Luteotrophic effects of relaxin, chorionic gonadotrophin and FSH in common marmoset monkeys (Callithrix jacchus)

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Abstract

In early pregnant primates, relaxin (RLX) is highly upregulated within the corpus luteum (CL), suggesting that RLX may have an important role in the implantation of the blastocyst. Therefore, the aim of the present study was to investigate the local effects of RLX and gonadotrophins on the maintenance of the CL using an in vitro microdialysis system. CLs of common marmoset monkeys were collected by luteectomy during different stages of the luteal phase and early pregnancy. Each CL was perfused with either Ringer’s solution alone or Ringer’s solution supplemented with either porcine RLX (250, 500 and 1000 ng/ml) or gonadotrophins (50 IU/ml). Application of RLX provoked a significant luteal response of progesterone (P4) and oestradiol (E2) secretions during the mid-luteal phase (500 ng/ml: P4 54 ± 42%, E2 24 ± 11%; 1000 ng/ml: E2 16 ± 13%), and especially during the late luteal phase (250 ng/ml: P4 53 ± 10%; 500 ng/ml: P4 44 ± 15%; 1000 ng/ml: P4 62 ± 15%, E2 18 ± 7%). The effects of RLX on steroid secretion were irrespective of the RLX dosages. While treatment with human chorionic gonadotrophin did not affect luteal steroid or RLX secretion, the application of FSH resulted in a significant increase in the secretion of both P4 (20 ± 8%) and E2 (37 ± 28%), and a prominent rise in RLX during early pregnancy. In conclusion, our results demonstrate that RLX and FSH have a luteotrophic function in the marmoset monkeys; moreover, FSH has a function beyond its traditional role just as a follicle-stimulating hormone.

Reproduction (2010) 139 923–930

Introduction

In the common marmoset monkey (Callithrix jacchus), relaxin (RLX) is produced within the corpus luteum (CL; Steinetz et al. 1995, Einspanier et al. 1997, 1999). Once the CL is formed, there is a steadily increasing level of RLX gene and protein expression throughout the luteal phase with a highest level in the CL of early pregnancy; whereas the regression CL shows a decline in RLX expression (Einspanier et al. 1997). The fact that RLX is an important factor for CL maintenance could be supported by the significant increase in peripheral RLX levels in conceptive cycles compared with the non-conceptive cycles (Einspanier et al. 1999), suggesting a possible role for RLX in the maintenance and establishment of the CL during early pregnancy. A recently published paper provided strong evidence that RLX is an important factor for the implantation of the blastocyst in the common marmoset monkey (Einspanier et al. 2009), because both RLX and its receptor are highly upregulated in the uterus during the time of implantation. Since the plasma concentrations of RLX remain relatively low (1 ng/ml) throughout implantation in primates, we hypothesized that RLX may act in a paracrine fashion in the CL and the placenta. While the role of RLX in parturition (uterine source) is well described, the diverse actions of RLX in the CL are still unknown. The role of RLX in the CL needs further investigation, especially in light of the newly described functions of RLX. The novel functions include RLX as a promoter of the decidualization of the endometrial stromal cells (Bani et al. 1995), as a stimulant of angiogenesis in the uterus (Unemori et al. 1999) and as a remodelling factor for the extracellular matrix (Samuel et al. 2007). This remodelling action of RLX has been reported for different organs, such as the reproductive tract (Bryant-Greenwood et al. 2007), kidneys (Samuel et al. 2007), heart (Dschietzig et al. 2006), arteries (Novak et al. 2006) and mammary glands (Binder et al. 2004). A similar function of RLX in CL maintenance is therefore conceivable.

Although chorionic gonadotrophin (CG) seems to be of fundamental importance for human pregnancy (Keay et al. 2004), it is only one of the factors necessary for a healthy pregnancy. In humans, 62% of the pregnancies diagnosed with increased CG do not reach term (Edmonds et al. 1982). Therefore, the aim of the present
study was to examine the local effect of RLX on the secretion of ovarian steroid hormones. We examined this effect by perfusing an explant of a CL from a common marmoset monkey with RLX during different stages of the luteal phase using an in vitro microdialysis system (MDS). Furthermore, our studies examined whether the luteal secretions of steroid hormones and RLX are influenced by the gonadotrophins. These gonadotrophins include human CG (hCG) and FSH. The MDS is a well-established system, which has been used in many species for investigating the autocrine and paracrine mechanisms in the CLs, for which cell-to-cell contacts are a paramount feature allowing proper function.


