Gonadotropin-induced expression of pancreatitis-associated protein-III mRNA in the rat ovary at the time of ovulation

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Abstract

The ovulatory process in mammals involves gross physiological events in the ovary that cause transient deterioration of the ovarian connective tissue and rupture of the apical walls of mature follicles. This gonadotropin-induced process has features similar to an acute inflammatory reaction that affects most of the ovary. The present study reveals that the ovulatory events include induction of mRNA for pancreatitis-associated protein-III (PAP-III). Immature Wistar rats were primed with 10 IU equine chorionic gonadotropin s.c., and 48 h later the 12-h ovulatory process was initiated by 10 IU human chorionic gonadotropin (hCG) s.c. Ovarian RNA was extracted at 0, 2, 4, 8, 12 and 24 h after the animals were injected with hCG. The RNA extracts were used for RT-PCR differential display to detect *PAP-III* gene expression in the stimulated ovarian tissue. Northern blotting showed that

transcription was significantly greater at 4–12 h after the ovaries had been stimulated by hCG. *In situ* hybridization indicated that PAP-III mRNA expression was limited mainly to the hilar region of the ovarian stroma, with most of the signal emanating from endothelial cells that lined the inner walls of blood vessels, and from small secondary follicles. Treatment of the animals with ovulation-blocking doses of indomethacin (an inhibitor of prostanoid synthesis) or epostane (an inhibitor of progesterone synthesis) revealed that ovarian transcription of PAP-III mRNA was moderately dependent on ovarian progesterone synthesis. In conclusion, the present evidence of an increase in *PAP-III* gene expression in gonadotropin-stimulated ovaries provides further evidence that the ovulatory process is comparable to an inflammatory reaction.

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Introduction

Pancreatitis-associated protein (PAP) was discovered in 1984 as a secretory product of the pancreas after the experimental induction of acute inflammation in the rat pancreas (Keim *et al.* 1984, 1994). Two years later, a homologous rat protein called peptide 23 was identified as a secretory product from cultured hypophyseal somatotropes that had been stimulated by growth hormone-releasing hormone (GHRH) (Yokoyama & Friesen 1986). Subsequently, other homologs of PAP were found in a variety of tissues, including the gastric pyloric gland (Yamamoto *et al.* 1992), the small intestine (Iovanna *et al.* 1993, Chakraborty *et al.* 1995a), and the uterus (Chakraborty *et al.* 1995c). Collectively, these related proteins are products of the C-type lectin supergene family (Katsumata *et al.* 1995). They are especially abundant in the inflamed pancreas, in parts of the gastrointestinal tract and in GHRH-stimulated somatotropes, yet their physiological role remains unknown (Frigerio *et al.* 1993, Bowers 1995, Chakraborty *et al.* 1995a).

In the present study, we characterized the temporal and spatial patterns of expression of PAP-III mRNA in the ovaries of gonadotropin-primed immature rats during the ovulatory process. Expression of mRNA for this protein was detected by RT-PCR differential display of RNA extracts from rat ovaries at specific intervals after the injection of human chorionic gonadotropin (hCG) to induce ovulation in the experimental animals. The temporal pattern of PAP-III mRNA expression during the periovulatory period was characterized by northern blot analysis and the spatial distribution of ovarian PAP-III mRNA was determined by *in situ* hybridization. In addition, the effects of inhibition of ovarian prostaglandin (PG) and progesterone synthesis on PAP-III mRNA production were assessed. The results reveal a significant increase in expression of the *PAP-III* gene in certain areas of the ovarian stroma, in secondary follicles, and rarely in limited regions of mature ovarian follicles in response to an ovulatory dose of hCG, a homolog of luteinizing hormone.

