7 to high levels in late pregnancy on days 19–21 (Fig. 2B).

TyrP of STAT5 (early to mid-pregnancy)

Since it is generally accepted that STATs increase SOCS expression, and then SOCS in turn act to limit further STAT signaling, it was of interest to examine endogenous STAT5 signaling in the ovary from early to mid-pregnancy. We therefore performed IP/WB on whole-cell extracts of ovaries from the same animals used for quantitative real-time (qPCR) above. Results show low amounts of TyrP STAT5 on day 7, while on days 10 and 13 TyrP STAT5 was readily detectable (Fig. 3A). Quantitation of all replicates revealed significantly ($P<0.05$) higher levels of TyrP on both days 10 and 13 compared with day 7 (Fig. 3A).

Does activation of PRLR in early pregnancy activate STAT5 and upregulate SOCS expression in the ovary?

At mid-pregnancy, increased endogenous TyrP of STAT5 together with increased SOCS expression in the ovary above is most likely a consequence of increasing

Figure 2 Ovarian SOCS3 and CISH protein levels increase during pregnancy and are maximal in late pregnancy. SOCS3 and CISH protein levels were determined by western blot on whole-cell extracts as described in Materials and Methods. Membranes were then stripped and reprobed for β -actin to confirm loading. Representative blots showing SOCS3 (A) and CISH (B) levels from day 7 to 21 and corresponding β -actin in same samples. Histograms show quantitation of western blots for all replicates. Results show the ratio of SOCS3 to β -actin and normalized relative to day 7 of pregnancy. Different superscripts denote a significant ($P<0.05$) difference. Bars represent means $\pm$ S.E.M. ($n=4-6$ rats per day).

secretion of PL acting via the PRLR. Since manipulating PL is difficult, we examined whether activation of the PRLR by exogenous PRL in early pregnancy (day 7) increased TyrP STAT5 and SOCS expression.

In vehicle-treated rats on day 7, TyrP STAT5 again was low and difficult to detect (Fig. 4A). However, at 30 and 60 mins after 125 μ g ovine PRL (oPRL) injection, prominent TyrP STAT5 was observed (Fig. 4A). Quantitation of all samples at each time point revealed that oPRL significantly ($P<0.01$) induced TyrP STAT5 compared to vehicle-treated animals at 30 and 60 mins, but the effect was transient with no significant treatment effect observed at 120 mins (Fig. 4B).

Figure 3 Tyrosine phosphorylation of STAT5 in rat ovary increases from early to mid-pregnancy, but declines during late pregnancy. Whole-cell extracts were immunoprecipitated for STAT5 before western blot with anti-phosphotyrosine antibody 4G10 as described in Materials and Methods. (A) Representative blots showing TyrP of STAT5 from individual animals (upper panel) and STAT5 loading (lower panel). (B) Quantitation of western blots for all replicates. Results show the ratio of TyrP STAT5 to STAT5 loading, and normalized relative to either day 7 or day 19 of pregnancy. Different superscripts denote a significant ($P < 0.001$) difference. Bars represent means $\pm$ S.E.M. ($n = 5$ –6 rats per day).

In the same animals, we also examined *SOCS* mRNA expression and surprisingly exogenous PRL had no effect on the mRNA expression of any *SOCS* at either 30 or 60 mins (Fig. 5). However, significant ($P < 0.05$) increases in *Socs1*, *Socs2*, and *Cish* mRNA expression were observed later at 120 mins, and notably *Cish* mRNA expression was increased more than 7.5-fold. In contrast, *Socs3* mRNA expression was not significantly induced by exogenous PRL up to 120 min after injection.

TyrP of STAT5 (mid- to late pregnancy)

As pregnancy proceeds, increasing levels of endogenous *SOCS* expression possibly function to negatively regulate responses to high circulating levels of PL. Indeed, previous studies have shown diminished responses to exogenous PRL after mid-pregnancy (Russell & Richards 1999). To confirm these results in our model, we examined TyrP of STAT5 in response to exogenous PRL in mid- and late pregnancy. In contrast to our results in early pregnancy (Fig. 4A and B), exogenous PRL did not increase TyrP of STAT5 60 min after injection on either day 13 or day 19 (Fig. 4C). Quantitation of all replicates revealed no significant effect on either day 13 (vehicle 1.00 ± 0.07 versus oPRL 0.84 ± 0.06) or day 19 (vehicle 1.00 ± 0.36 versus oPRL 0.91 ± 0.23) of pregnancy.

