Kisspeptin is involved in ovarian follicular development during aging in rats

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Abstract

We have previously reported that kisspeptin (KP) may be under the control of the sympathetic innervation of the ovary. Considering that the sympathetic activity of the ovary increases with aging, it is possible that ovarian KP also increases during this period and participates in follicular development. To evaluate this possibility, we determined ovarian KP expression and its action on follicular development during reproductive aging in rats. We measured ovarian KP mRNA and protein levels in 6-, 8-, 10- and 12-month-old rats. To evaluate follicular developmental changes, intraovarian administration of KP or its antagonist, peptide 234 (P234), was performed using a mini-osmotic pump, and to evaluate FSH receptor (FSHR) changes in the senescent ovary, we stimulated cultured ovaries with KP, P234 and isoproterenol (ISO). Our results shows that KP expression in the ovary was increased in 10- and 12-month-old rats compared with 6-month-old rats, and this increase in KP was strongly correlated with the increase in ovarian norepinephrine observed with aging. The administration of KP produced an increase in corpora lutea and type III follicles in 6- and 10-month-old rats, which was reversed by P234 administration at 10 months. In addition, KP decreased the number and size of antral follicles in 6- and 10-month-old rats, while P234 administration produced an increase in these structures at the same ages. In ovarian cultures KP prevented the induction of FSHR by ISO. These results suggest that intraovarian KP negatively participates in the acquisition of FSHR, indicating a local role in the regulation of follicular development and ovulation during reproductive aging.

Key Words

- kisspeptin
- follicular development
- ovarian aging

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Introduction

Reproductive senescence in mammals is poorly understood. Over the last few decades, women have increasingly postponed pregnancy until their 30s. The decrease in the birth rate index with a concomitant delay in motherhood has resulted in decreased fertility and unsuccessful IVF programs (Faddy *et al.* 1992, O'Connor *et al.* 1998, te Velde *et al.* 1998). Because of these problems, it is necessary to understand the mechanisms of follicular development during the subfertility period and ovarian aging. One of the key players in the regulation of reproductive function is kisspeptin (KP). This peptide acts through its receptor Kiss1R (or GPR54), which is a G-protein coupled receptor (Pinilla *et al.* 2012). KP participates in the ovulation process through the activation of GnRH

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neurons (Navarro *et al.* 2004, Seminara 2005). It has been demonstrated that the intracerebroventricular administration of KP induces luteinizing hormone (LH) secretion (Thompson *et al.* 2004, Castellano *et al.* 2005). Additionally, mRNA and immunohistochemistry analyses have demonstrated the presence of Kiss1R and KP in the stroma of GnRH neurons in the preoptic area and in axon terminals at the median eminence (Yamada *et al.* 2007, Uenoyama *et al.* 2011). Furthermore, the presence of KP and its actions have been demonstrated in many peripheral organs (Vikman & Ahren 2009, Pinilla *et al.* 2012) such as the ovary. KP has been found in the ovaries of different species, including humans and rats (Castellano *et al.* 2006, Ricu *et al.* 2012). For example, ovarian KP mRNA levels change throughout the estrus cycle of the rat, with the highest levels occurring during proestrus (Castellano *et al.* 2006). Previously, we showed that the *in vivo* administration of a KP antagonist (P234) to the ovarian bursa of 22- to 50-day-old rats delayed vaginal opening and decreased the percentage of estrous cyclicity (Ricu *et al.* 2012). These results supported a role for locally produced ovarian KP in the ovulatory process (Gaytan *et al.* 2009). In contrast, rat luteal cells stimulated *in vitro* with KP secreted progesterone via ERK1/2 signaling (Peng *et al.* 2013). Similar studies were performed in chicken granulosa cells. In these studies, it was also demonstrated that the presence of KP increased progesterone production, in addition to increasing the level of mRNA for StAR, P450scc and 3β-HSD, all of which are steroidogenesis regulatory proteins (Xiao *et al.* 2011). Recently, it was demonstrated that KP receptor haplo-insufficiency in mice caused premature ovarian failure, resulting in the progressive loss of oocytes, preantral and antral follicles (Gaytan *et al.* 2014). Altogether, these data suggest a potential role for KP in the local control of ovarian function. By contrast, it has been described that with ovarian aging, women present a diminished follicular reserve (Faddy *et al.* 1992, O'Connor *et al.* 1998) and an increase in ovarian sympathetic nerves (Heider *et al.* 2001). The rat model also presented an increase in ovarian adrenergic sympathetic tone with aging (Chavez-Genaro *et al.* 2007, Acuna *et al.* 2009), and this finding was correlated with an increase in the cystic structures and a decrease in ovulation (Acuna *et al.* 2009), effects that were reversed under pharmacological β-adrenergic blockade (Fernandois *et al.* 2012). Because we recently described through *in vitro* studies that KP levels increased under β-adrenergic stimulation (Ricu *et al.* 2012), it is possible that the ovarian KP content also increases during ovarian aging. Therefore, because it was postulated that KP plays a role in folliculogenesis, this potential increase in KP may participate in changes in the follicular dynamics that occur during the subfertility period.