**Results**

**Dialyzed corpora lutea**

We used CLs of the mid-luteal phase (days 11–13) and late luteal phase (days 14–15) as well as regressing CLs (days 14–17) and CLs of pregnancy (days 15–40). A detailed assignment of the CLs to the luteal phases is described in the Materials and Methods part. Morphological examinations of the perfused tissue samples, which were harvested after 8.3 h of the MDS experiments, showed an intimate association of the perfusion membrane with the luteal tissue (Fig. 1A). The luteal cells that were juxtaposed to the microdialysis membrane were visually intact and intensively stained for 3β-hydroxysteroid dehydrogenase (HSD3B, Fig. 1B–D) activity; however, the cells of the CL that were at an advanced regression stage did not show HSD3B staining.

Over the dialysis period of 8.3 h, all the control CLs showed a steady progesterone (P₄) and oestradiol (E₂) secretion (data not shown), which demonstrates the validity of luteal explants in in vitro MDS experiments. The baseline P₄ secretion (means ± S.D.) of the perfused CLs was 73 ± 35, 83 ± 29 and 45 ± 34 pg/ml during the mid-luteal phase, late luteal phase and regression respectively. The E₂ secretion was 199 ± 132, 157 ± 24 and 195 ± 110 pg/ml respectively.

There was no significant correlation between plasma P₄ and baseline P₄ secretion of the CLs (n = 13 animals, correlation coefficient = 0.406, P = 0.168); moreover, there was no significant correlation between plasma E₂ and baseline E₂ secretion of the CLs (n = 14 animals, correlation coefficient = −0.045, P = 0.877).

**Relaxin application**

In general, we observed a significant effect of RLX on P₄ and E₂ hormone secretion in the CL. During the mid-luteal phase, RLX applications of 250, 500 and 1000 ng/ml resulted in a progressive increase in the secretion of P₄ compared with the baseline levels. RLX applications of 250, 500 and 1000 ng/ml increased luteal secretion of P₄ by 38 ± 44% (P = 0.187, n = 4), 54 ± 42% (P = 0.045, n = 5) and 102 ± 86% (P = 0.058, n = 5) respectively (Fig. 2). The most likely time for...
implantation is around day 11 after ovulation, which corresponds with the mid-luteal phase. During this time, we found that the highest RLX dose (1000 ng/ml) resulted in the highest mean P₄ secretion (102%); however, ANOVA did not show significant differences between the effects of the various RLX dosages on P₄ secretion. On days 14 and 15 of the luteal phase, all the different RLX concentrations resulted in significant increases in P₄ secretion compared with the baseline levels (250 ng/ml: 53 ± 10%, P = 0.012, n = 3; 500 ng/ml: 44 ± 15%, P = 0.039, n = 3; 1000 ng/ml: 62 ± 15%, P = 0.018, n = 3). In the regressed CL, RLX application caused a significant change in P₄ secretion only when 500 ng/ml of RLX was used (250 ng/ml: 20 ± 31%, P > 0.05, n = 3; 500 ng/ml: 18 ± 15%, P = 0.035, n = 6; 1000 ng/ml: 43 ± 63%, P = 0.153, n = 6). Importantly, in all CL stages, no significant differences were observed in P₄ secretion between the baseline levels just before the RLX applications of 250, 500 and 1000 ng/ml and the secretion levels after the end of the RLX applications (data not shown).

During the RLX applications, the increase in E₂ secretion by the CL was less pronounced than the increase in P₄ secretion (Fig. 3). In contrast to the effect of RLX on P₄ secretions, the response in E₂ secretion appeared to be more sensitive to RLX as the lowest concentration had already evoked the maximal, but not significant, response in the mid-luteal phase CL (250 ng/ml: 31 ± 24%, P = 0.157, n = 3; 500 ng/ml: 24 ± 11%, P = 0.009, n = 5; 1000 ng/ml: 16 ± 13%, P = 0.050, n = 5). However, the resulting differences between the effects of the various dosages of RLX on E₂ secretion did not reach the level of significance (ANOVA, P > 0.05). During the late luteal phase, only 1000 ng/ml RLX (18 ± 7%, P = 0.042, n = 3) had a significant effect on E₂ secretion. During regression, no significant changes in E₂ secretion were observed irrespective of the RLX concentration (250 ng/ml: 5 ± 20%, P > 0.05, n = 3; 500 ng/ml: 3 ± 28%, P > 0.05, n = 6; 1000 ng/ml: 11 ± 27%, P > 0.05, n = 6). In contrast to the secretion of P₄, a prolonged rise in E₂ secretion could be observed in the mid-luteal phase CL after the end of 1000 ng/ml RLX application (9 ± 7%, P = 0.045), and in the regressed CL after the end of 500 ng/ml RLX application (10 ± 6%, P = 0.009) compared with the baseline levels. In all other cases, no prolonged rise in E₂ was detectable after the end of RLX application (data not shown).