In late pregnancy, we propose that a reduction in luteotrophic support for the CL through STAT5 signaling is one of the mechanisms by which PGF_{2 α} induces luteolysis (Curlewis *et al.* 2002). Therefore, we examined that endogenous TyrP of STAT5 in the late pregnant animals also used for qPCR analysis of *SOCS* mRNA expression (day 19, 20, and 21 samples). For comparison, we also obtained samples from other animals on the

Figure 4 Exogenous PRL induces tyrosine phosphorylation of STAT5 in the ovary in early pregnancy (day 7), but not in mid- (day 13) or late (day 19) pregnancy. Rats on day 7 of pregnancy were given an s.c. injection of either vehicle or 125 μ g oPRL, and ovaries were collected at 30, 60, and 120 min thereafter. Similarly in mid- (day 13) and late pregnancy (day 19), rats were treated with oPRL but ovaries were collected only at 60 min post-injection. Ovarian whole-cell extracts were immunoprecipitated for STAT5 before western blot with anti-phosphotyrosine antibody 4G10 as described in Materials and Methods. (A and C) Representative western blots showing TyrP of STAT5 (upper panel) and STAT5 loading (bottom panel) from vehicle- or oPRL-treated animals. (B) Quantitation of western blots on day 7 of pregnancy. Results shown are the ratio of TyrP STAT5 to STAT5 loading, and normalized relative to vehicle. Different superscripts denote a significant ($P < 0.05$) difference. Bars represent means $\pm$ S.E.M. ($n = 4$ rats per treatment and time).

morning of day 18 of pregnancy. Representative blots show that TyrP of STAT5 was readily detectable on both days 18 and 19 (Fig. 3C). Quantitation of all replicates revealed that TyrP of STAT5 was not significantly different between days 18 and 19, but thereafter levels significantly ($P < 0.01$) declined on day 20, and further on day 21 (Fig. 3D).

Discussion

SOCS proteins are well established as negative regulators of cytokine signaling, with *SOCS1*–3 and *CISH* being important regulators of PRLR signal transduction via JAK2/STAT pathways. In this study, we have shown that expression of these *SOCS* significantly increases in the ovary as pregnancy progresses. Furthermore, in late pregnancy around the time of functional luteolysis, we observed reduced TyrP of STAT5 concurrent with maximal mRNA expression of *SOCS*. Given the critical

Figure 5 Exogenous PRL induces SOCS mRNA expression in the ovary during early pregnancy. Day 7 pregnant rats were given an s.c. injection of either saline vehicle or 125 µg oPRL, and ovaries were collected 30, 60, and 120 mins later. Total RNA was isolated, reversed transcribed, and SOCS genes amplified using real-time qPCR as described in Materials and Methods. Data are expressed relative to β -actin and normalized relative to vehicle treatment. Different superscripts denote a significant ($P < 0.05$) difference. Bars represent means $\pm$ S.E.M. ($n = 4$ rats per treatment and time).

importance of PRLR signal transduction through STAT5 in maintaining progesterone output by the CL of pregnancy in rodents, the present results are consistent with the idea that upregulation of SOCS proteins are a key element in PGF $_{2\alpha}$ -induced luteolysis.

