KiSS-1 in the mammalian ovary: distribution of kisspeptin in human and marmoset and alterations in KiSS-1 mRNA levels in a rat model of ovulatory dysfunction

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THE HYPOTHALAMIC-PITUITARY-OVARIAN AXIS is a complex neuro-hormonal system responsible for the regulated secretion of ovarian hormones and the cyclic release of fertilizable oocytes at ovulation (39, 47). The latter is triggered by the preovulatory surge of LH, evoked by the positive feedback effects of estrogen at midcycle (22). This hormonal surge induces the sequential expression of a series of genes in the ovary (8, 23, 50), which finally leads to the release of the cumulus-oocyte complex (COC) to the periovulatory space. The central mechanisms responsible for the generation of the preovulatory surge of gonadotropins (22, 28), and the subsequent molecular events in the ovary, have been actively investigated recently.

Proteolytic degradation of the extracellular matrix is mandatory for successful ovulation, and several proteolytic enzymes, including plasminogen activator-plasmin and matrix metalloproteinase (MMP) systems, have been involved in this process (4, 32, 52). Additionally, protease inhibitors, such as plasminogen activator inhibitors (PAIs) and tissue inhibitors of MMPs (TIMPs), are also concomitantly expressed during ovulation (4, 52). Indeed, the balance between proteolytic and antiproteolytic activities appears essential for the selective rupture of follicular apex, and conditions suspected to disturb such a balance are known to cause ovulatory dysfunction. Thus in cycling rats treated with the nonsteroidal anti-inflammatory drug indomethacin, a potent inhibitor of cyclooxygenase-1 (COX-1) and COX-2, follicle rupture occurs frequently at the basolateral follicular areas, with the COC being released to the ovarian stroma (16, 17, 19). In this model, degradation of the perifollicular tissues, invasion of blood and lymphatic vessels, degradation of the ovarian bursa, and invasion of the periovular fat pad can be observed (17, 19). Such limited but detectable invasive capacity of granulosa and cumulus cells after COX blockade partially resembles tumor dissemination and trophoblast invasion of the maternal decidua. Whether the genes restraining cell invasion in tumor metastasis and placentation formation also play also a role in preventing perifollicular invasion during normal ovulation is yet to be evaluated.

KiSS-1 was originally identified as a metastasis-suppressor gene that encodes a family of structurally related peptides, termed kisspeptins, which acting via the G protein-coupled receptor 54 (GPR54) were shown to prevent tumor spread (33, 36, 37, 40). In addition, a role for kisspeptins in controlling trophoblast invasion was suggested (2). However, such anti-metastatic and anti-invasive activities were recently “eclipsed”...
by the demonstration of the fundamental roles of KiSS-1/ kisspeptins and GPR54 in reproductive physiology (36, 37, 40). Indeed, in the last 4 yr, KiSS-1 neurons in the forebrain have been demonstrated to play key functions in the dynamic control of gonadotropin secretion along the life span (20, 36, 37, 40), kisspeptins being very potent elicitors of LH and FSH secretion in different mammalian species, including humans (5, 6, 21, 27, 29, 30, 37, 42). Specifically in the female, the hypothalamic KiSS-1/GPR54 system seems to operate as a central conduit not only for the negative, but also for the positive, feedback regulation of gonadotropins, thereby playing a substantial role in the generation of the preovulatory surge of LH (24, 38, 44).

While the contention that kisspeptins act primarily at central (hypothalamic) levels to regulate ovarian function is well defined, the possibility of additional effects at other sites of the hypothalamic-pituitary-ovarian axis cannot be ruled out on the basis of the published experimental evidence (38). In fact, initial analyses in rodents suggested the expression of KiSS-1 and GPR54 genes in the rat ovary (48). In addition, discernible kisspeptin and GPR54 immunoreactivity was recently demonstrated in ovarian tissue sections from cyclic rats, where KiSS-1 gene expression was shown to fluctuate in a cycle-dependent manner under the regulation of pituitary LH (3). Likewise, cyclic KiSS-1 gene expression and kisspeptin-IR has been demonstrated in rat oviduct (18). Moreover, ovarian expression of GPR54 has been also recently documented in fish (10, 31). Taken together, these observations are suggestive of a potential (and, likely, evolutionary conserved) role of kisspeptins in the local control of ovarian function (38). Yet, the physiological relevance of such a role, if any, and whether the elements of the KiSS-1/GPR54 system are also expressed in the primate ovary remain unexplored to date. In this context, we report herein expression analyses of KiSS-1 and GPR54 in the human and marmoset monkey ovary and present evidence for altered ovarian expression of KiSS-1 gene in a rat model of ovulatory dysfunction due to systemic administration of indomethacin.

