Expression of epiregulin and amphiregulin in the rat ovary

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Abstract

We have previously reported that the epidermal growth factor (EGF) family growth factor, epiregulin, is expressed in rat ovarian granulosa cells by induction with pregnant mare serum gonadotropin (PMSG). In this study, we report that amphiregulin, another member of the EGF family, was also induced in the rat ovary by gonadotropin treatment. Northern blot analysis revealed that PMSG treatment induced the expression of both epiregulin and amphiregulin mRNA after 24 h, but the expression then decreased 48 h after treatment. Further treatment with human chorionic gonadotropin (hCG) rapidly induced the expression of both epiregulin and amphiregulin genes and maximal levels were reached 4 h after hCG treatment. A marginal increase in amphiregulin mRNA levels was also observed 6 h after PMSG treatment. *In situ* hybridization revealed that epiregulin and amphiregulin mRNAs were localized in the granulosa cells of large antral follicles. These spatio-temporal expression patterns were similar to those of cyclo-oxygenase-2 (COX-2) and progesterone receptor (PR). In adult cycling rats, epiregulin and amphiregulin were strongly induced at 1800 and 2000 h on proestrus coinciding with the preovulatory LH surge. An *in situ* hybridization study also showed that epiregulin and amphiregulin mRNAs were detectable in the granulosa cells of preovulatory ovarian follicles at 2000 h on proestrus, where transcripts of COX-2 and PR were co-localized with those of epiregulin and amphiregulin. These observations suggested that the EGF family members, epiregulin and amphiregulin, may play a role in the ovulatory process of cycling rats as well as in the induction of ovulation in immature rats.

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Introduction

While pituitary luteinizing hormone (LH) and follicle-stimulating hormone (FSH) play central roles in the regulation of normal reproductive development and functions, a large number of growth factors serve as paracrine and autocrine factors in a gonadotropin-dependent or -independent fashion (Leung & Steele 1992, Richards 1994). The epidermal growth factor (EGF) family growth factors, EGF and transforming growth factor- α (TGF- α), are also thought to be involved in this process. Both act as mitogens (Roy & Greenwald 1991, Liang *et al.* 1994) and modulators of FSH-stimulated steroid secretion (Hsueh *et al.* 1981) in ovarian granulosa cells. Furthermore, EGF induces cumulus expansion and

oocyte maturation that usually occurs after the preovulatory LH surge (Downs 1989, Das *et al.* 1992, Prochazka *et al.* 2000).

We previously isolated hundreds of clones of FSH-inducible genes from rat ovarian granulosa cells by subtraction cloning (Kameda *et al.* 1999), and an EGF growth factor family member, epiregulin, was included in these clones (Sekiguchi *et al.* 2002).

Epiregulin has been identified as a growth-inhibitory factor from the conditioned medium of a fibroblast tumor cell line, NIH-3T3/clone T7 (Toyoda *et al.* 1995a). The gene expression profiles of epiregulin in human (Toyoda *et al.* 1997) and mouse (Toyoda *et al.* 1995b) tissue have been reported. Northern blot analyses have shown that the expression of human epiregulin occurs mainly

A

a

b

B

in peripheral blood macrophages and the placenta. In contrast, the mouse epiregulin gene has been detected in the uterus of an adult mouse and a 7-day-old mouse embryo by Northern blot analysis. In addition, several studies have reported on the epiregulin mRNA expression in bovine (Robert *et al.* 2001) and rat (Espey & Richards 2002, Burns *et al.* 2003) ovarian granulosa cells.

In addition, we report here for the first time that another EGF family growth factor, amphiregulin, is also induced in the rat ovary by gonadotropin treatment. Amphiregulin was originally isolated from the conditioned medium of a human breast carcinoma cell line MCF-7 (Shoyab *et al.* 1988). In the rat, amphiregulin was purified from the growth-conditioned medium of the JS1 Schwann cell line (Kimura *et al.* 1990), and therefore is referred to as Schwannoma-derived growth factor. High levels of rat amphiregulin transcripts have been detected in the lungs of newborn animals, and lower levels were found in fetal and adult brains, as well as in sciatic nerves, as evidenced by Northern blot analysis. Several studies have reported that amphiregulin is associated with lung branching morphogenesis in the mouse (Schuger *et al.* 1996) and epithelial branching in the embryonic mouse kidney (Lee *et al.* 1999).