Materials and methods

Animals

A total of 129 female Sprague-Dawley rats (six (26 animals), eight (44 animals), ten (21 animals) and 12 months old (eight animals)) and 30 rats (5 days old) were used in this study. The animals were maintained in individual cages at 23 °C in a 12 h light:12 h darkness cycle. Food and water were available *ad libitum*. The rats were sacrificed at the end of each procedure on the morning of estrus by decapitation. The ovaries of 6- and 10-month-old rats were removed and fixed in Bouin's fixative solution. The ovaries of 8-month-old rats were immediately incubated in DMEM/F-12 or frozen at −80 °C. The Institutional Ethics Committee of the Faculty of Chemistry and Pharmaceutical Sciences of the Universidad de Chile, under protocol CBE2012 and in accordance with the guidelines of the CONICYT (Guide for the Care and Use of Laboratory Animals), approved all of the animal procedures.

Intraovarian administration of KP or P234

The administration of KP or P234 (a KP antagonist) to the ovaries was performed according to the procedure previously described (Ricu *et al.* 2012). In summary, mini-osmotic ALZET pumps (model 2004, 0.25 μl/h, Alza Corp. Palo Alto, CA, USA) were connected to the underlying bursa of the right ovary with SILASTIC (Dow Corning Corp, Midland, MI, USA) tubing for 28, 29 or 30 days. The pumps were loaded with 100 mmol/200 μl of P234 (Phoenix Pharmaceutical, Inc., Mountain View, CA, USA), or 0.1 pmol/200 μl of KP-10 (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA). These doses are known to produce a local effect and do not alter hypothalamic function (Thompson *et al.* 2004, Pineda *et al.* 2010, Ricu *et al.* 2012). The left ovaries were removed at the time of pump implantation. The sham rats underwent the same surgery (hemiovarectomization of the left ovary) but without a pump implantation and only the tubing was inserted into the right ovary. The rats were subjected to the surgery at 5 and 9 months and then sacrificed by decapitation at 6 and 10 months in the morning of the first estrus from the day 28 of infusion onwards. Estrous cyclicity was monitored by daily vaginal lavage.

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We ovariectomized all rats implanted with mini osmotic pumps because unpublished results have shown that contralateral ovaries compensate the effect of the treatment and could mask the results.

Real-time PCR

Total RNA was extracted using an E.Z.N.A Total RNA kit (Omega-Biotek, Norcross, GA, USA). A total of 2 µg of RNA was subjected to RT. For real-time PCR, we used Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, Inc., Santa Clara, CA, USA) in an IQ5 thermocycler (Bio-Rad Laboratories, Inc.). Primer-specific amplification and quantification cycles were run at 95 °C for 20 s, 62.5 °C for 20 s for KP (60 °C for FSH receptor (FSHR) and 18S), 72 °C for 20 s and a final extension of 72 °C for 10 min. To normalize the quantification of Kiss1 and Fshr mRNA, we measured the amount of ribosomal 18S mRNA in each protocol using a commercially available RT primer pair (Ambion, Inc., Austin, TX, USA). The corresponding standard curve for each gene was obtained by serial dilution of known copy numbers of the purified amplification PCR product of each gene using Diffinity RapidTips (Diffinity Genomics, Inc., West Henrietta, NY, USA). The primers used for each product were the following: Fshr: sense 5′-CAT CAC TGT GTC CAA GGC CA-3′, antisense 5′-TGC GGA AGT TCT TGG TGA AAA-3′; Genebank access AF095642; and for KP: sense 5′-CCGGACCCCAG-GAACTCG T-3′, antisense 5′-CGTAGCGCAGGCCAAAG-GAG-3′; Genebank access NM_181692.

Western blot of KP

Detection of ovarian KP and the internal control GAPDH by western blot at different ages was performed, following separation by SDS–PAGE on 15% polyacrylamide gels under reducing conditions. The proteins were transferred to PVDF membranes, blocked with 5% milk for 1 h and probed with either rabbit polyclonal anti-KP antibody (ab19028, Abcam, Inc., Cambridge, MA, USA) in a 1:50 dilution overnight or in a 1:40 000 dilution of rabbit polyclonal anti-GAPDH (antibody G9545, Sigma–Aldrich Co.) for 1 h. The antibody complexes were detected using goat anti-rabbit IgG Fc (HRP) (ab97200, Abcam, Inc.) at 1:10 000, and for chemiluminescence, an EZ-ECL Enhanced Chemiluminescence Detection Kit (Biological Industries, KBH, Israel) was used. Chemiluminescence was captured using a G-Box Syngene system (Syngene Headquarters, MD, USA).