MATERIALS AND METHODS

Tissue (Human/Marmoset) Samples

Human samples. Sections from normal human cyclic ovaries were obtained from the files of the Department of Pathology of University of Córdoba, upon approval of the local Committee of Bioethics. Tissues had been fixed in phosphate-buffered formaldehyde and routinely processed for paraffin embedding. Ovaries from oophorectomized women due to uterine pathology, who did not show ovarian disease, were not undergoing hormonal therapy, and displayed normal cyclicity, were used. The phase of the cycle was assigned by considering the menstrual history and dating of the endometrium and cyclicity, were used. The phase of the cycle was assigned by considering the menstrual history and dating of the endometrium and

Experimental (Rat) Studies

Adult Wistar female rats bred in the vivarium of University of Córdoba were used. The animals were maintained under constant conditions of light (14 h of light, from 0700) and temperature (22°C). Experimental procedures were approved by Córdoba University Ethical Committee for animal experimentation and conducted in accordance with the European Union normative. In all experiments, the animals were monitored for estrous cyclicity by daily vaginal cytology; only rats with at least two consecutive regular 4-day estrous cycles were used for subsequent studies. The animals were killed by decapitation at the end of the experimental procedures, when ovaries were removed and processed for morphometric analysis or RNA isolation (see below). Unless otherwise stated, all reagents were obtained from Sigma (St. Louis, MO).

As model of ovulatory dysfunction, female rats were treated with inhibitors of COX-1 and/or COX-2 (16, 17), and ovaries were collected for gene expression analysis and/or assessment of ovulation. In experiment 1, the effects of indomethacin, a nonselective COX inhibitor, on the expression levels of a panel of ovulation-related genes in the ovary were studied at the periovulatory period. Groups of cyclic female rats (n = 6) were subcutaneously injected with indomethacin (1.0 mg/rat) or vehicle in the morning of proestrus (1000) of proestrus, and ovarian samples were obtained upon decapsulation of the animals in the evening of proestrus (2100) or early morning of estrus (0200), the latter being roughly coincident with ovulation. Ovarian tissue was obtained also from cyclic females (not treated with indomethacin) at the morning of proestrus and estrus. In all groups, ovaries were dissected out of the surrounding fat pad, snap frozen in liquid nitrogen, and stored at −80°C until used for mRNA analyses.

Considering that previous data from our group demonstrated the ability of choriogonadotropin (hCG) to acutely increase KiSS-1 mRNA levels in rat ovary (3), and given the inhibitory effects of indomethacin on KiSS-1 mRNA levels detected in our initial experiments, in experiment 2, the effects of indomethacin on hCG-induced KiSS-1 mRNA expression in the ovary were explored. Cyclic female rats were subcutaneously injected in the morning of proestrus (900) with an effective dose of the potent gonadotropin-releasing hormone (GnRH) antagonist ORG 30276 (GnRH-ANT, 5 mg/kg; Organon, Oss, The Netherlands) and were subsequently treated at 1200 with an ovulatory bolus of hCG (25 IU/rat sc). Thereafter, groups of GnRH-ANT/hCG-treated animals (n = 6) were subcutaneously injected with indomethacin (1.0 mg/rat) or vehicle and were sequentially killed by decapsulation after 1, 3, 6, and 12 h for ovarian sampling.

The effects of selective COX inhibitors on ovulatory efficiency and KiSS-1 gene expression were compared with those of indomethacin in experiment 3. Groups (n = 6) of cyclic female rats, treated in the morning of proestrus (1100) with an effective dose of GnRH-ANT, were subsequently injected subcutaneously at 1300 with indomethacin...
(1.0 mg/rat), the selective inhibitor of COX-1 SC560 (10 mg/kg), or the selective inhibitor of COX-2 NS398 (10 mg/kg). The animals received at 1700 an ovulatory dose of hCG (25 IU/rat sc) and were killed by decapitation after 3 h (2000). In addition, groups of treated females (n = 6) were maintained until the morning of estrus, when ovaries were embedded in paraffin and serially sectioned for morphometric assessment of ovulation efficiency (17).