However, the roles of rat epiregulin and amphiregulin in the ovary are completely unknown. In this study, we report on an examination of the hormonal regulation and precise distribution of epiregulin and amphiregulin in the rat ovary, using cycling adult rats as well as immature rats in which superovulation was induced by treatment with gonadotropins. We have demonstrated here that the ovarian expression of rat epiregulin and amphiregulin coincides with those of cyclo-oxygenase-2 (COX-2) and progesterone receptor (PR), both of which are known to play integral roles in ovulation. These observations suggest that rat epiregulin and amphiregulin are induced by the preovulatory LH surge, and may be associated with ovulatory events.

Materials and methods

Materials

Pregnant mare serum gonadotropin (PMSG) was obtained from Teikokuzouki, Inc. (Tokyo, Japan). hCG was obtained from Sankyo Co. Ltd (Tokyo, Japan). T7 and SP6 RNA polymerase were obtained from Roche Molecular Biochemicals (Mannheim, Germany). The ToRizol reagent, Superscript II reverse transcriptase, and cloning vector pSPORT1 were purchased from Invitrogen (Carlsbad, CA, USA). α - 32 P dCTP (111 TBq/mmol) and 35 S-CTP (46.2 TBq/mmol) were obtained from NEN Life Science Products (Wilmington, DE, USA). The pGEM-T Easy vector was purchased from Promega (Madison, WI, USA). The EX Taq DNA polymerase and Bca BEST Labeling Kit were purchased from TaKaRa Shuzo (Kyoto, Japan).

Animals and hormone treatments

Immature, kwl: Wistar female rats (21 days old) were injected with 30 IU PMSG, and 48 h later with 30 IU hCG. Rats were killed at various time-points, and the ovaries were removed for Northern blot analysis or *in situ* hybridization analysis. Adult Iar: Wistar–Imamichi female rats (body weight 260–290 g) were housed in a photoperiod of 12 h light and 12 h darkness, with the lights on at 0800 h. Estrous cycle stages were determined by a daily examination of vaginal cytology, and only those animals demonstrating at least two consecutive 4-day cycles were used in the experiments. Rats were killed and the ovaries were removed at 1400 h on each day of the cycle, and at 1600, 1800, 2000, 2200, and 2400 h on proestrus. Ovaries were rapidly frozen on dry ice for RNA isolation or fixed for *in situ* hybridization. At all times, the animals were treated according to NIH guidelines.

Figure 1 Expression pattern of rat epiregulin in the ovary. (A) Northern blot analysis of rat epiregulin mRNA in the ovary. Total RNA was isolated from ovaries removed at the indicated times. Rats were treated with (a) PMSG and (b) PMSG followed by hCG. Total RNA (10 μ g) from each run was loaded onto the lanes. After electrophoresis, the RNA was transferred to nylon membranes, which were hybridized with 32 P-labeled cDNAs of epiregulin or 36B4. (B) *In situ* hybridization of epiregulin in immature rat ovaries. Ovaries from 21-day-old rats were dissected, sectioned, and hybridized with 35 S-labeled antisense (a, b, and c) or sense (d) epiregulin probes. Rats were treated as follows: (a) no treatment, (b) PMSG for 24 h, and (c and d) hCG for 4 h after 48 h of PMSG treatment. Left panels are bright-field exposures (BF); right panels are dark-field exposures (DF). A scale bar of 500 μ m is included.