Uptake and release of NE

The procedure was performed as previously described (Wakade 1980, Greiner *et al*. 2008, Ricu *et al*. 2008, Acuna *et al*. 2009). The rats were sacrificed at 10 months of age. The right ovaries were incubated with ^{3H}NE for 30 min and then placed in a perfusion chamber. After 3 min of lavage, the ovaries were subjected to a train of monophasic electrical pulses (80 V, 10 Hz, 10 ms/pulse for 1 min), delivered through a parallel set of platinum electrodes; the pulses were generated using a Grass S-4 stimulator (Grass Instruments, Quincy, MA, USA). One-minute fractions were collected for an additional 5 min. The total amount of neurotransmitters released was calculated as the area under the curve after stimulation minus that of basal efflux (Ferruz *et al*. 1991, Greiner *et al*. 2008, Ricu *et al*. 2008). The release percentages of ^{3H}NE previously incorporated in 6-, 8- and 12-month-old rats were obtained from a previous publication by our laboratory (Acuna *et al*. 2009).

Organ culture

The ovaries used to examine the potential effects of KP on FSHR formation were at least 8 months old. The ovaries were dissected under aseptic conditions, placed on sterile lens paper, and cultured on plastic grids at the air/culture medium interface, as previously described (George *et al*. 1987, Barra *et al*. 2014), under an atmosphere consisting of 95% O_2 and 5% CO_2 and at a temperature of 37 °C. One ovary per well was cultured for 8 h in 24-well plates; each well contained 750 µl DMEM/F-12 50:50 v/v (12500, Gibco, Life Technologies). The medium was supplemented with glucose (4.5 g/l), penicillin (100 U/ml), and streptomycin (100 µg/ml), as previously reported (Mayerhofer *et al*. 1997, Romero *et al*. 2002). Six experimental conditions were assayed: control (DMEM/F-12); KP (100 ng/µl); KP+ISO (100 ng/µl+20 µM); KP+P234 (1 µg/µl+100 µg/µl); ISO (20 µM); and ISO+P234 (20 µM+1 µg/µl). In summary, the ovaries from 36 rats were incubated in the presence of every condition, and 36 ovaries (6 per condition) were collected at the end of this period and stored at −80 °C until RNA extraction. The other 36 ovaries were washed in fresh media and incubated for another 24 h with human FSH (F4021, 7000 IU/mg, Sigma Chemical Co.) at a final concentration of 10 IU/ml in incubation media, and the ovaries were fixed to perform morphometric analysis of the follicles, as described previously (Barra *et al*. 2014).

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Ovarian morphology

Fresh ovaries were immersed in Bouin's fixative, embedded in paraffin, cut into 6-μm sections, and stained with hematoxylin and eosin. The presence of secondary, antral, type III and cystic follicles was analyzed according to the work presented in Acuna *et al.* (2009), Cruz *et al.* (2012), Fernandois *et al.* (2012) and Lara *et al.* (2000). All of the slices were analyzed. Briefly, the antral follicles were defined as follicles with antral cavity and with 2 or more layers of granulosa cells. Atretic follicles were defined as those in which more than 5% of the cells had pyknotic nuclei in the largest cross-section and showed shrinkage and an occasional breakdown of the germinal vesicle. The antral follicles were counted when the nucleus of the oocyte was visualized. The cystic follicles were defined as those follicles that were devoid of oocytes and displayed a large antral cavity, an enlarged thecal cell layer and a thin (mostly monolayer) granulosa cell compartment that contained apparently healthy cells. The type III follicles were also defined according to the criteria proposed by Brawer *et al.* (1989). These follicles are large; contain 4 or 5 layers of small, densely packed granulosa cells surrounding a very large antrum; and display a seemingly normal thecal compartment, but presents abnormal basal membrane. For each follicle, two perpendicular diameters were measured using an ocular micrometre. Corpora lutea was analyzed in every section and it was included for counting, when the corpus luteum reaches the largest cross-section. For every follicle the measurement of each diameter was obtained from basement membrane to basement membrane, and the average of the two diameters was then calculated for each follicle.

Anti-Müllerian hormone assay

Serum anti-Müllerian hormone levels were measured using an ELISA for rat antimullerian hormone (AMH), following the manufacturer's instructions (Cusabio Biotech Ltd, Hubei, China). The AMH sensitivity was 0.6–150 ng/ml.

