ORIGINAL ARTICLE: REPRODUCTIVE ENDOCRINOLOGY

Variation in circulating antimüllerian hormone precursor during the periovulatory and acute postovulatory phases of the human ovarian cycle

Michael W. Pankhurst, Ph.D.[a] and Yih Harng Chong, M.B.Ch.B., Ph.D.[a,b]

[a] Department of Anatomy, Otago School of Medical Sciences and [b] Department of Medicine, Dunedin School of Medicine, University of Otago, Dunedin, New Zealand

Objective: To determine whether the relative quantity of circulating AMH precursor (proAMH) declines relative to levels of the active form ($AMH_{N,C}$) in the periovulatory phase of the ovarian cycle.
Design: Longitudinal study.
Setting: Local community.
Patient(s): Sixteen women aged between 18 to 30 years with regular menstrual cycles between 25 to 35 days long.
Intervention(s): None.
Main Outcome Measure(s): Serum concentrations of proAMH and total AMH (proAMH and $AMH_{N,C}$ combined) measured by immunoassay, with relative levels of proAMH expressed as the AMH prohormone index (API = [ProAMH]/[Total AMH] × 100).
Result(s): The mean API in the 11 eligible women fell from 20.7 during the luteinizing hormone (LH) surge period to 18.7 during the acute postsurge period. No statistically significant differences in the API were observed among samples taken at single time points in the early follicular, midfollicular, midluteal, and late luteal phases.
Conclusion(s): This study suggests that activation of AMH by proteolytic enzymes is largely stable throughout the ovarian cycle. However, there is a subtle but robust decrease in the level of proAMH relative to $AMH_{N,C}$ in the acute postovulatory period. This may indicate that periovulatory increases in prohormone convertases cause increases in proAMH cleavage rates. Alternatively, rapid changes in the hierarchy of follicle developmental stages during ovulation may result in changes in the relative ratios of proAMH and $AMH_{N,C}$. (Fertil Steril® 2016;■:■–■. ©2016 by American Society for Reproductive Medicine.)
Key Words: Menstrual cycle, ovarian cycle, prohormone convertases, total AMH

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Antimüllerian hormone (AMH) is a gonadal glycoprotein hormone of the transforming growth factor β (TGF-β) superfamily that is produced by granulosa cells in nonatretic developing ovarian follicles (1–3). The ovarian functions of AMH include regulation of primordial follicle transition rate and regulation of follicle-stimulating hormone (FSH) sensitivity in the smaller antral follicles (4–6). Serum AMH concentrations vary during the ovarian cycle (7), but the degree of variation appears to be small relative to other ovarian hormones (8). However, it has recently been shown that there are two forms of AMH in blood: the inactive precursor (proAMH) and the receptor-binding form ($AMH_{N,C}$). Commercially available assays measure both forms without discrimination (9–11), so most studies to date have described total AMH ([proAMH] + [$AMH_{N,C}$]).

ProAMH is converted to $AMH_{N,C}$ by subtilisin/kexin-type prohormone convertases (PCSKs) (12), which generate an N-terminal (AMH_N) and C-terminal (AMH_C) cleavage fragment. $AMH_{N,C}$, a noncovalently associated complex of

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Reprint requests: Michael W. Pankhurst, Ph.D., Department of Anatomy, University of Otago, P.O. Box 913, Dunedin, 9054, New Zealand (E-mail: michael.pankhurst@otago.ac.nz).

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the two fragments, is capable of activating AMH receptors via direct interaction with the AMH_C fragment (13, 14). Free AMH_C can also activate AMH receptors (14), but it has not been detected in blood (15) and thus may not be a physiological species.

Although $AMH_{N,C}$ is the most abundant form of AMH in blood (15), proAMH is the predominant form in the ovarian follicular fluid of mono-ovulatory species, using sheep as a model (16). This raises the possibility that proAMH is converted to $AMH_{N,C}$ after secretion. Antimüllerian hormone is a proven substrate for two cleavage enzymes, furin (PCSK3) and PCSK5 (12). Both enzymes exhibit increased mRNA expression in the rat ovary after stimulation with human chorionic gonadotropin (hCG), presumably acting via luteinizing hormone (LH) receptors (17, 18). This raises the possibility of neuroendocrine regulation of ovarian PCSK expression throughout the ovarian cycle.