Finally, we aimed to provide functional evidence for the association between altered prostaglandin synthesis (after COX-2 inhibition) and disturbed KiSS-1 mRNA expression. To this end, in experiment 4, cyclic female rats (n = 6), injected with indomethacin (1.0 mg/rat) in the morning (1000) of proestrus, were subsequently treated with two subcutaneously boluses of PGE2 (500 μg/injection; at 1900 and 2000) or vehicle, and ovarian samples were obtained in the evening of proestrus (2100). Ovarian tissue from preovulatory females not treated with indomethacin served as controls. In addition, in experiment 5, ovarian KiSS-1 mRNA levels were assayed in another model of ovulatory failure, the RU486-treated female rat. Cyclic female rats (n = 6) were subcutaneously injected in the morning (1000) of proestrus with an effective dose of RU486 (2 mg/rat) or vehicle, and ovarian samples were obtained in the evening (2100) of proestrus.

Analysis of RNA Expression by RT-PCR

Total RNA was isolated from human ovarian fragments and cultured granulosa-lutein cells using TRIzol reagent, following the instructions of the manufacturer. Samples of rat ovarian tissue were processed for RNA isolation as described previously (3). In human samples, qualitative detection of KiSS-1 and GPR54 mRNA was conducted by RT-PCR, using primer pairs and conditions included in Table 1; coamplification of the 240-bp fragment of S11 ribosomal protein mRNA was used as internal control. In rat ovarian samples, expression of KiSS-1 and GPR54 mRNAs was assessed by RT-PCR, optimized for semiquantitative detection, using primer pairs and conditions described in Table 1, in keeping with our previous reference (3). Expression of the genes encoding COX-2 and progesterone receptor (PR-AB) was also assayed, following previously published protocols (see Table 1), as these genes are induced in rat ovary by the preovulatory surge of LH (25, 35). As an internal control for RT and reaction efficiency, amplification of a 290-bp fragment of L19 ribosomal protein mRNA was carried out. For semiquantitative analyses, the number of amplification cycles was chosen for each target based in our previous optimization curves, testing different numbers of PCR cycles, to attain exponential amplification conditions. Specificity of PCR products was confirmed by direct sequencing (Central Sequencing Service, University of Cordoba, Cordoba, Spain). When relevant, quantification of the intensity of RT-PCR signals was carried out by

Table 1. Oligo-primer pairs and PCR conditions used for amplification of KiSS-1, GPR54, and RP-S11 mRNAs in the human ovary, as well as KiSS-1, GPR54, COX-2, and PR-AB transcripts in the rat ovary

<table>
<thead>
<tr>
<th>Target</th>
<th>Oligo Primers</th>
<th>Expected Size</th>
<th>PCR Cycles/Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human KiSS-1</td>
<td>Sense</td>
<td>350 bp</td>
<td>38/56°C</td>
</tr>
<tr>
<td>Sense</td>
<td>Antisense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human GPR54</td>
<td>Sense</td>
<td>118 bp</td>
<td>37/63°C</td>
</tr>
<tr>
<td>Sense</td>
<td>Antisense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human RP-S11</td>
<td>Sense</td>
<td>240 bp</td>
<td>26/58°C</td>
</tr>
<tr>
<td>Sense</td>
<td>Antisense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat KiSS-1</td>
<td>Sense</td>
<td>202 bp</td>
<td>34/62.5°C</td>
</tr>
<tr>
<td>Sense</td>
<td>Antisense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat GPR54</td>
<td>Sense</td>
<td>197 bp</td>
<td>34/62.5°C</td>
</tr>
<tr>
<td>Sense</td>
<td>Antisense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat COX-2</td>
<td>Sense</td>
<td>244 bp</td>
<td>28/55°C</td>
</tr>
<tr>
<td>Sense</td>
<td>Antisense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat PR-AB</td>
<td>Sense</td>
<td>326 bp</td>
<td>33/57.5°C</td>
</tr>
<tr>
<td>Sense</td>
<td>Antisense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat RP-L19</td>
<td>Sense</td>
<td>290 bp</td>
<td>24/55°C</td>
</tr>
<tr>
<td>Sense</td>
<td>Antisense</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For each target, the primer pair used for amplification is included. In addition, the expected size of the generated cDNA products, number of cycles selected for exponential amplification, and annealing temperature for RT-PCR analysis are indicated for each signal. KiSS-1, kisspeptin gene; GPR54, G protein-coupled receptor 54; PR-AB, progesterone receptor; COX-2, cyclooxygenase-2.
densitometric scanning using an image analysis system (1-D Manager; TDI, Madrid, Spain), and values of the specific targets were normalized to those of internal controls. In all assays, liquid controls and reactions without RT resulted in negative amplification.