Statistical analysis

The differences between age groups were assessed using an ANOVA, followed by the Student–Newman–Keuls multiple range test for unequal replications or the Kruskal–Wallis test followed by Dunn's multiple comparison. A correlation was obtained with Pearson's test. The difference between size groups were assessed using two-way ANOVA, followed by the Student–Newman–Keuls multiple range test for unequal replications. $P<0.05$ was considered to be statistically significant. The statistical analyses were performed using GraphPad Prism software, version 6.0 (GraphPad Software, San Diego, CA, USA).

Results

Changes in ovarian KP during aging

Figure 1 shows the changes in the mRNA levels of Kiss1 and KP peptide expression due to ovarian aging in the rat. Panel A shows an increase in ovarian KP mRNA levels in 10- and 12-month-old rats compared with 6-months-old rats. Panel B shows that the levels of KP peptide followed the same pattern as the mRNA levels. Because KP

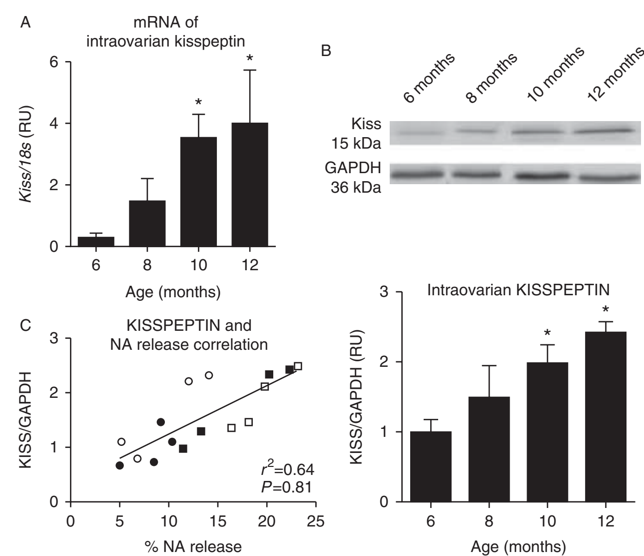

Figure 1
Kisspeptin increases during reproductive aging. (A) Kisspeptin transcript levels in the ovaries of Sprague-Dawley rats were quantified by RT-qPCR. mRNA levels were normalized to the 18S gene. $n=11$ for 6 months old, $n=8$ for 8 months old, $n=6$ for 10 months old and $n=8$ for 12 months old. (B) Protein levels were quantified by western blot; each sample was assessed three times in triplicate. Kisspeptin values were normalized to GAPDH. Pixels were counted using Image-J software; $n=4$ for each bar. (C) Correlation of kisspeptin with noradrenaline. The percentage of noradrenaline release induced by electric depolarization. The results are expressed as the total ^{3}H-NA released by electric depolarization and the uptake of NA. Correlations were calculated between kisspeptin protein levels shown in panel B. Black circles correspond to 6-month-old measurements, open circles to 8 months, black squares 10 months and open squares to 12 months. The results are plotted as the mean±S.E.M. Statistics: for graph A, the significance was obtained with the Kruskal–Wallis test, followed by Dunn's multiple comparison; for B, the significance was obtained using a one-way ANOVA, followed by a multiple comparisons test. *$P<0.05$. Correlations were determined using Pearson's test, with a correlation factor of 0.8059. Asterisks indicate differences compared with the conditions at 6 months old.

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expression can be induced by an adrenergic stimulus (Ricu *et al*. 2012), we performed a correlation analysis (panel C) between KP and the release of norepinephrine from the ovary, as a reflection of adrenergic tone. This analysis showed a high correlation between the levels of KP peptide and noradrenaline (NA) release from the ovary (the Pearson correlation factor was 0.81).

The effect of KP on follicular development

To evaluate the effect of KP on ovarian follicular development due to aging, we implanted a mini-osmotic pump directly into the ovary to deliver KP or the KP antagonist P234 for 28 days. Figure 2 shows that ovaries infused with KP at 6 months (panel A) and 10 months (panel B) had fewer total antral follicles than sham ovaries. The opposite effect was observed in ovaries that were infused with P234, showing an increase in the number of antral follicles compared with the controls. Figure 2C and D shows that the infusion of KP increased the number of type III follicular structures (Brawer *et al*. 1989) and the number of corpora lutea (Fig. 2E and F) in 6- and 10-month-old rats. On the contrary, P234 at 6 months did not affect either the type III follicular structures or the number of corpora lutea (Fig. 2C and E), but at 10 months (Fig. 2D and F), P234 infusion produced a decrease in type III follicular structures and corpora lutea. Figure 2G and H shows that KP-infused ovaries has an increase in corpora lutea ≥850 μm; these may correspond to the last ovulated cycle. None of these changes altered the precyst and cyst follicles (Supplementary Figure 1, see section on supplementary data given at the end of this article). As a marker of the preantral follicle population, we assessed the plasma levels of AMH. Figure 3A shows that KP increased plasma AMH in 6- and 10-month-old rats; by contrast, P234 administration resulted in a decrease in 10-month-old rats, thus indicating a possible contrary effect. Because it was reported that AMH is primarily secreted by the secondary and small antral follicles (Baarends *et al*. 1995), we analyzed the size of the healthy antral follicle population. Figure 3B and C shows that KP at 6 and 10 months, respectively, generated an accumulation of small-sized antral follicles (100–199 μm) and a decrease in the number of follicles between 200 and 299 μm compared with the controls.