**Gonadotrophin application**

The influence of the gonadotrophins hCG and FSH on luteal hormone secretion was tested from early pregnancy through day 40 of pregnancy (Fig. 4). We found that recombinant human FSH had a significant luteotrophic effect on both P₄ (20 ± 8%, P = 0.009, n = 5) and E₂ (37 ± 28%, P = 0.018, n = 5) secretions. On the other hand, hCG did not have a significant effect on P₄ (9 ± 17%, P > 0.05, n = 3) or on E₂ (26 ± 15%, P > 0.05, n = 3) secretion. There was a prominent rise in RLX secretion (239 ± 334%, P > 0.05, n = 3) during FSH application; however, this was not statistically significant. This is probably due to the high variability in the results in...
Discussion

In this study, we showed that RLX has a luteotrophic action on in vitro microdialyzed marmoset CLs throughout the different stages of the luteal phase. The highest RLX-induced P₄ secretions occurred in the mid-luteal and late luteal phase CLs, which is important because the mid-luteal phase is the most likely time for the implantation to occur. Whereas the adaptable nature of the CL begins to regress with tissue reorganization during the late luteal phase, it retains a functional lifespan during pregnancy of ~144 days in the common marmoset monkey. Despite being less pronounced, the E₂ response to RLX in the mid-luteal phase CL was more sensitive than that of P₄, as even the lower RLX dosages gave a maximal response compared with the highest RLX dosage, which resulted in the lowest response. This sensitivity of E₂ under RLX application seems to be necessary for successful implantation, while the balanced and temporal secretion of this steroid is important for angiogenesis and immunotolerance (Segerer et al. 2008).

Multiple studies have shown that P₄ from the CL is required to maintain early pregnancy. P₄ has many roles, one of which is the remodelling of the proliferating endometrium, where RLX is also involved. The importance of luteal RLX in the marmoset is also implied by circulating RLX levels: low levels are observed during the follicular phase and increasing levels are observed during the luteal phase. Moreover, significantly higher RLX levels are detectable during the mid-luteal and late luteal phases of animals in concepive cycles compared with the animals in the non-conceptive cycles (Einspanier et al. 1999). These results are in accordance with our MDS data, suggesting that sufficient levels of ovarian RLX must be secreted during the mid-luteal and late luteal phases in order to allow appropriate placental development and implantation of the blastocyst. The luteotrophic stimulus of RLX may augment the local effects of P₄ on luteal cells, such as the suppression of apoptosis to maintain CL function (Okuda et al. 2004) and activation of angiogenesis (Jeyabalans et al. 2007).

Moreover, P₄ also induces immunotolerance (Segerer et al. 2008), which can be further modulated by oestrogens (Segerer et al. 2008). Therefore, the luteal E₂ produced in our mid-luteal and late luteal phase-dialyzed CLs during RLX application is likely to have an additive effect on the immunotolerance. Ravindranath & Moudgal (1990) demonstrated that oestrogens are necessary for a successful establishment of pregnancy during the peri-implantation period in the bonnet monkey (Macaca radiata). Adding E₂ to P₄ for luteal support resulted in a significant increase in implantation and pregnancy rates in human patients undergoing IVF cycles (Farhi et al. 2000). Luteal effects of E₂ in the marmoset monkey are supported by the presence of the oestrogen receptor in the ovaries (Saunders et al. 2001) and the expression of 17β-hydroxysteroid dehydrogenase type 7 (HSD17B7) in the CL (Husen et al. 2003), an enzyme which converts oestrone to E₂.