**Kisspeptin Immunohistochemistry**

Archival samples of cyclic human ovaries, as well as fixed marmoset ovaries, were used. Processing of marmoset ovaries was as described in detail elsewhere (3, 49), followed by standard paraffin embedding. Ovarian sections (5-μm thick) were incubated overnight with a primary anti-human kisspeptin-13 (4–13) antibody (Bachem, Bubendorf, Switzerland) that recognizes all forms of kisspeptins sharing the common 10 amino acid terminal region. Final dilution of the antibody was 1:800 in keeping with previous studies in the rat ovary (3). The sections were processed according to the avidin-biotin-peroxidase complex technique, as described elsewhere (46). As positive controls, first trimester human placental samples (generously supplied from the archives of the Pathology Department of the

![Fig. 2. Immunolocalization of kisspeptins in the cyclic human (A–E) and marmoset (F–J) ovaries. In human follicles (A), cytoplasmic immunostaining was restricted to steroidogenic theca cells, whereas in mature corpora lutea (B) strong immunoreactivity was observed in both theca-lutein and granulosa-lutein cells. Intense kisspeptin immunoreactivity was also detected in secondary interstitial cells (C), hilar cells (D), and ovarian surface epithelium (E). In the marmoset monkey ovary, kisspeptin immunostaining was detected in the theca layer of antral follicles (F and G), luteal tissue (F and H), secondary interstitial cells (I), and ovarian surface epithelium (J) in a pattern similar that found in the human ovary (hematoxylin counterstaining).](http://ajpendo.physiology.org/)
University of Cordoba) were used, as previously described (3). In addition, immunohistochemical controls included omission of the primary anti-kisspeptin antibody and reactions after preabsorption of the antiserum overnight at 4°C with 1 μg/ml of mouse kisspeptin (KiSS-1 (112–121)-NH₂; Phoenix Pharmaceuticals, Belmont, CA); procedures that completely abolished kisspeptin immunolabeling.

GPR54 Immunohistochemistry

The peptide sequence CVLGEDNAPL in the carboxyl terminal tail domain of the human GPR54 was custom synthesized by EZ Biolab (Westfield, IN) as immunogen. This sequence is not present in any other proteins in the human genome. The peptide was conjugated via the cysteine to keyhole limpet hemocyanin, and 1 mg was injected into two rabbits in Freund’s complete adjuvant, followed by further booster immunizations of 1 mg in Freund’s incomplete adjuvant at 2, 4, and 7 wk. The rabbits were bled out (30 ml) at 10 wk and sera precipitated with 33% (NH₄)SO₄, reconstituted in PBS, dialyzed against PBS, and lyophilized. The titer in ELISA was >1:6,000 for both antisera. Antiserum 1210 was used for the findings reported, but antiserum 1212 gave the same results (data not shown). The specificity of the antisera was demonstrated by absence of staining in different ovarian compartments after preabsorption of antisera with the peptide immunogen and when the preimmune serum was used.

Immunohistochemistry on ovarian tissue was conducted as follows. Tissue was fixed in 4% neutral buffered formaldehyde or 4% paraformaldehyde before paraffin waxing. Five-micrometer paraffin sections were cut and mounted onto superfrost plus slides (Thermo Shandon). Sections were dewaxed and rehydrated following standard procedures. After quenching of endogenous peroxidase activity, slides were incubated overnight with primary antibody at a 1:750 dilution. Sections were then serially incubated with biotinylated swine anti-rabbit IgG (Dako, Glostrup, Sweden; 1:500 dilution) and streptavidin-peroxidase (Dako; 1:1,000 dilution) and were visualized with diaminobenzidine, as per manufacturers instructions.