Materials and Methods

Animals and treatments in vivo

Immature Wistar rats were induced to superovulate by equine chorionic gonadotropin (eCG) and hCG treatment as described previously (Espey *et al.* 2000b). In this experimental model, the ovaries begin to ovulate at 12 h after hCG, and ovulation is essentially complete by 16 h after hCG (Tanaka *et al.* 1989). Ovarian RNA was extracted at the periovulatory intervals of 0, 2, 4, 8, 12 and 24 h after hCG. At each interval, the ovaries from 10 animals were pooled for extraction of the total RNA. These nucleic acid extracts were used for differential display and for northern blotting. In parallel groups of animals, indomethacin and epistane were injected s.c., also as described previously (Espey *et al.* 2000b). These anti-ovulatory agents were administered at 3 h after hCG in doses of 1.0 mg and 5.0 mg respectively, because these are the intervals after hCG at which these agents are known to block ovarian PG and progesterone effectively (Espey *et al.* 1990, Tanaka *et al.* 1992). The ovarian RNA was extracted from the treated animals at 8 h after hCG, and northern analyses were performed to determine whether blockage of ovarian synthesis of PG or progesterone, or both, affected the transcription of ovarian PAP-III. The ovulation rate in the various experimental animals was determined by a procedure that also has been described previously (Espey *et al.* 2000b). For the determination of ovulation rate and the extraction of ovarian RNA, rats were killed by exposure to CO₂. The animals were acquired, retained and used in compliance with the National Institutes of Health (NIH) Guide and with the approval of the institutional animal care review committee.

Differential display to detect PAP-III mRNA transcription

The steps of the differential display were carried out as described previously (Espey *et al.* 2000b). In brief, RNA was extracted by a standard guanidine isothiocyanate/cesium chloride procedure from ovaries taken at the six different periovulatory intervals identified above. RT-PCR was performed using primers from an RNAimage Kit (G508, GenHunter Corporation, Nashville, TN, USA). The primer set that yielded a differentially expressed fragment of cDNA for PAP-III was comprised of the poly-T primer 5'-HTTTTTTTTTG-3' and the random primer 5'-HGCAAGTT-3' (i.e., primer set G62), with 'H' representing the attachment of a HindIII restriction site to the primers.

Northern analysis to confirm differential expression of PAP-III mRNA

After extraction and re-amplification of the differentially expressed cDNA, a standard northern analysis was performed to confirm that the expression of the parent

mRNA for PAP-III increased significantly during the ovulatory process. This unique cDNA fragment was subcloned using a pCR-TRAP Cloning System (P404, GenHunter), and cloning colonies containing the PAP-III cDNA were identified by secondary northern analyses. The northern blots included the same six periovulatory intervals indicated above. The different blots were all prepared from the same pool of RNA that was extracted from 10 ovaries at each of the designated intervals after administration of hCG to the animals.

Sequencing for the PAP-III cDNA fragment

Samples of the cloning colonies that contained PAP-III cDNA were cloned further in LB medium containing 10 µg tetracycline/1.0 ml medium. The harvested plasmid DNA was purified using a Wizard Minipreps DNA Purification System (A750, Promega). Manual sequencing of the cDNA was performed using a Sequenase Version 2.0 DNA Sequencing Kit (US70140, Amersham Pharmacia Biotech, Inc.). The nucleic acid sequence was analyzed by a BLASTn search of the database server at the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>).

In situ hybridization for assessment of spatial distribution of PAP-III mRNA

In situ hybridization was performed as described previously (Espey *et al.* 2000b). Ovaries were removed from three individual rats at each of the six periovulatory intervals described above and were fixed overnight in 4% paraformaldehyde. The ovarian sections were routinely hybridized with sense probe, as an internal control to detect any background signal.

Statistical analysis

The intensity of the signals from the northern blots was analysed by the NIH image densitometry program, as described previously (Espey *et al.* 2000b). Numerical data are presented as means ± S.E.M., based on data from three northern blots that were prepared from the RNA extracts of pools of ovaries from 10 animals at each time point. The significance of the differences among the six principal time points of 0, 2, 4, 8, 12 and 24 h after hCG was determined by the Duncan's multiple range test after a completely randomized one-way analysis of variance of the means of the groups. The probability value used as the cutoff between 'significant' and 'not significant' was $P=0.05$.