SOCS proteins are acutely upregulated in response to cytokine signaling, and essentially act to limit further signaling through JAK2/STAT pathways. In the present study, the significant increase in *Socs1–3* and *Cish* mRNA levels in the ovary at mid-pregnancy (day 10 or 13) is most likely due to increasing secretion of PL. Around day 10 of pregnancy, luteotrophic support for the CL changes from PRL to PL, with termination of the twice-daily surges of pituitary PRL and replacement with increasing continuous release of PL (Freeman & Neill 1972). This change in ligand and/or pattern of secretion most likely increases PRLR-mediated signal transduction and SOCS expression. Indeed, we have found that increased SOCS expression at mid-pregnancy in the present study is associated with a significant increase in endogenous TyrP STAT5. This latter result is in accord with earlier studies that show increased constitutive STAT5 activity in the rat CL from day 10 of pregnancy (Russell *et al.* 1996, Russell & Richards 1999). Therefore, PL most likely increases PRLR signal transduction via STAT5 and increases SOCS expression. While no study has directly shown regulation of SOCS expression by PL, in late pregnancy, increased SOCS expression in the arcuate nucleus of the hypothalamus has been noted in response to both PRL (PL) and estrogen (Steyn *et al.*

2008). However, this induction of SOCS in the hypothalamus is dependent upon declining progesterone levels. This is marked contrast to our observations in the ovary, where increased SOCS expression is associated with high endogenous levels of PL, estrogen, and progesterone.

In the present study, we examined whether activation of the PRLR in early pregnancy would upregulate SOCS expression in the ovary. As expected, we observed that exogenous PRL rapidly (within 30 mins) induced TyrP of STAT5 in ovarian extracts on day 7 of pregnancy. However, surprisingly, the activation of STAT5 did not result in upregulation of SOCS mRNA expression by 60 mins, rather only later at 120 mins. Nevertheless, upregulation of SOCS mRNA expression at 120 mins was associated with decreased TyrP of STAT5 at this time, consistent with the idea that SOCS are negative regulators of STAT signaling. Most surprising was a lack of PRL-induced SOCS3 expression and rather minor (2.5-fold) responses in SOCS1 and SOCS2. Overall, these results contrast markedly with an earlier study (Tam *et al.* 2001), where oPRL injection rapidly (within 30 mins) and substantially (7- to 20-fold) induced ovarian SOCS mRNA expression. Differences in results likely reflect the animal models used, with the earlier study using non-pregnant lactating rats given bromocriptine to suppress endogenous PRL. In the present study, we did not use bromocriptine, but rather sampled animals in early pregnancy at a time of day (1100–1200 h) when circulating PRL concentrations are low, avoiding the endogenous PRL surges. Low endogenous SOCS expression in early pregnancy may be particularly important, as upregulation of ovarian SOCS1 and SOCS3 expression in the CL by proinflammatory cytokines has been recently linked to early pregnancy failure (Erlebacher *et al.* 2004).

As normal pregnancy progresses, increasing levels of endogenous SOCS expression in the CL may function to negatively regulate the cellular response to PL and thereby prevent overstimulation of the JAK2/STAT signal transduction pathways. Previous studies have established that administration of exogenous PRL does not increase STAT5 DNA binding in the CL after mid-pregnancy, when constitutive activity is present (Russell & Richards 1999). Moreover, constitutive nuclear STAT5 binding is itself reduced from day 17 (Russell & Richards 1999), despite continued elevated levels of PL around this time (Robertson & Friesen 1981). Essentially, as pregnancy progresses, the CL appears to develop a PRL-resistant state, at least in terms of PRLR signal transduction via STAT5. Our current SOCS results provide a possible physiological basis for this loss of STAT5 responsiveness in the CL. Previous work has shown that the luteal expression of key PRLR-signaling molecules, JAK2 and STAT5, is not limiting up to day 21 of pregnancy (Russell *et al.* 1996). Indeed, these authors conclude that in late pregnancy, the decrease in responsiveness of luteal

cells to PRL occurs at a point in the signaling pathway between the receptor and STAT proteins. We now suggest that the progressive increases in SOCS3 and CISH expression during pregnancy together with maintained (mid-pregnancy) levels of SOCS1 and SOCS2 expression, as noted in the present study, limit the responsiveness of the CL to PL as pregnancy proceeds.