The effects of KP on FSHR expression

Because FSHR is responsible for follicular growth, we evaluated the effects of KP on the expression of FSHR

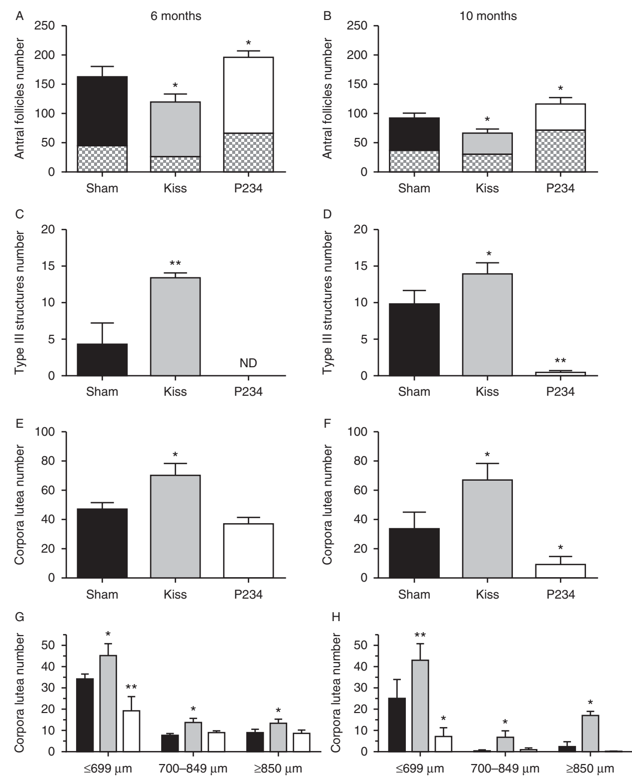

Figure 2
Effects of intraovarian kisspeptin or antagonist (P234) administration on follicular dynamics. The quantification of follicles by microscopic observation. The graphs show the total number of follicles per ovary. (A) Total antral follicles (healthy plus atretic) at 6 months old and (B) 10 months old. Lower squares fill indicates the mean of atretic follicles in each graph bar. (C) Type III follicles at 6 months old and (D) 10 months old. (E) Corpora lutea at 6 months old (F) 10 months old. (G and H) Corresponds to corpora lutea quantification by microscopic observation, classified by size at 6 and 10 months old, respectively. The results are expressed as the mean ± S.E.M. of $n=5$ ovaries. Significance was obtained using a one-way ANOVA followed by a multiple comparisons test. *$P<0.05$ and **$P<0.01$. Asterisks represent differences against control sham ovaries. ND stands for non detected structures.

induced by an adrenergic stimulus. For this purpose, we performed a whole ovary *ex vivo* incubation, stimulating ovaries with KP, P234 and isoproterenol (ISO), a β-adrenergic agonist that is capable of inducing the functional expression of FSHR (Mayerhofer *et al*. 1997). Figure 4A shows that KP alone was not capable of inducing FSHR gene expression; rather, when KP was co-incubated with ISO, there was a significant decrease in the mRNA levels of FSHR compared with those of ISO. Incubation of ovaries from 5-day-old rats corroborated these results (Supplementary Figure 2, see section on supplementary data given at the end of this article). By contrast, when ISO was co-incubated with P234, this peptide potentiated the