Results

Differential display of PAP-III cDNA during the ovulatory process

After RT-PCR, the sub-populations of radioactively labeled cDNAs that were generated from RNA extracts at

Figure 1 Autoradiograph of differentially displayed PAP-III cDNA (arrows). Note that the cDNA bands representing PAP-III are mainly visible in the 4-, 8- and 12-h lanes.

each of the six stages of the periovulatory period were separated from one another by electrophoresis on polyacrylamide gels. The autoradiograph of these PAGE results revealed a faint, but differentially expressed, cDNA band that was more intense at 4, 8 and 12 h after hCG, and was minimal at 0 and 24 h into the ovulatory process (Fig. 1). Sizing on an agarose minigel revealed that this differentially displayed cDNA was approximately 260 bp in length (including both primers). The most intense cDNA band (i.e., the band in the 8-h lane) was excised from the acrylamide gel and re-amplified for use as a probe in northern analysis.

Northern analysis of PAP-III mRNA expression during ovulation

The northern blots revealed a temporal pattern of expression of mRNA (eventually identified as PAP-III mRNA) during ovulation that was comparable to the expression of cDNA on the differential display autoradiograph (Fig. 2). In order to compare the intensity of the signals from the northern blots with other data on gene expression during ovulation, the intensity of the signal from the 8-h lane was arbitrarily set at 100%, and the densities at the other times during the periovulatory period were expressed as fractions of that maximum. Accordingly, the NIH-image program was used to digitize all of the bands on three northern

Figure 2 Intensity of Northern blot signals at the six intervals of the periovulatory period after administration of hCG to the rats. The signal density at 8 h was arbitrarily set at 100%, and the other points on the graph represent the mean values from three northern blots prepared from pooled extracts of RNA. Bands taken from a representative northern blot with PAP-III cDNA, along with the β -actin control, are shown below the linear graph. Note that the greatest intensity is at 8 h after hCG.

blots, and the ratio of the density of each experimental band to its corresponding β -actin control band was calculated for each lane. Means ($\pm$ S.E.M.) of the signal densities at 0, 2, 4, 8, 12 and 24 h after hCG were $2.9 \pm 2.9\%$, $9.1 \pm 2.8\%$, $33.7 \pm 4.7\%$, 100%, $51.2 \pm 7.3\%$ and $19.3 \pm 4.8\%$ respectively. Thus *PAP-III* gene expression increased approximately 34-fold within 8 h after the ovulatory process was initiated by the injection of hCG into the animals. Subsequently, at 24 h after hCG (i.e., during early luteal development), *PAP-III* gene expression declined to a level that was close to the level of the 0-h control value.

Sequence of the cDNA fragments for PAP-III

After the hCG-induced expression of the *PAP-III* gene was confirmed by northern analysis, the cDNA fragment of this gene was subcloned and sequenced. The NCBI accession number for the fragment is AF159102. This cDNA is essentially identical to a segment of a *PAP-III* gene reported for the Wistar rat (NCBI accession No. L20869; Frigerio *et al.* 1993) and the Sprague–Dawley rat (NCBI accession No. U09193; Dusetti *et al.* 1995). It is also homologous to the regenerating-III (*reg-III*) gene in the mouse (NCBI accession No. D63362; Narushima *et al.* 1997).

Figure 3 Comparison of the percentage of signal from northern blots containing RNA extracted at 8 h after hCG from animals that were also treated with either 1 mg indomethacin (Indo) or 5 mg epostane (Epo) administered at 3 h after hCG. Bar graphs are based on NIH image analyses of three northern blots that were probed with PAP-III cDNA. An additional group of rats received epostane plus progesterone (E+P) as described in Materials and Methods. The signal from the 8-h control lane (Ctrl) was arbitrarily set at 100% optical density. In parallel groups of rats, the ovulation rate (expressed as ova/rat) was determined at 24 h after hCG.