In late pregnancy, the relatively high expression of all SOCS examined, together with a significant decline in TyrP STAT5 at this time, suggests that SOCS proteins may be an important element in PGF_{2α}-induced luteolysis. It is of interest to consider the pattern of SOCS expression relative to the time at which endogenous PGF_{2α} levels begin to increase. Most studies in rats indicate that PGF_{2α} is increased on day 19 or 20 of pregnancy and then further increases close to term (Labhsetwar & Watson 1974, Shaikh *et al.* 1977, Wilson *et al.* 1982). However, the exact timing of increased uterine PGF_{2α} secretion is difficult to determine due to the pulsatile pattern of PGF_{2α} release. Interestingly, some studies have shown small but significant increases in PGF_{2α} levels as early as day 16 of pregnancy (Puri & Garfield 1982). In relation to our SOCS results, we observed significant increases in both *Socs3* and *Cish* mRNA expression from day 16 to 19, then a further significant increase from day 19 to 20 for CISH. Furthermore, our western blot data show that SOCS3 and CISH protein levels are maximal in late pregnancy (days 19–21). In contrast, *Socs1* and *Socs2* mRNA expression did not significantly change at these times, remaining at mid-pregnancy levels. Therefore, these results suggest that PGF_{2α} may specifically upregulate SOCS3 and CISH expression in the CL. In support of this hypothesis, we have previously shown by immunohistochemistry that both SOCS3 and CISH are predominantly localized to the CL in the rat ovary (Tam *et al.* 2001, Curlewis *et al.* 2002), and moreover that administration of the PGF analog cloprostenol to rats on day 19 causes a rapid and sustained increase in SOCS3 mRNA and protein expression in luteal cells (Curlewis *et al.* 2002). However, cloprostenol did not increase *Cish* mRNA expression. While this may suggest that high levels of endogenous CISH expression observed in the present study are not due to PGF_{2α}, it should be noted that here we quantitated *Cish* mRNA by qPCR, a far more sensitive method than by northern blot used in our previous study (Curlewis *et al.* 2002). Future studies examining the induction of SOCS in luteal cells in response to PGF_{2α} would be useful. Nevertheless, functional luteolysis may require the combined inhibitory actions of a number of SOCS proteins. Interestingly, a line of SOCS2-deficient mice exhibits a small delay (about 1 day) in parturition, but otherwise gives birth normally (Cargill *et al.* 2000, Corva *et al.* 2004). In contrast, CISH null mice display no obvious reproductive phenotype, while an essential role of

SOCS3 in luteolysis remains unknown, as SOCS3 null mice die *in utero* (Marine *et al.* 1999). Further work closely examining the luteal function in SOCS knock-out mice, particularly mice deficient in several SOCS genes, may reveal redundant roles for individual SOCS proteins in luteolysis. Also characterization of endogenous SOCS expression in the CL of pregnant PGF_{2α} receptor knockout mice would be helpful.

One mechanism of action of SOCS proteins, which may be important in functional luteolysis, is the targeting of PRLR for proteasomal degradation (Zhang *et al.* 1999). Indeed, as mentioned earlier, downregulation of PRLR appears to be an important part of PGF_{2α}-induced luteolysis. However, this only occurs immediately prior to parturition (Telleria *et al.* 1997). Furthermore, while PRLR downregulation undoubtedly limits the luteotrophic effects of PRL/PL, we suggest that a reduction in PRLR is an event subsequent to and a consequence of increased SOCS expression. We have previously shown that cloprostenol induces SOCS expression and inhibits TyrP and nuclear translocation of STAT5 some 8 h before any loss in PRLR expression (Curlewis *et al.* 2002). In the present study, we show that endogenous TyrP of STAT5 is initially reduced on day 20 of gestation, 1 day before any decrease in PRLR (Telleria *et al.* 1997). Also PRLR expression itself is thought to be a downstream effect of PRLR signal transduction by STAT5 (Galsgaard *et al.* 1999). Therefore, our hypothesis is that the inhibition of PRLR signal transduction through STAT5 is caused initially by PGF_{2α}-induced SOCS expression, followed by a downregulation of PRLR expression and these events reduce luteotrophic support for the CL.