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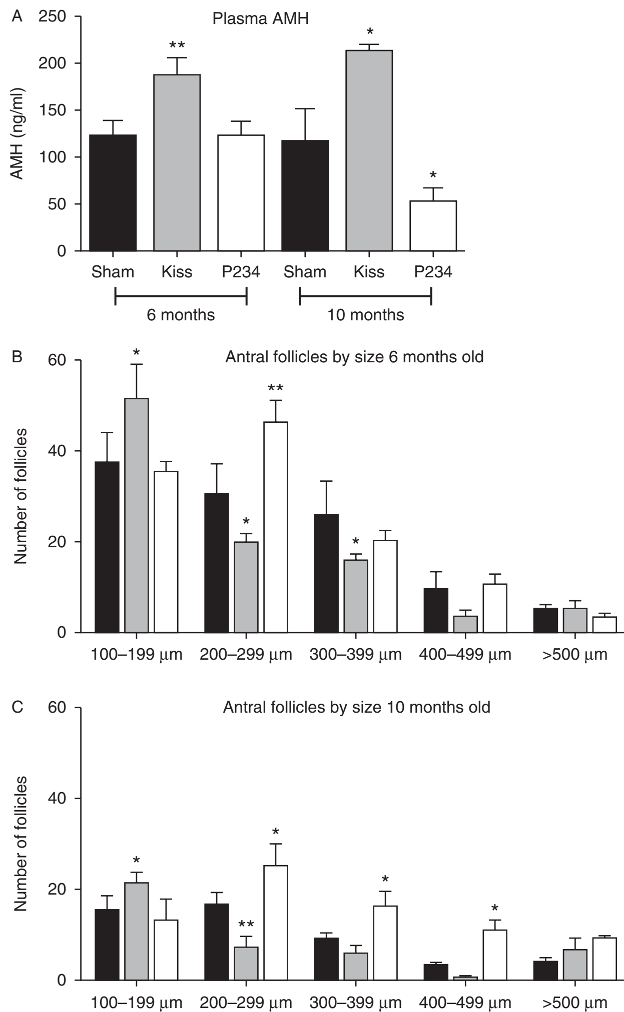

Figure 3
Effect of intraovarian kisspeptin or antagonist (P234) infusion on AMH secretion. (A) Anti-Müllerian hormone serum levels measured by ELISA; (B and C) represent healthy antral follicular quantification by microscopic observation, classified by size at 6 and 10 months old, respectively. The results are expressed as the mean ± S.E.M. of $n=5$ ovaries. Significance was obtained using a one-way ANOVA in A, and two-way ANOVA in B and C followed by a multiple comparisons test. *$P<0.05$ and **$P<0.01$. Asterisks represent differences against control sham ovaries.

effects of ISO. Figure 4B shows the stimulation of ovaries with FSH and the quantification of secondary follicles under each experimental condition. FSH stimulation increased the number of secondary follicles in the ISO group compared with the controls. Additionally, we observed a decreased number of secondary follicles in the ISO+KP group compared with the ISO group, and an increased number in the ISO+P234 group compared with the control and ISO group. In summary, Fig. 4B shows that the FSHR mRNA expression observed in Fig. 4A was functional and that KP could prevent the FSHR expression induced by ISO. In addition, as shown in Fig. 4C, we observed that the ISO and ISO+P234 groups increases the newest and small-sized secondary follicles (<75 μm) compared with the control group, while ISO+KP prevented this change showing a decrease compared with the ISO group.

Discussion

KP has been implicated in the control of puberty onset and the generation of LH peaks, leading to ovulation by controlling hypothalamic function (Thompson *et al*. 2004, Castellano *et al*. 2005, 2006, Pinilla *et al*. 2012). Because KP

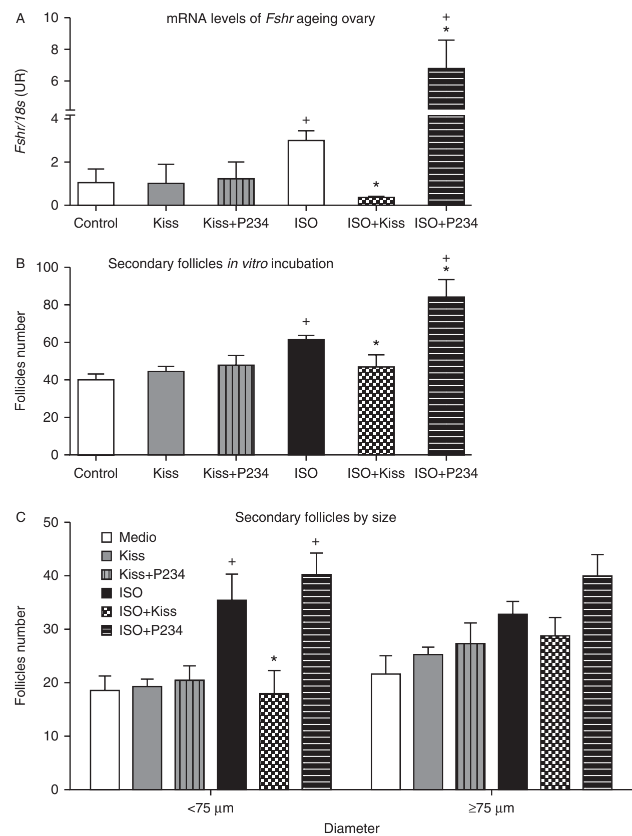

Figure 4
Effects of kisspeptin and NA on FSHR. (A) mRNA levels of Fshr were measured *ex vivo* in ovaries at 8 months old; the ovaries were incubated with either kisspeptin 100 ng/ml (kiss), isoproterenol 20 mM (ISO) or P234 1 μg/μl, or with the combinations indicated. FSHR mRNA levels were normalized to 18S, $n=6$. (B) The number of secondary follicles by microscopic observation after pharmacological stimulation as in A and then 24 h with FSH. (C) Secondary follicular quantification classified by size. The results are expressed as the mean ± S.E.M. of $n=6$ ovaries for each condition. Significance was obtained using a one-way ANOVA followed by a multiple comparisons test. *$P<0.05$ and $^{+}$. Asterisks represent differences compared with ISO conditions, and the plus sign represents differences compared with controls.