Another enzyme for steroidogenesis, HSD3B, which is necessary for P₄ synthesis, was also detectable in the dialyzed CL at the end of the perfusion, supporting the idea that luteal tissues are still functional after MDS experiments. Cellular integrity of marmoset CLs following MDS experiments was also observed by electron microscopy, where luteal cells lying in a sheath of five to seven cell layers around the dialysis tubing were shown to be intact and interconnected by gap junctions (Fehrenbach et al. 1995). Despite the parallel increases in RLX and CG levels during early pregnancy, the precise relationship between both hormones is still uncertain. Exogenous applications of hCG to women (Quagliarello et al. 1980) or rhesus monkeys (Ottobre et al. 1984, 1991, Duffy et al. 1996) resulted in an increase in RLX secretion, which was partly delayed. These results contrast the findings obtained here as well as those reported for human luteal cell culture experiments (Schmidt et al. 1986) or in vivo studies with marmosets (Einspanier et al. 1999) failing to demonstrate hCG-induced RLX secretion. This inconsistency may indicate that a constant hCG
influence is needed, and that different CG preparations, purity or concentrations have been used.

While treatment of CLs with hCG did not affect the RLX, P₄ or E₂ secretion, the application of FSH resulted in a significant increase in both P₄ and E₂ secretions during the dialysis of the early pregnant CLs. Moreover, FSH provoked a high, but statistically not significant, increase in RLX secretion. The high sample variability might be caused by CLs being taken from animals, which were in the range of days 15–40 of pregnancy. Thus, our results clearly demonstrate that FSH acts as a gonadotrophin with a luteotropic effect, and that FSH has a function beyond its conventional allocation as a FSH only, as suggested by several authors, e.g. Kumar (2009). This assumption is supported by the detection of the FSH receptor in the human CL (Minegishi et al. 1997).

Furthermore, bioactive FSH levels were shown to be elevated only in the mid-luteal to late luteal phase of the human menstrual cycle (Christin-Maitre et al. 1996). Therefore, it seems likely that FSH takes over some LH functions in the primate CL. For example, in the hamster, FSH stimulates the LH receptors in the CL (Yuan et al. 1995), and FSH as well as LH stimulates P₄ production in hamster luteal cells (Yuan et al. 1995).

To discuss the high sample variability conclusively, we also calculated alongside the present analysis (relative change in secretion in percentage) the absolute change in secretion in pg/ml. Both methods yielded similar results; however, stronger significance was obtained by the results of the relative changes. However, a high variability still occurred, which might be due to interindividual endocrine variation or the range of days of luteectomy for each group, and therefore, different maturations of the CL. Interestingly, the plasma steroid concentrations of each marmoset also show this high variability within each group. In conclusion, our results demonstrate that the CL responds to RLX by secretion of P₄ and E₂ during the mid-luteal and late luteal phases. We propose that RLX and E₂ support early pregnancy in conjunction with P₄ to orchestrate the processes that allow for successful implantation, and to retain a functional CL thereafter. Our findings also highlight the importance of prolactin and estrogen in the marmoset CL during early pregnancy, suggesting that FSH may perform some of the functions usually ascribed to LH in other mammals.

Materials and Methods

Animals

Female marmoset monkeys (C. jacchus; n=17; mean±s.d., age 5.0±1.8 years, body weight 427±66 g) were paired with male partners. Animals were housed in individual cages under controlled and standardized conditions (12 h light:12 h darkness; temperature 25 °C and humidity 63%) at the German Primate Centre (Göttingen, Germany). The nutrition for the monkeys was provided in the form of marmoset pellets (SSNIFF, Soest, Germany) in combination with seasonal fruits and vegetables.

The marmoset monkey is a polyovulatory species (Tardif et al. 1993) with a cycle length of 28 days (10 days of follicular phase, 18 days of luteal phase, term of pregnancy ~144 days; Hodges et al. 1983). On average, implantation occurs on day 11 after ovulation (Hearn et al. 1988). For the ovarian cycle analysis and pregnancy detection, 0.2-ml blood samples were taken from the vena femoralis twice per week. Plasma P₄ concentrations were determined by direct enzyme immunoassay (Heiestermann et al. 1993, 1998) in order to monitor the ovarian cycles. Ovulation occurs shortly before the P₄ level rises above 10 ng/ml (Harlow et al. 1984). A luteolytic dosage of prostaglandin F₂α (0.8 µg cloprostenol, Estrumate; Pitman-Moore Inc., Mundelein, IL, USA) was administered between days 10 and 14 of the luteal phase to induce luteolysis, and to help identify the timing of the subsequent ovulation accurately (Summers et al. 1985). Before the start of the experiments, the ovaries and the uterus were examined and measured by ultrasound using a transducer with 11–13 MHz (Logiq 400; General Electrics, Fairfield, CT, USA) as described previously by Einspanier et al. (2006).