Materials and Methods

Materials

oPRL-20 was obtained from the National Hormone and Peptide Program (Baltimore, MD, USA). Goat anti-SOCS3 (sc 7009), rabbit anti-STAT5 (sc 835X), and rabbit anti-CISH (sc 15344) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Mouse monoclonal anti-β-actin (A 5441) was obtained from Sigma-Aldrich. ECL peroxidase anti-mouse antibody (NA931VS) and ECL peroxidase anti-rabbit antibody (NA931VS) were obtained from Amersham Pharmacia Biotech. HRP-conjugated rabbit anti-goat antibody (31402) was purchased from Pierce Chemical Co. (Rockford, IL, USA). Anti-phosphotyrosine 4G10 (28818) was obtained from Upstate (Lake Placid, NY, USA). Analytical reagent grade chemicals were purchased either from the Sigma-Aldrich or Ajax Chemical Co. (Auburn, NSW, Australia).

Animals

Wistar rats were housed at 24 °C with a 12 h light:12 h darkness cycle (lights on 0500–1900 h) and allowed free excess to rat chow and water. For mating, females (8–10 weeks of age) were housed overnight with males and checked the following morning

for a vaginal plug (designated as day 1 of pregnancy). In our colony, Wistar rats normally give birth on day 21 or 22. Rats were killed by spinal dislocation at various times during pregnancy (between days 7 and 21) between 1100 and 1200 (morning) to avoid the endogenous PRL surges. In late pregnancy, additional samples were obtained between 2300 and 2400 h (evening). In some experiments, pregnant rats were given a s.c. injection of 125 µg oPRL or vehicle (saline) at 1000–1030 h and killed 30–120 min later. Following death, the ovaries were quickly removed and trimmed of adhering fat and connective tissue. Ovaries were then dissected in half by counting corpora lutea and then one half from each ovary was pooled together for either RNA or protein extraction. During dissection of the ovary, most interstitial tissue was removed before the remaining tissue (principally corpora lutea) was frozen on solid CO₂ and stored at –80 °C until required. Animals with less than five corpora lutea on an ovary were not used. These experiments were approved by the University of Queensland Institutional Animal Ethics Committee according to National Health and Medical Research Councils (Australia) guidelines.

RNA extraction and RT

Total RNA was isolated from ovarian tissue using TRIzol Reagent (Gibco BRL) as previously described (Curlewis *et al.* 2002). Samples were DNase treated before RT using Superscript II preamplification kit according to the manufacturer's instructions (Gibco BRL) with random hexamer primers.

Quantitative real-time PCR

For qPCR, cDNA was diluted 1:20 in water and PCR performed using 5 µl diluted cDNA, 3 µM and 2 µM Taqman probes for β-actin, and each SOCS respectively 2 µM primers and 13 µl Taqman Master Mix in a 25 µl volume. *Cish* forward primer 5'ccgactggactccaattgct3', *Cish* reverse primer 5'ctgcacaaggctgaccacat3', *Cish* probe 6-FAM-agacctgaatcctgccttcca3'-TAMRA; *Socs1* forward primer 5'ccgtgggtcgcgagaac3', *Socs1* reverse primer 5'aaggaaactcaggtagtcacggagta3', *Socs1* probe 6-FAM-tggcgcgcacatcccttaacc3'-TAMRA; *Socs2* forward primer 5'aagcacgcgaggtcctttg3', *Socs2* reverse primer 5'gatgagtaacacgtccaagaca3', *Socs2* probe 6-FAM-ccccagctcggcgaccac3'-TAMRA; *Socs3* forward primer 5'aaatccagc-cccaatgg3', *Socs3* reverse primer 5'ggcctgaggaagaagcctatc3', *Socs3* probe 6-FAM-cggtagctcccagttagcccagcag3'-TAMRA; β-actin forward primer 5'ttaacaccccagccatgt3', β-actin reverse primer 5'cagtgtacgaccagaggcata3', β-actin probe 6-FAM-cgtagccatccagctgtgtgtccc3'-TAMRA. Each sample was analyzed in triplicate by qPCR using the ABI prism 7000 Taqman Real-Time PCR machine (Applied Biosystems, Foster City, CA, USA). Negative controls were included in all qPCR runs by substituting cDNA with RNase-free water. Analysis was performed by normalizing the SOCS signal to β-actin for each sample, then levels were expressed as the relative change (comparative C_t method) compared with levels on either day 7 (early pregnancy) or day 19 (late pregnancy), or vehicle treatment depending on the experiment. Coefficients of variation within sample triplicates were <1%.