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and its receptors are expressed in the ovaries (Castellano *et al*. 2006, Gaytan *et al*. 2009, Ricu *et al*. 2012), we wanted to assess whether KP directly regulates the ovarian follicular development of senescent rats through a paracrine mechanism. Interestingly, we found that ovarian KP expression increased with aging and was correlated with an increase in norepinephrine release. In addition, we found that KP could negatively regulate the effects of the adrenergic agonist ISO on FSHR expression and hence regulate the development of secondary follicles *in vitro*. In *in vivo* experiments, we found that KP infused pharmacologically into the ovary affects the growth of antral follicles and thus modifies the transition of antral follicles into corpora lutea and type III follicles. These results suggest that KP regulates follicular development. By affecting the action of KP in the ovary, follicular development could be pharmacologically manipulated in women during the subfertility period.

Ovarian KP increases during aging and is correlated with ovarian noradrenaline release

In the present work, our initial finding was that KP expression in the ovary is elevated during aging. Although this data is significant to describe a physiological role for KP, it is important to consider that several changes occur in the distribution of ovarian follicles while age increases. The decrease of all developing follicles could lead to a decrease in 18S constitutive gene because not all of these cells are associated with KP and hence we can get an artificial increase in Kiss1/18S ratio. Taking into account this limitation, further experiments are needed to confirm that each follicular cell express more KP with the aging. Beside this, we agreed, along with other authors, that both the noradrenaline content of the ovary and its release increase during aging (Chavez-Genaro *et al*. 2007, Acuna *et al*. 2009). Because we have also described the induction of KP by ISO (adrenergic agonist) *in vitro* (Ricu *et al*. 2012), it is possible that the rise in adrenergic activity in the ovary that occurs naturally with aging provokes the increase in KP observed here. The high correlation between NA release and the observed increase in intraovarian KP supports this idea. Additionally, we found that sympathetic denervation of the ovary decreases the expression of ovarian KP in senescent rats (data not shown), which is in line with our hypothesis. Together, these results suggest that ovarian KP is under sympathetic control and could thus participate together with NA in the regulation of follicular dynamics.

KP and follicular development

Although KP has been traditionally related to the hypothalamic control of ovarian function, recently, the role of KP as a paracrine regulator of follicular development is beginning to be elucidated (Dorfman *et al*. 2014, Gaytan *et al*. 2014). As shown previously, (Acuna *et al*. 2009) a progressive decrease in antral follicles is observed with increasing age. This decay in follicular count coincides with the increase in ovarian KP observed in our work and is probably not related to changes in hypothalamic function because the function of rat hypothalamus fails after we observed the decline in antral follicles (Lu *et al*. 1979, Steger *et al*. 1980). We hypothesized that the increase in ovarian KP is directly associated with the decline in the follicular pool that occurs during aging. For this reason, we proposed an *in vivo* experiment in which we attempted to either advance the decay in the number of antral follicles by administrating KP or delay this decay by administering the KP antagonist P234. In this experiment, we administered KP in a constant rate and not following estrous cycle variations, which represent more a pharmacologic than a physiologic modulation of the KP/Kiss1R system. We found that KP infusion into the ovary decreased the number of total antral follicles (healthy+atretic) while P234 increased the number of total antral follicles both at 6 and 10 months. Because we observed a decrease in the growth of antral follicles in both control intact aging rats and rats administered with KP, we hypothesized that KP could decrease the sensitivity of follicles to FSH. To assess this possibility, we conducted an *in vitro* experiment in which we measured the expression of FSHR and the growth of secondary follicles in response to FSH under stimulation with KP. Our results indicate that KP alone does not affect FSHR expression or the number of secondary follicles. However, because ovarian aging courses with an increase in adrenergic tone (Acuna *et al*. 2009) and NA induces FSHR expression through β-adrenergic receptors (Mayerhofer *et al*. 1997), we also incubated KP plus ISO (a β-adrenergic agonist). Surprisingly, we found that KP prevents the increase in FSHR expression produced by ISO, acting as a functional antagonist. Furthermore, incubation with P234 potentiated the effect of ISO on FSHR expression. To evaluate whether the change in FSHR expression reflects the follicular sensitivity to FSH, we stimulated the ovaries with FSH and counted the number of secondary follicles in each condition. We found that ISO increased the number of secondary follicles, as shown previously