Northern blot analysis and RT-PCR

Total RNA was isolated by the acid guanidium thiocyanate extraction method (Chomczynski & Sacchi 1987). For the Northern blot analysis, 10 µg total RNA from each sample was separated by electrophoresis on denaturing agarose gels and subsequently transferred to a nylon membrane (Biodyne; ICN, Glen Cove, NY, USA). Rat epiregulin (MN-021689) cDNA fragment corresponding to bases 1–510 and rat amphiregulin (NM-017123) cDNA fragment corresponding to bases 313–820 were radiolabeled by the random primer labeling method and used as a probe for RNA blot hybridization. Auto-radiographic bands were quantified by means of a fluoro-image analyzer (BAS2000; Fuji Co. Ltd, Kanagawa, Japan). The blots were striped and rehybridized with a randomly radiolabeled probe for a 954 bp fragment of the pGEM-T Easy-36B4 encoding acidic ribosomal phosphoprotein P0 (36B4) (Yamada *et al.* 2003) to normalize for the equivalent loading of RNA. For RT-PCR, 1 µg total RNA was reverse-transcribed and a portion (1/20) of the reaction mixture was subjected to the PCR reaction. Primers for rat epiregulin were 5'-CGCTTTGCGAGCTGCACCGA-3' (5'-primer: nucleotide (nt) 128/147) and 5'-GCGATTTCTGTACCATCTGC-3' (3'-primer: nt 517/536) respectively. Primers for rat amphiregulin were 5'-CGCTGCTGGTCTTAGGCTCA-3' (5'-primer: nt 216/235) and 5'-GAA ACTTGGCGGCACACGGA-3' (3'-primer: nt 580/599) respectively. Primers for rat COX-2 were 5'-AGGAGAGACGATCAAGATAGT-3' (5'-primer: nt 1093/1113) and 5'-ATACAGTTCCATGGCATCGA-3' (3'-primer: nt 1575/1594) respectively. Primers for rat PR were 5'-CATGTTCAGTGGACAGATGCT-3' (5'-primer: nt 2278/2297) and 5'-ACTTCAGACATCATTTCCGG-3' (3'-primer: nt 2686/2705) respectively (Knauth *et al.* 1996). Primers for rat

36B4 were 5'-GAACAACCCCGCTCTGGAGA-3' (5'-primer: nt 283/302) and 5'-GGCCTGCTCTGTGATGTCCA-3' (3'-primer: nt 684/703) respectively. The reaction conditions were 30 cycles for amphiregulin, and 25 cycles for epiregulin, COX-2, PR, and 36B4 respectively, by denaturing at 94 °C for 20 s, annealing for 30 s and extending at 72 °C for 1 min using the EX Taq DNA polymerase. Ten microliters of the PCR products were electrophoresed on a 1.5% agarose gel and subsequently visualized by ethidium bromide staining.

In situ hybridization

In situ hybridization was performed as described (Sekiguchi *et al.* 2002) with minor modifications using paraffin-embedded tissues instead of frozen tissues. Rat ovaries were fixed in freshly prepared 4% paraformaldehyde (PFA) at 4 °C for 12 h, dehydrated with ethanol, cleared in xylene, and embedded in paraffin. Sections (5 µm thick) were mounted on (3-aminopropyl) triethoxysilane-coated glass slides for *in situ* hybridization. The sections were deparaffinized, rehydrated, fixed in 4% PFA, treated with proteinase-K, post-fixed in 4% PFA, and acetylated before hybridization. Rat epiregulin cDNA fragment corresponding to bases 1–510 and rat amphiregulin cDNA fragment corresponding to bases 313–820 were subcloned into the pSPORT1 vector and pGEM-T Easy vector respectively. Antisense or sense ³⁵S-CTP-labeled RNA probes were synthesized using T7 or SP6 RNA polymerase. Hybridization with the ³⁵S-labeled cRNA probes was performed at 60 °C for 6 h, and the sections were then washed under conditions of high stringency and autoradiographed using an NTB2 emulsion (Eastman Kodak Co., Rochester, NY, USA). After developing, all slides were counterstained with hematoxylin, dehydrated, and mounted.

Figure 2 Expression pattern of rat amphiregulin in the ovary. (A) Northern blot analysis of rat amphiregulin mRNA in the ovary. Total RNA was isolated from ovaries removed at the indicated times. Rats were treated with (a) PMSG or (b) PMSG followed by hCG. Total RNA (10 µg) was loaded onto the lanes. After electrophoresis, RNA was transferred to nylon membranes, which were hybridized with ³²P-labeled cDNAs of amphiregulin or 36B4. (B) *In situ* hybridization analysis of rat amphiregulin in an ovarian section. Localization of amphiregulin mRNA was examined by a ³⁵S-labeled antisense (a, b, and c) or sense (d) amphiregulin probe. Ovaries were taken from 21-day-old immature rats treated as follows: (a) no treatment, (b) PMSG for 24 h, and (c and d) hCG for 4 h after 48 h of PMSG treatment. Left panels are bright-field exposures (BF); right panels are dark-field exposures (DF). A scale bar of 500 µm is included.

Results

We have previously shown that the expression of the epiregulin gene is strongly induced in rat ovarian granulosa cells by PMSG *in vivo*. In this study, we examined the hormonal regulation and precise distribution of epiregulin and amphiregulin in the rat ovary using cycling adult rats as well as immature rats in which superovulation was induced by treatment with gonadotropins.