Presentation of Data and Statistics

For gene expression analysis in rat studies, semiquantitative RT-PCR assays were carried out in duplicate from at least four independent RNA samples of each experimental group. Semiquantitative data are presented as means ± SE. Results were analyzed for statistically significant differences using one- or two-way ANOVA, followed by Student-Newman-Keuls multiple range test (SigmaStat 2.0). P < 0.05 was considered significant.

RESULTS

KiSS-1/GPR54 Gene Expression and Pattern of Kisspeptin- and GPR54 Immunoreactivity in Human and Marmoset Ovary

Expression of KiSS-1 and GPR54 genes was demonstrated in the cyclic human ovary, as well as in cultured granulosa-lutein cells (Fig. 1). RT-PCR amplification of human ovarian RNA, using a primer pair designed to amplify a 350-bp fragment of KiSS-1 mRNA, resulted in the generation of an amplicon of expected size, the identity of which was confirmed.
by direct sequencing. Likewise, positive amplification, of correct size and sequence, was obtained using a primer pair spanning a 118-bp fragment of GPR54 cDNA. Similar RT-PCR profiles were obtained for KiSS-1 and GPR54 transcripts in RNA samples from cultured human granulosa-lutein cells.

The presence and pattern of cellular distribution of kisspeptins in the human and marmoset ovary were demonstrated by immunohistochemistry, using a specific polyclonal antibody raised against human kisspeptin-10, an antiserum that was used previously for immunodetection of kisspeptins in human placenta and rat ovary (3). This antibody recognizes all forms of kisspeptin sharing the C-terminal region. As a positive control, strong cytoplasm staining was detected in syncytiotrophoblast cells of placental villi from 3-mo-old human placenta (data not shown).

Clear-cut kisspeptin-IR was detected in the human cyclic ovary, where specific signals were mainly located at parenchymal steroidogenic cells. In detail, in growing follicles, kisspeptin-IR appeared in the theca layer of growing follicles, whereas granulosa cells were negative (Fig. 2A). After ovulation, im-

Fig. 4. Expression of KiSS-1, GPR54, progesterone receptor (PR)-AB, and cyclooxygenase-2 (COX-2) genes in the rat ovary along the proestrus-to-estrus transition, after systemic administration of indomethacin (INDO) to cyclic female rats. A: representative RT-PCR reactions are shown of ovarian samples taken at the evening of proestrus (P2100) and the early morning of estrus (E0200), from animals treated or not with indomethacin, are shown. For comparative purposes, reactions from samples taken from cyclic females at the morning of proestrus (P1000) and the morning of estrus (E1000) are also presented. For each group, 3 representative independent samples are shown. Parallel amplification of L-19 ribosomal protein mRNA served as internal control. B: semiquantitative values of mRNA levels of the different targets are means ± SE of at least 6 independent determinations. Groups with different superscript letters are statistically different (P < 0.05, ANOVA followed by Student-Newman-Keuls multiple range test). OD, optical density.
immunostaining was also present in the nonfully luteinized granulosa cells of just-ruptured postovulatory follicles. Similarly, both theca-lutein and granulosa-lutein cells in mature corpora lutea showed intense immunostaining (Fig. 2B). Otherwise, the stromal component of the corpus luteum was negative. In addition, strong kisspeptin-IR was present in androgen-producing cells, i.e., in the hypertrophic theca cells in atretic follicles (data not shown) and its derivatives, the secondary interstitial cells (Fig. 2C), as well as in hilus Leydig cells (Fig. 2D). Finally, while the ovarian stroma was negative, strong immunostaining was detected in the ovarian surface epithelium (Fig. 2E).

In the marmoset ovary, the cellular pattern of kisspeptin-IR was grossly similar to that of the human ovary. Strong immunostaining was present in the theca layer of antral follicles (Fig. 2, F–G) and, after ovulation, in steroidogenic luteal cells (Fig. 2, F and H). Kisspeptin-IR was also detected in interstitial cells derived from atretic follicles (Fig. 2I), as well as in the ovarian surface epithelium (Fig. 2J). In contrast, the ovarian stroma was negative for kisspeptin-IR.