Protein extraction, immunoprecipitation, and western blots

Frozen ovarian tissue was homogenized in TK lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton-X-100, 30 mM NaF, 10 mM Na₂P₂O₇, 10 mM EDTA, and 1 mM Na₂VO₃) with complete protease inhibitor cocktail (#1697498, Roche). Homogenates were centrifuged at 15 000 g for 15 mins at 4 °C and the supernatant removed, and stored at –80 °C until required. Total protein in tissue lysates was determined using the bicinchoninic acid protein assay as per the manufacturer's instructions (Pierce Chemical).

Protein A beads were washed twice in PBS and blocked in 1% BSA in TBS with 0.1% Tween-20 (TBST) for 1 h at room temperature with rotation. Beads were washed in PBS and in TK lysis buffer without inhibitors (TK–), and 2 µg of STAT5 antibody (sc 835X) in TK– were bound overnight at 4 °C with rotation. Following this, beads were washed in PBS and twice in TK–, before the addition of 0.5 mg of cell supernatant protein in TK+ buffer for 2 h at 4 °C with rotation. Beads were again washed thrice in TK+, all traces of liquid were removed from the beads, and bound proteins were eluted in sample buffer (15 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 10 mM dithiothreitol) at 100 °C for 5 mins. Samples were loaded onto 8% SDS-PAGE gels and electrophoresed for 2 h at 90 V in 1×SDS Lamelli buffer.

After electrophoresis, gels were equilibrated in transfer buffer (15% methanol in 1×SDS buffer) before being transferred onto PVDF membrane by an electroblotter (Amersham Pharmacia Biotech). Membranes were blocked in 2% BSA in TBST for 1 h at room temperature, before being incubated overnight with primary antibody (phosphotyrosine antibody, 4G10) at 1:1000 dilution in blocking buffer with rocking at 4 °C. Membranes were then washed in TBST, and incubated with secondary antibody (ECL peroxidase anti-mouse antibody at 1:10 000) for 2 h at room temperature with rocking. Membranes were washed as before, and ECL Plus Western Blot detection system (Amersham Pharmacia Biotech) used as per the manufacturer's instructions to develop signal. To determine STAT5 protein loading, membranes were incubated in stripping buffer (2.8 ml 1 M Tris, 42.2 ml deionized water, 5 ml of 20% SDS, and 350 µl β-mercaptoethanol, pH 6.8) for 20 mins at 60 °C, extensively washed in TBST, then reprobed with primary STAT5 antibody (sc 835X) at 1:10 000 overnight at 4 °C before secondary antibody (ECL peroxidase anti-rabbit antibody at 1:5000), and developed as described above.

For SOCS3 and CISH western blots, 50 µg of ovarian lysates were electrophoresed on a 12% SDS-PAGE gel and then transferred on to PVDF membranes. The membranes were blocked with 5% skin milk in TBST, before being incubated with primary goat anti-SOCS3 antibody (sc 7009) at 1:500 or rabbit anti-CISH (sc 15344) at 1:1000 both diluted in blocking buffer. Thereafter, membranes were incubated with either HRP-conjugated rabbit anti-goat (1:5000) or ECL peroxidase anti-rabbit (1:10 000) secondary antibody diluted in blocking buffer. To ensure equal loading of protein in each sample, membranes were stripped and reprobed with anti-β-actin (A 5441) antibody, followed by secondary ECL peroxidase anti-mouse antibody (1:10 000) and development as described above.

Statistical analysis of data

One-way ANOVA was used to analyse the data, followed by *post hoc* Newman–Keuls tests to examine individual comparisons. Data were log-transformed before analysis to remove heterogeneity of variance where appropriate. Values were considered statistically significant at $P < 0.05$.

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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