For the induction of superovulation, immature rats were treated with 30 IU PMSG for 48 h, followed by 30 IU hCG. A Northern blot analysis revealed that PMSG treatment induced the expression of epiregulin mRNA after 24 h, but this expression was decreased and became undetectable 48 h after the treatment. Further treatment with hCG rapidly induced the epiregulin gene expression again, and the maximal level was reached 4 h after the hCG treatment (Fig. 1A).

Using ovaries removed after 24 h of PMSG treatment or 4 h after hCG treatment (see Materials and methods) where strong expressions of the epiregulin gene were observed, *in situ* hybridization was performed. In both sets of ovaries (24 h after PMSG treatment or 4 h after hCG treatment), epiregulin mRNA was localized in the granulosa cells of large antral follicles (Fig. 1B).

To determine whether EGF-like growth factors other than epiregulin are also induced by gonadotropin treatment in the rat ovary, we examined the expression of various EGF family growth factor genes, and found that only amphiregulin mRNA was induced in ovaries stimulated with PMSG (data not shown).

The temporal expression pattern of amphiregulin mRNA in immature rats treated with PMSG followed by hCG was very similar to that of epiregulin (Fig. 2A). The only difference was that a small induction of amphiregulin mRNA was observed 6 h after PMSG treatment (Fig. 2A). We next examined amphiregulin mRNA localization in the ovary by *in situ* hybridization. In ovaries removed 24 h after PMSG treatment or 4 h after

hCG treatment, amphiregulin mRNA was also found to be localized in the granulosa cells of large antral follicles, as shown in Fig. 2B.

To characterize the expression patterns of these growth factors, they were compared with those of COX-2 and PR, both of which are known to be related to ovulation. As shown in Fig. 3A, COX-2 and PR transcripts were detected 24 h after PMSG treatment and decreased by 48 h after PMSG treatment. Further treatment of PMSG-primed rats with hCG rapidly induced these transcripts again with a peak at 4 h. These temporal expression patterns were similar to those reported for epiregulin and amphiregulin. Furthermore, *in situ* hybridization analysis showed that all transcripts, COX-2, PR, epiregulin, and amphiregulin, were found in the same follicles (Fig. 3B). These results indicated that the spatio-temporal expression patterns of these growth factors were very similar to those of PR and COX-2.

We next examined whether these growth factors were also induced in cycling adult rats. RT-PCR was performed to detect epiregulin and amphiregulin mRNA in ovaries isolated from rats at various times during the 4-day estrous cycle. It is well known that a peak of the preovulatory LH surge occurs in the afternoon of proestrus. As shown in Fig. 4A, rat epiregulin and amphiregulin were induced at high levels at 1800 and 2000 h on proestrus. Transcripts of COX-2 and PR were also detected at high levels at 1800 and 2000 h on proestrus.

To determine the localization of these growth factors, we examined the distribution of these mRNAs in ovaries removed at 2000 h on proestrus. As shown in Fig. 4B, rat epiregulin and amphiregulin mRNAs were detected in preovulatory follicles, in which transcripts of COX-2 and PR were also induced.

Discussion

A number of studies have reported that EGF or TGF- α are associated with ovarian functions

Figure 3 Gene expression of epiregulin, amphiregulin, COX-2, and PR in immature rat ovaries. (A) Northern blot analysis of epiregulin, amphiregulin, COX-2, and PR mRNAs in the rat ovary. Ovaries were removed at the indicated times after treatment with PMSG or PMSG followed by hCG. (B) *In situ* hybridization analysis of these mRNAs on adjacent ovarian sections. Ovaries were taken from 21-day-old immature rats treated with PMSG for 48 h and followed by hCG for 4 h. Ovaries were dissected, sectioned, and hybridized with ³⁵S-labeled antisense epiregulin (a), amphiregulin (b), COX-2 (c), and PR (d) probes. A scale bar of 500 μ m is included.

(Hsueh *et al.* 1981, Downs 1989, Roy & Greenwald 1991, Das *et al.* 1992, Liang *et al.* 1994, Prochazka *et al.* 2000). However, most of these data were obtained from *in vitro* studies, and the physiological functions of these growth factors continue to remain unclear.