In addition to the ligand, immunodetection of the canonical kisspeptin receptor GPR54 was conducted in ovarian samples using a polyclonal antiserum raised against the human receptor. Of note, this antibody did not appear to properly recognize the antigen in marmoset tissues. This could be due to the fact that the amino acid sequence CVLGEDNAPL in the C-terminal region of the human GPR54, used as immunogen, corresponds to CVLGEAGAPL in the monkey, where the heavily charged DN sequence is substituted by the neutral AG residues (underlined). Thus our analyses were solely conducted in human ovaries. Moreover, immunostaining for GPR54 seemed highly sensitive to tissue fixation conditions, and archival material gave barely detectable signal and rather variable results. Accordingly, immunostaining was carried out in freshly isolated, adequately fixed sparse follicles. In these preparations, theca cells of growing follicles showed clear-cut GPR54 immunoreactivity, whereas the granulosa layer appeared negative (Fig. 3A). Intense GPR54 immunoreactivity was also detected in mature corpora lutea (Fig. 3B). In detail, strong cytoplasmic immunostaining was observed in steroidogenic granulosa-lutein cells, while the signal was negligible in theca-lutein cells (Fig. 3C). Specificity of GPR54 immunoreactivity was confirmed by omission or preadsorption (Fig. 3D) of the primary antibody, which consistently abolished luteal immunostaining. Of note, intense immunolabeling was also noticed in irregularly shaped stromal cells that were particularly abundant at the inner border of the granulosa-lutein layer (Fig. 3C), cells that corresponded to macrophages, as indicated by parallel immunostaining with the specific marker CD68 (Fig. 3E). Yet, the nature of this signal is not totally clear as, in contrast to other ovarian cell types and compartments, staining of macrophages was not fully prevented by preadsorption of the primary antibody with the peptide. The fact that our antiserum did not properly recognize marmoset GPR54 made it impossible for us to check if this phenomenon is also detectable in other species.

**Expression of KiSS-1 and Ovulation-Related Genes in a Rat Model of Ovulatory Dysfunction**

In addition to expression analyses in the human and marmoset, we conducted functional studies aimed at providing (indirect) evidence for the putative roles of the ovarian KiSS-1/GPR54 system in the local control of ovulation. To this end, the expression levels of KiSS-1, GPR54, and other ovulation-related genes, such as COX-2 and progesterone receptor (PR-AB), were explored in a rat model of ovulatory dysfunction, namely the cyclic female treated with indomethacin (19). Changes in mRNA levels of target genes were selectively assayed at the periovulatory period, namely at 2100 of proestrus and 0200 of estrus. For reference purposes, expression analyses were also conducted in ovaries from control females at the morning (1000) of proestrus and estrus. As shown in Fig. 4, relative levels of KiSS-1 and GPR54 mRNAs in control ovaries remained rather invariant along the periovulatory period. Conversely, the expression of the transcript encoding both A and B forms of progesterone receptor, as well as COX-2 mRNA levels, rose significantly at the periovulatory period and were dramatically suppressed thereafter. Of note, treatment of cyclic females with indomethacin in the morning of proestrus failed to induce any detectable change in the pattern of ovarian GPR54, PR-AB, and COX-2 mRNA expression along the proestrus-to-estrus transition. In striking contrast, relative expression levels of KiSS-1 in the ovary were dramatically suppressed at 2100 proestrus and 0200 estrus by pretreatment with indomethacin (Fig. 4).

Given the clear-cut inhibitory effects of indomethacin on ovarian KiSS-1 mRNA levels, we explored its ability to blunt hCG-induced KiSS-1 overexpression. Injection of hCG at 1200 proestrus resulted in the expected increase in KiSS-1 mRNA levels in the ovary (3), which peaked at 3 h and progressively declined thereafter. In clear contrast, cotreatment with an effective dose of indomethacin fully prevented the rise in ovarian expression of KiSS-1 mRNA after hCG stimulation (Fig. 5).