Effects of indomethacin and epostane on PAP-III gene expression

For this experiment, northern blots were prepared from RNA that was extracted either from control ovaries at 0 and 8 h into the ovulatory process or from experimental ovaries taken at 8 h after hCG from rats that had been treated 5 h earlier with an ovulation-inhibiting dose of either epostane or indomethacin. These experimental intervals were selected in order to compare the present data with related studies using epostane and indomethacin (Espey *et al.* 2000a, b). The signal density (normalized against the β -actin control) of the 8-h control lane was arbitrarily set at 100% (Fig. 3). There was minimal

expression of PAP-III mRNA at 0 h, but substantial expression at 8 h. In animals treated with the anti-ovulatory agent indomethacin, which blocks PG synthesis (Espey *et al.* 1991), the signal density was $74.2 \pm 14.1\%$, but this mean was not statistically different from the 8-h control value. In animals treated with the anti-ovulatory agent epostane, which blocks progesterone synthesis (Espey *et al.* 1990), the signal density of $48.2 \pm 7.3\%$ was moderately, but significantly ($P \leq 0.05$), lower than the 8-h control value. In contrast, the ovaries from a parallel group of gonadotropin-treated animals that were administered (by a procedure described previously (Espey *et al.* 2000a)) 10 mg of exogenous progesterone before the epostane injection exhibited a recovery of the ovarian PAP-III mRNA level to $101.5 \pm 10.0\%$ of the 8-h control value (Fig. 3). The ovulation rates (expressed as ova/rat) in parallel groups of animals that were treated with indomethacin or epostane alone were significantly inhibited, but there was recovery of the ovulation rate in the epostane-treated group that received progesterone (Fig. 3).

Localization of ovarian PAP-III mRNA expression by *in situ* hybridization

In situ hybridization confirmed the temporal pattern of PAP-III mRNA expression that was observed in the differential display autoradiographs and in the northern blots. There was minimal signal from the 0-h control ovaries, an increasing signal up to 8 h after hCG treatment, and a declining signal by 24 h after the animals were injected with hCG (Fig. 4). Hybridization was localized primarily in stromal tissue in the vicinity of the ovarian hilus and, to a much lesser extent, in thecal tissue between some of the larger follicles. There was not a distinct pattern of distribution of PAP-III mRNA in the stromal tissue. Closer examination of the *in situ* data showed that most of the signal was emitted from presumptive endothelial cells of ovarian blood vessels and from a limited number of secondary follicles that were visible in the ovarian sections (Fig. 5). Small, concentrated areas of PAP-III mRNA signal could also be detected sporadically along the antral border of the granulosa layer of follicles that had grown to a mature size (Fig. 6).

Discussion

'PAP' is a term that is commonly (but not consistently) used to connote members of a closely related group of lectin-type proteins/genes that have substantial homology with PAP-I, which was initially detected in the inflamed pancreas of rats 6 h after the experimental induction of pancreatitis (Keim *et al.* 1984). The segment of PAP mRNA that has been identified in the present differential display study is identical to PAP-III, which was first discovered 8 years ago during screening of a rat jejunal

Figure 4 Change in intensity of the *in situ* hybridization signal during the six periovulatory intervals after hCG administration. Light-field micrographs on the left show the histology of ovarian sections stained with hematoxylin and eosin, and the dark-field micrographs of the same sections show the localization of PAP-III mRNA as detected by hybridization of a ^{35}S -labeled anti-sense probe derived from the PAP-III cDNA.

cDNA library (Frigerio *et al.* 1993). The complete mRNA sequence of PAP-III is 773 nucleotides, with a single open-reading frame of 174 codons (Frigerio *et al.* 1993). The sequence is 66% homologous to PAP-I and 63% homologous to PAP-2. It is also homologous to the so-called 'regeneration protein' (*reg*) found in regenerating islets of Langerhans of the rat pancreas (Terazono *et al.* 1988, Narushima *et al.* 1997). Other, related, members of

Figure 5 Closer view of the distribution of the PAP-III probe in the ovary. Arrows pointing to the left identify secondary follicles that emitted relatively strong signal. Arrows pointing to the right single out a large blood vessel that was outlined by the PAP-III signal.

the lectin superfamily include pancreatic stone protein (Chakraborty *et al.* 1995a), pancreatic thread protein (Katsumata *et al.* 1995), and hepatocarcinoma-intestine-pancreas protein (Bartoli *et al.* 1998).