We have previously shown that the EGF family growth factor, epiregulin, is expressed in rat ovarian granulosa cells by induction with PMSG *in vivo* (Sekiguchi *et al.* 2002). Espey & Richards (2002) also reported that epiregulin is one of 20 rat ovulation-specific genes identified by the molecular procedure known as differential display. In addition to epiregulin, we have demonstrated here that amphiregulin, another EGF-type growth factor, is also induced in the rat ovary as the result of gonadotropin treatment. A Northern blot analysis demonstrated that EGF, TGF- α , heparin-binding-EGF (HB-EGF), betacellulin, and neuregulin transcripts were expressed weakly in the ovary but were not induced by either PMSG or hCG treatment (data not shown). These observations suggest that epiregulin and amphiregulin may participate in ovarian functions.

However, several reports have demonstrated that EGF family members are expressed in the ovary. Using an immunohistochemical procedure, EGF-positive cells were detected in the granulosa layer and the antrum of follicles (Fukumatsu *et al.* 1995). Kudlow *et al.* (1987) indicated that TGF- α is synthesized in the ovary, probably in the theca-interstitial compartments. It has also been reported that TGF- α is expressed in theca cells and is induced in rat follicle cultures by FSH treatment (Wang *et al.* 2002). Yeh *et al.* (1993), however, reported that TGF- α mRNA is expressed in cultured granulosa cells. The discrepancies between these and our observations need to be clarified in future experiments.

Our findings suggest that epiregulin and amphiregulin may be associated with phenomena induced by the preovulatory LH surge in the rat

ovary. It is well known that cumulus expansion, a process necessary for the release of mature oocytes into the oviduct, is induced by an LH surge (Dekel & Phillips 1979, Dekel *et al.* 1979, Robker *et al.* 2000) and that oocyte maturation, the re-initiation of meiosis that is signified by germinal vesicle breakdown, is also triggered by an LH surge (Channing *et al.* 1978). *In vitro* studies have shown that cumulus expansion (Channing *et al.* 1978, Eppig 1979, Salustri *et al.* 1990, Tirone *et al.* 1997) and oocyte maturation (Downs *et al.* 1988, Das *et al.* 1991, Kalous *et al.* 2003) could be induced by EGF. Because epiregulin and amphiregulin share common receptors with EGF (Prigent & Lemoine 1992, Jones *et al.* 1999), it would be expected that these growth factors could also induce cumulus expansion and oocyte maturation. In addition, it has been reported that the expression of LH receptor mRNA was very low or undetectable in the cumulus cells of preovulatory follicles (Eppig *et al.* 1997). Therefore epiregulin and amphiregulin may serve as mediators of the LH surge which, in turn, serves as a signal for cumulus expansion and oocyte maturation. It is noteworthy that, in the gastric epithelial RGM1 cell line, epiregulin causes a significant increase in the expression of COX-2 mRNA and the corresponding protein (Sasaki *et al.* 1998). It can be easily concluded that epiregulin as well as amphiregulin may also be involved in the expression of COX-2 gene in the rat ovary. Further study will be needed to clarify the ovarian function of these growth factors further.

In summary, we report here that epiregulin and amphiregulin among EGF family members are induced in the rat ovary by gonadotropin treatment. These inductions appear to be associated with ovulation, because the temporal and spatial expression of these growth factor genes coincided with those of COX-2 and PR, both of which are known to be ovulation-related genes. We also found that epiregulin and amphiregulin transcripts were strongly induced in cyclic rats during the

Figure 4 Gene expression of epiregulin, amphiregulin, COX-2, and PR in cycling adult rats. Ovaries were removed at the indicated times (metestrus (Me), diestrus (Di), proestrus (Pro) and estrus (Est)) from adult female rats exhibiting at least two consecutive 4-day cycles. N, negative control. (A) Ovaries from various stages of cycling rats were used for RNA isolation and subsequent RT-PCR assays for epiregulin, amphiregulin, COX-2, and PR gene expression. (B) Ovaries were removed at 2000 h on proestrus, and were used for *in situ* hybridization to localize epiregulin, amphiregulin, COX-2, and PR mRNAs on adjacent sections. Ovaries were dissected, sectioned, and hybridized with ³⁵S-labeled antisense epiregulin (a), amphiregulin (b), COX-2 (c), and PR (d) probes. A scale bar of 500 μ m is included.

preovulatory LH surge, further supporting the roles of these growth factors in the ovulatory process.

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