**Effects of Selective COX Inhibitors on Ovarian KiSS-1 Gene Expression and Ovulation**

To dissect out the relative contribution of COX-1 and COX-2 pathways to the above phenomenon, the effects of
indomethacin were compared with those of the selective inhibitor of COX-1 SC560 and the selective blocker of COX-2 NS398. In this experiment, the effects of the inhibitors on KiSS-1 mRNA levels in the ovary (at 3 h after administration) were correlated with their impact on ovulatory efficiency (21 h after treatment). As observed in control cyclic females, rats treated with the selective COX-1 inhibitor displayed no alterations of the ovulatory process, with a preserved number of effective ovulations (i.e., oocytes released at the oviduct) and no oocytes being trapped in corpora lutea or released to the ovarian stroma. In clear contrast, in all rats treated with indomethacin or the selective inhibitor of COX-2, a significant decrease in the percentage of effective ovulations was noticed, the magnitude of which was higher in indomethacin-treated rats (>50% decrease vs. ~25% reduction in females treated with the selective COX-2 inhibitor). Likewise, trapping of expanded COCs inside luteinized follicles and, to a lesser extent, oocytes released to the ovarian stroma were frequently seen after indomethacin or NS398 treatments (Fig. 6). Of note, these results in adult females are roughly analogous to those obtained previously in gonadotropin-primed immature rats (13) and provide the basis for our gene expression analysis. In keeping with ovulatory data, ovarian KiSS-1 mRNA levels remained unaltered after systemic administration of SC560 but were equally and significantly decreased after treatment with either indomethacin or the COX-2 inhibitor NS398 (Fig. 7A).

Effects of PGE₂ on Ovarian KiSS-1 Expression

Replacement experiments, testing the effects of PGE₂ administration to indomethacin-treated rats, were also carried out. As shown in Fig. 7B, indomethacin injection in the morning of proestrus evoked the expected decrease in ovarian KiSS-1 mRNA levels at proestrus evening. Such a lowering in KiSS-1 levels was fully prevented by coadministration of two boluses of an effective dose of PGE₂ (15). Further proof for the selectivity of the effects of COX-2 inhibitors was provided by the demonstration that ovarian KiSS-1 expression is not altered in proestrus rats after treatment with the anti-progestagen RU486 (Fig. 8), a treatment known to induce ovulatory defects distinct from those of indomethacin (13).

DISCUSSION

To our knowledge, this study provides the first comprehensive overview of the pattern of expression of KiSS-1/kisspeptins and GPR54 in the human and nonhuman (marmoset)
primate ovary. As we (3) previously demonstrated in the rat, KiSS-1 and GPR54 genes are expressed in the cyclic human ovary, as well as in human granulosa-lutein cells. In good agreement, kisspeptin- and GPR54 immunoreactivities were also detected in human (kisspeptin/GPR54) and marmoset (kisspeptin) ovaries. Of note, the pattern of cellular distribution of kisspeptin-IR within ovarian tissues was grossly similar between the human and monkey. Moreover, the location of kisspeptin and GPR54 immunoreactivity seems to also be similar to that in the rat ovary (3). The above commonalities are not only suggestive of a conserved function of the KiSS-1/GPR54 pair in the local control of ovarian function but also support the usefulness of our experimental approach where joint expression analyses in primates and functional studies in rodent models might help to provide an integral view of the ovarian KiSS-1/GPR54 system in mammals.

The physiological relevance of ovarian expression of KiSS-1 and GPR54 is yet to be fully defined but based on its pattern of cellular distribution and hormonally regulated expression in the rat ovary, a potential role of kisspeptins in the local modulation of ovulation was hypothesized (3, 38). This possibility was further suggested by the previous demonstration of the ability of kisspeptins to regulate protease activity in certain cell systems (2, 51), matrix proteases being crucially involved in the ovulatory process (4, 32). To shed light on such putative role, functional expression analyses of KiSS-1 and GPR54 genes were implemented in a rat model of ovulatory dysfunction induced by systemic administration of COX inhibitors (17, 19). The pivotal role of COX-2 in ovulation has been well established by a number of experimental studies that demonstrated that ovarian expression of COX-2 gene is induced by the preovulatory surge of gonadotropins (43, 45) and pharmacological blockade of COX-2 disrupts ovulation (19). Of note, detailed morphometric analyses proved recently that, instead of merely preventing follicular rupture, indomethacin causes dysorganization of the process of selective proteolysis of follicular apex (16, 17, 19). Given the documented ability of kisspeptins to modulate MMP-2 and MMP-9 expression in other cellular systems (2, 51), we found this model specially suited for the assessment of potential roles of the KiSS-1/GPR54 system in the local modulation of ovulation.