All animal experiments were approved by the local ethics committee for animal rights protection Brunswick, Germany, in accordance with German legislation on animal rights and welfare (file reference number 509.42502/0803.99).

Tissue collection

As the marmoset is polyovulatory, two or three CLs are usually present in each cycle. Therefore, if possible, the CLs of an individual animal were allocated to each of the three different RLX concentrations or gonadotrophins as described in the following paragraph ‘in vitro MDS’. The collection of the CLs (n=46 from 17 animals) was carried out between days 11 and 17 during the luteal phase or till day 40 of early pregnancy. The animals were sedated with an i.m. injection containing a mixture of ketamine (Parke Davis, USA; 5 mg/350 g body weight) and xylazine (Rompun, Bayer; 1 mg/350 g body weight), and narcotized with a halothane–nitrous oxide–oxygen mixture (Baxter, Unterschleißheim, Germany). Laparotomy was performed, and the tissue was removed by luteectomy as described by Webley et al. (1989). The CLs were staged retrospectively to the different luteal phases by plasma P₄ determination twice per week after ovulation, and the ensuing analysis of the plasma P₄ on the day of luteectomy. Stable or increasing plasma P₄ concentrations after at least 10 days of ovulation dated the explanted CLs to the mid-luteal or late luteal phase. Declining P₄ concentrations presented regressing CLs at least 14 days after ovulation. On the day of luteectomy, the mean±s.d. plasma P₄ concentration was 395±276, 225±118 and 83±34 ng/ml for the mid-luteal phase (days 11–13), late luteal phase (days 14–15) and regression (days 14–17) respectively. Pregnancy (days 15–40) could be detected with ultrasound as early as day 15, as pregnant monkeys exhibit an increased uterus lumen from day 15 forward (Nubbemeyer et al. 1997).
analyzed by direct enzyme immunoassay for P4, E2 and RLX physiological concentrations in the luteal tissues (Einspanier & Hodges 1994). After the application of the test substances, the CLs were dialyzed for another 3 h to observe the secretion pattern after the application of the test substances. To check the validity of the luteal explants in the MDS experiment, seven control CLs were dialyzed with Ringer’s solution and LDL only. The collected fractions were stored at −20 °C until analyzed by direct enzyme immunoassay for P4, E2 and RLX (Heistermann et al. 1993, 1998, Einspanier et al. 1999). RLX was only examined in the group of CLs where we examined luteal stimulation by the gonadotrophins. To control the half-life of RLX, all RLX test substances of each experimental run were analyzed in the RLX assay thereafter.

After perfusion, the CLs were fixed in 3.7% neutral buffered formalin. The functional integrity of the tissue was confirmed histologically by haematoxylin and eosin staining, and immunohistochemically by examining the protein expression of HSD3B in the cytoplasm, one of the essential enzymes in the P4 synthesis pathway. A detailed description of processing and sectioning of the tissue samples has been published by Einspanier et al. (1997).

**Statistical analysis**

All hormone values presented in the text are given as mean ± 1 s.d. of the indicated groups’ data. An unpaired, two-tailed Student’s t-test was used to evaluate the statistical differences between two groups, while a one-way ANOVA was used for testing for differences between more than two subgroups. For all tests, a P < 0.05 was considered statistically significant.

In order to test for potential correlation between plasma P4 and baseline P4 secretion of the perfused CL, measurements of P4 secretion at baseline were averaged over all CLs and all fractions in each animal. Pearson’s correlation analysis was performed including all animals irrespective of the group. Test for correlation between plasma E2 and baseline E2 secretion of the CLs was performed analogously.

**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.

**Funding**

The project was supported by DFG grant Ei 333/11-3.

**Acknowledgments**

The authors would like to thank Dr Monika Ziegler (German Primate Centre) and Susanne Rensing (Covance) for the veterinary support, and Kerstin Fuhrmann (German Primate Centre), Angelika Jurdzinski (German Primate Centre), Alexandra Marten (German Primate Centre) and Susanne Tätzner (University of Leipzig) for technical assistance.

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Received 23 June 2009
First decision 27 August 2009
Revised manuscript received 5 January 2010
Accepted 15 February 2010