The principal cellular origins of PAP proteins have not been completely defined. In the inflamed pancreas, they reportedly are produced in cells of the islets (Terazono *et al.* 1988, Katsumata *et al.* 1995), or in acinar cells close to the islets (Chakraborty *et al.* 1995a). In the anterior pituitary of GHRH-treated rats, or in treated cell cultures, they are in somatotropes (Yokoy & Friesen 1986, Yamamoto *et al.* 1992, Bowers 1995, Chakraborty *et al.* 1995a, b). In the small intestine, PAP proteins/mRNAs are constitutively expressed in the columnar epithelial cells of the ileum, jejunum and duodenum (Frigerio *et al.* 1993, Masciotra *et al.* 1995). Also, they have been identified in goblet cells and Paneth cells of the jejunum (Masciotra *et al.* 1995). (Paneth cells are immune cells in the intestinal mucosa that contain bright eosinophilic granules with a variety of antimicrobial proteins (Ouellette & Selsted 1996).) In the uterus of estrogen-stimulated pregnant rats, peptide23/PAP mRNA was localized in the luminal epithelial cells of the endometrium (Chakraborty *et al.*

Figure 6 Another high-magnification view of the distribution of PAP-III mRNA in the ovary. Most of the signal in the field is emitted from presumptive small blood vessels in the stroma of this ovary. The arrow pointing downward in the lower right corner of the field marks a longitudinal segment of a larger blood vessel. Arrows pointing to the left identify isolated spots along the antral surface of the granulosa that emitted relatively strong signal. Notice that the granulosa in the large follicle in the upper left corner of the field is void of any comparable signal.

1995c). In contrast, in the present study, PAP-III mRNA was conspicuous in luminal endothelial cells in parts of the ovarian vasculature. In addition, the *in situ* signal for this message was localized in the epithelial (granulosa) cells of secondary follicles and in a few presumptive anomalies of folliculogenesis that were rarely observed along the antral surface of the granulosa layer of large tertiary follicles. These limited structures in the granulosa layer might be related to the Call-Exner bodies, or to 'microfollicles,' which represent aberrant entities that occasionally become entrapped in growing follicles, without having any known deleterious effect (Gosden *et al.* 1989, Meng *et al.* 1994, Rodgers *et al.* 2000).

Although it is not clear whether the various PAP proteins have a common function, at least a few distinct characteristics of these members of the lectin superfamily have been established. Firstly, it is evident that the experimental induction of acute inflammation in the pancreas results in more than a 300-fold increase in PAP

mRNA within 12 h (Iovanna *et al.* 1991). Under such acute conditions, PAP comprises about 5% of the proteins secreted from the pancreas. However, this protein reportedly has a half-life of only about 4·8 min (Chakraborty *et al.* 1995b), and its secretion returns to undetectable levels when the pancreas has totally recovered (Iovanna *et al.* 1991). It should be noted that, in contrast to many of the other proteins secreted by the pancreas, PAP does not have any proteolytic activity (Heller *et al.* 1999). Secondly, GHRH induces the expression of PAP (i.e., peptide 23) by hypophyseal somatotropes, whereas somatostatin inhibits such secretion (Yokoy & Friesen 1986, Bowers 1995, Bartoli *et al.* 1998). However, even though the secretion of pituitary PAP is affected by the same factors that regulate growth hormone (GH) secretion, this protein is not homologous to GH (Katsumata *et al.* 1995). These observations have led to the suggestion that PAP may be involved in cell proliferation, as GHRH is known to stimulate somatotrope hyperplasia *in vitro* (Bowers 1995, Katsumata *et al.* 1995). Thirdly, in quite a different pattern from the above examples, PAP is expressed continuously by the small intestine (but not by the colon) (Frigerio *et al.* 1993, Chakraborty *et al.* 1995a, Maschiotra *et al.* 1995). However, as in other tissues, the function of PAP in the gastrointestinal tract has not been established. Fourthly, the role of PAP in luminal epithelial cells of the uterus has not been established. This substance is not produced by the uteri of immature rats, and its secretion in adult rats is limited mainly to the later stages of pregnancy, with maximum expression on day 12 of gestation (Chakraborty *et al.* 1995c).