Our studies demonstrated that both periovulatory (Fig. 4, A and B) and hCG-induced (Fig. 5A) expression of KiSS-1 gene in the rat ovary is strongly inhibited by indomethacin, i.e., after blockade of COX activity. This effect seems to be specific, as...
it was rescued by PGE$_2$ replacement but not observed in an unrelated model of ovulatory failure, as in the RU486-treated rat. Moreover, indomethacin failed to alter the expression of other ovulation-related genes, such as COX-2 itself and progesterone receptor, the mRNA levels of which were clearly induced at the time preceding ovulation, in keeping with previous observations (35, 43), but were not disturbed by COX inhibition. Likewise, GPR54 mRNA levels remained unaltered after indomethacin injection. Although not exhaustive, this set of data is suggestive of the potential association between dysregulated KiSS-1 expression at the time preceding ovulation and the severe impairment of ovulatory efficiency after treatment with indomethacin. This effect seems to be selectively mediated by inhibition of COX-2, as its blockade, but not that of COX-1, mimicked the actions of indomethacin. The molecular mechanisms underlying the potential COX-2-mediated regulation of KiSS-1 expression in the ovary, and its subsequent impact on the ovulatory process, remain to be settled. Yet, from a physiological standpoint, the demonstration of the concurrent decrease in ovarian KiSS-1 expression and disturbed ovulation, despite otherwise normal neuroendocrine events, as evidenced by preserved induction of COX-2 and progesterone receptor genes at the proestrous-to-estrus transition, indirectly points out that temporal and spatial dynamics in local kisspeptin expression might play a role in the tuning of the ovulatory process.

The indispensable role of hypothalamic KiSS-1 and GPR54 in the neuroendocrine control of reproduction in general, and ovulation in particular, has been well settled in the last years (37); a major hierarchical role that is not disputed by our present data. Nonetheless, the fact that GnRH/gonadotropin replacement seemed sufficient to rescue the profound hypogonadal state of GPR54-null humans and mice might have “obscured” additional, less prominent, roles of kisspeptins and GPR54 at other levels of the gonadotropic axis. Concerning the ovary, the relevance of local kisspeptin/GPR54 signaling is questioned by the fact that women and mice with GPR54 inactivation were successfully induced to ovulate after standard gonadotropin priming (mouse) or GnRH therapy (human) (34, 41), observations that preclude an indispensable role of direct kisspeptin actions for ovulation. However, the efficiency of the ovulatory process after GnRH/gonadotropin priming in the absence of GPR54 signaling has not been thoroughly studied. Thus it remains possible, although yet to be proven, that in spite of detectable oocyte release after protocols of pharmacological stimulation, GPR54-null females may show subtle (qualitative and/or quantitative) alterations in the ovulatory process, such as a decreased percentage of successful ovulation and/or dysregulated follicular rupture. Indeed, the conserved patterns of kisspeptin and GPR54 expression in the primate and rodent ovary, as well as the regulated expression of KiSS-1 gene by key players in the control of ovulation, such as gonadotropins and COX-2, strongly suggest that, although not strictly indispensable for oocyte release, ovarian kisspeptin may participate in the fine tuning of some facets of the ovulatory process.

Besides their potential involvement in the control of ovulation, the patterns of tissue distribution of kisspeptins and GPR54 in primate and rodent ovaries are strongly suggestive of regulatory roles on other ovarian functions, the nature and actual relevance of which remain, as yet, unexplored. These might include 1) active tissue remodeling of the follicular and luteal compartments of the ovary along the cycle (7, 45); 2) cell migration and remodeling at the ovarian surface epithelium during postovulatory wound repair (1); and 3) hormone production by steroidogenically active cells of the human ovary, such as theca and luteal cells, as well as the interstitial gland and hilus cells. Detailed structural and functional analyses of the ovaries of gonadotropin-primed GPR54$^{-/-}$ mice might help to unravel the roles, if any, of local kisspeptins in the modulation of these functions in the female gonad.

In summary, we present herein compelling evidence for the expression, at the gene and peptide levels, of KiSS-1 and GPR54 in the human and nonhuman primate ovary, with obvious similarities with the patterns reported previously in the rat ovary (3). In addition, we document the capacity of inhibitors of COX-2, which induce severe alterations of the ovulatory process, to selectively suppress KiSS-1 expression in the rat ovary at the time of ovulation or after gonadotropin stimulation. Although the indispensable role of the hypothalamic KiSS-1 system in the central control of ovarian function remains undisputed, our data are suggestive of a conserved, likely subordinated, role of local KiSS-1 in the direct control of ovarian physiology in mammals, the relative importance of which awaits to be fully elucidated.

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