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(Mayerhofer *et al*. 1997), and that KP prevented this increase while P234 potentiated it. If we separate the secondary follicles into newly formed secondary follicles (<75 μm) and large secondary follicles (≥75 μm), we observed that ISO notably increased the number of smaller ones and KP prevented this increase, demonstrating that KP negatively participates in the acquisition of FSHR and hence attenuates the initial follicle recruitment (primary to secondary) by FSH. In addition, by dissecting the population of healthy antral follicles based on size in the *in vivo* experiment, we observed that at both ages (6 and 10 months old) KP administration specifically increased the small antral follicles (<200 μm). This accumulation in small antral follicles could be responsible for the increase in serum AMH levels observed in rats with KP stimulation, as AMH is principally produced by the secondary and small antral follicles in the rat (Baarends *et al*. 1995). The cyclic recruitment of these small antral follicles is performed by FSH and may be also being prevented by KP. To confirm the effect of KP in preventing FSHR expression in early preantral follicles without interference of other follicles we repeated the *in vitro* experiment in 5-day-old neonate ovaries (Supplementary Figure 2), which only contain preantral follicles. In this experiment, we reproduced the effect of KP in preventing ISO-induced Fshr mRNA expression.

In summary, KP decreases the recruitment of follicles by decreasing their sensitivity to FSH and thus participates in the reduction of developing follicles observed during aging. The mechanism of this effect could involve a functional antagonism of NA. Our findings coincide with those of Gaytan *et al*. (2014), who showed that KISS1R haplo-insufficient mice exhibit premature ovarian failure that was not attributable to a defect in gonadotropin secretion.

KP and ovulation

As discussed above, the effect of KP on follicular development *in vivo* appears to be a cumulative effect, in which a low recruitment of follicles (as demonstrated *in vitro*) is maintained by a tonic increase in KP infused into the ovary. In the same experiment, we observed an increase in total number of corpora lutea with an increase in newly generated (≥850 μm) corpora lutea, those that can correspond to the recently ovulated follicles, indicating that high KP conducts a higher proportion of large antral follicles to ovulation. This finding coincides with the fact that KP expression in the ovary increases specifically in late proestrus (Castellano *et al*. 2006) and also increases in response to human chorionic gonadotrophin (hCG) stimulation in a transient manner (Gaytan *et al*. 2009). Moreover, if ovulation is blocked by indomethacin, the induction of KP by hCG is prevented, indicating the close participation of KP in ovulation (Gaytan *et al*. 2009). However, the fact that KP promotes ovulation apparently contradicts the fact that while ovarian KP increases with age, corpora lutea decrease. In this sense, it is important to understand that during ovarian aging, fewer follicles are recruited, decreasing the number of healthy antral follicles available for ovulation. Despite this, the ratio of corpora lutea to antral follicles increases with age (Supplementary Figure 3, see section on supplementary data given at the end of this article), indicating that ovulation is more efficient. It is important to note that in addition to increasing the development of antral follicles, the infusion of P234 also decreases ovulation; therefore, its effect is the exact opposite of KP.

In the *in vivo* experiments, we observed that the increase in KP not only facilitates ovulation but also increases the number of type III follicles, which correspond to follicles in a state somewhere between an antral follicle and a follicular cyst (Lara *et al*. 2000). Due to this intermediate status, some studies have postulated that this type of follicle (those that still possess oocytes) intensely binds hCG (Brawer *et al*. 1989) and can be rescued from this status and be ovulated (Brawer *et al*. 1989, Convery *et al*. 1990). Therefore, it is possible that type III follicles originate from the antral preovulatory follicles.

Conclusion

KP can regulates follicular development by blocking the effect of adrenergic agonists on FSHR induction in early developing follicles. This mechanism could take more importance during ovarian aging because the previously described increase in ovarian noradrenergic tone during aging could be complementary to the putative increase in ovarian KP with age. KP also appears to increase the ovulation rate from a fewer number of antral follicles in aging rats, but more information is needed to complement this finding.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-15-0429.

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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Author contribution statement
D F: determination of KP, KISS1R, FSHR and 18S by real-time PCR; determination of KP and GAPDH by western blot; participation in the ovarian morphology analysis and discussion; analysis of the results and manuscript preparation. E N: participation in the ovarian administration of KP and ovarian morphology analysis; F C: participation in the ovarian administration of P234 and ovarian morphology analysis; H E L and G C: discussion, analysis of the results and manuscript review; A H P: noradrenaline release determination, contributions to the development, design, and coordination of the research, manuscript preparation and responsibility for the grant. All of the authors read and approved the final manuscript.

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