In the report by Chakraborty *et al.* (1995c) on PAP expression in the uterus, the investigators concluded that β -estradiol, rather than progesterone, is the major regulator of expression of this gene. Their conclusion was based in part on evidence that, during the sexual cycle of the rat, uterine PAP was maximal on the day of estrus and minimal on the day of diestrus. However, the study did not include an analysis of circulating steroids; instead, the investigators relied on an earlier research paper that analyzed steroid concentrations during the estrus cycle of the rat (Smith *et al.* 1975). In support of their hypothesis that β -estradiol rather than progesterone promotes PAP secretion, they cited that plasma β -estradiol concentrations are highest and progesterone lowest on the day of estrus (Chakraborty *et al.* 1995c). However, close examination of the cited reference indicates that β -estradiol is merely at baseline concentration on the day of estrus, whereas progesterone is declining, yet still significantly increased, on the morning of estrus (Smith *et al.* 1975). Clarification of this incongruity is necessary to interpret the present data on ovarian expression of PAP-III, because ovarian PAP mRNA increased in response to an ovulatory dose of gonadotropin in a pattern that was parallel to the well-known ovulatory increase in ovarian progesterone and concomitant with the well-known pause in ovarian

β -estradiol synthesis (Espey *et al.* 1990, 1991). Therefore, the present evidence that ovarian PAP-III mRNA is at least partially influenced by ovarian progesterone production is an observation that might not be contradictory to the reported effect of steroids on expression of PAP in the uterus.

The background information in the preceding paragraph is only marginally helpful in deciphering the significance of PAP-III mRNA expression in the gonadotropin-stimulated ovary. It is evident from the current northern blot data that ovarian PAP expression is transient – increasing only at the time when the mature follicles are undergoing an acute inflammatory reaction that disintegrates the follicles and causes them to rupture (Espey & Lipner 1994, Espey *et al.* 2000a). Also, it is evident from the present *in situ* hybridization data that most of the PAP was outside the larger ovarian follicles and spatially distributed in a pattern that suggested this agent may not be directly involved in the events that lead to degradation and rupture of the follicle wall. Instead, it would appear that the increase in ovarian PAP might be a response to, rather than a cause of, the physiological changes that occur in ovulatory follicles. In this regard, it would appear relevant that PAP has been proposed as a protective agent in leukocyte-mediated lung injury (Heller *et al.* 1999), and it has been considered as a potential component of a defense mechanism against infection (Frigerio *et al.* 1993). Therefore, it is possible that ovarian PAP-III mRNA is expressed in conjunction with a protective response to the hyperemia, exudation, proteolysis and inflammation that characterize the physiological process of ovulation (Espey & Lipner 1994). Such a hypothesis would be congruent with what is known about PAP expression in the inflamed pancreas. Conversely, such an explanation would be difficult to correlate with PAP expression in the anterior pituitary, unless it could be shown that this hypophyseal gland responds to stimulation by GHRH in a manner that is at least mildly comparable to the inflammatory reaction in gonadotropin-stimulated ovaries. In another direction, the present results do not rule out the possibility that ovarian PAP expression is associated in some way with the reported infiltration of neutrophils and macrophages into the ovary at the time of ovulation (Brannstrom *et al.* 1994). In any event, the existing information collectively suggests that the increase in ovarian PAP-III mRNA could be a protective response to the degradative events of the ovulatory process. Therefore, the tentative hypothesis arising from this study is that ovarian expression of PAP might function in some way to reduce trauma to the ovarian vasculature and to the developing secondary follicles during the acute inflammatory reaction that is characteristic of ovulation.

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