Studies investigating whether total AMH levels show variation during the ovarian cycle have yielded conflicting results (19). More recent studies have found significant declines between the late follicular and early luteal phases, and these findings may be attributable to the larger sample sizes (7), the precise techniques for defining the follicular-luteal transition (20), or study designs that accounted for the more pronounced variation apparent in younger women (7, 21). It is possible that subtle cyclical changes in total AMH affect ovarian function, but further complexity could arise if the rate of proAMH conversion to active $AMH_{N,C}$ is also variable.

We examined serum proAMH and $AMH_{N,C}$ concentrations during the ovarian cycle using a conventional commercially available total AMH assay (22) and a novel research-grade assay that is specific for proAMH (23). We examined the ratio of the two forms of AMH to determine whether the proAMH to $AMH_{N,C}$ conversion rate changes throughout the ovarian cycle. We hypothesized that there would be a relative decrease in proAMH and consequent increase in $AMH_{N,C}$ after the LH surge.

MATERIALS AND METHODS

Study Participants

Participants were recruited from Otago University via advertising by bulk email and leaflet distribution. The study involved 16 women aged between 18 and 30 years who had regular menstrual cycle lengths between 25 and 35 days, and were not taking any hormonal contraceptives. The cycle lengths were self-reported and were averaged over three menstrual cycles before the sampled cycle. The exclusion criteria were irregular menstrual cycle lengths, cycle length >35 days or <25 days, pregnancy or lactation, prior exposure to radiotherapy or chemotherapy, infertility or prior in vitro fertilization treatment, current steroid treatment (within the last 3 months), current smoker (within 12 months), or any history of reproductive, endocrine, or chronic illnesses, including polycystic ovary syndrome.

The project was approved by the University of Otago Human Ethics Committee (Health). All participants provided written, informed consent.

Sampling Regimen

The first day of menstruation (menstrual day 1) was self-reported by participants. Venous blood samples were taken on menstrual days 3 and 7, constituting the early and midfollicular phases, respectively. Periovulatory samples were collected on consecutive days in conjunction with urinary LH strip tests (Baby 4 You) beginning on menstrual day 11. This daily periovulatory sampling was concluded 2 days after the first positive LH test or up to a maximum of seven samples, whichever was sooner. Midluteal and late luteal samples were collected 7 and 11 days after the first positive LH strip test, respectively. If no positive LH strip test was obtained, samples were taken on menstrual days 21 and 25. All blood samples were taken between 08:00 and 10:00 hours and were allowed to clot for 1 to 2 hours at room temperature. The samples were centrifuged at $2,000 \times g$ for 5 minutes, and the serum aliquots were stored at $-80^{\circ}C$.

Assays

The levels of hormones, except for proAMH, were measured using commercial enzyme-linked immunosorbent assays (ELISA), according to the manufacturers' instructions. The ELISAs were as follows: total AMH (A79765; Beckman Coulter) for which the assay buffer was added to the serum before addition to the ELISA plate (in accordance with field safety notice FSN-20434-3, June 2013); LH (11-LUTHU-E01; ALPCO), FSH (11-FSHHU-E01;, ALPCO), inhibin B (A81303; Beckman Coulter), estradiol (11-ESTHU-E01; ALPCO), and progesterone (11-PROHU-E01; ALPCO).

ProAMH was assayed using a modification to the procedure for the AMH Gen II assay kit (A79765; Beckman Coulter) (23). The capture antibody in the AMH Gen II assay binds to AMH_C, and the detection antibody binds to AMH_N (10). Briefly, the proAMH assay works by dissociating the constituent fragments of $AMH_{N,C}$ with deoxycholate, which removes AMH_N and prevents binding of the detection antibody. ProAMH is unaffected by the treatment as the molecule is uncleaved and the AMH_N and AMH_C motifs do not dissociate (23). Two 15-minute, 150-μL wash steps of 0.2 %w/v sodium deoxycholate were inserted into the AMH Gen II protocol after the sample-binding step. Recombinant $AMH_{N,C}$ controls were included on two of the assay plates to assess the efficacy of the dissociation step. Cross-detection of $AMH_{N,C}$ was less than 4% on both assay plates.

The concentrations of the recombinant proAMH standards were determined with the AMH Gen II ELISA (cat. no. A79765; Beckman Coulter). The recombinant human proAMH standards have previously been shown to have the same dose-response characteristics as the AMH Gen II standards in the AMH Gen II ELISA (23).

The measurements of each hormone were conducted in single batches with each sample being measured in duplicate, which is in accordance with our prior recommendations for the proAMH assay (23). The reported intra-assay coefficients of variation (%CV) for the commercially available assays are as follows: total AMH, 5.3%–7.7%; LH, 2.9%–4.5%; FSH, 3.4%–5.8%; progesterone, 10.2%–10.6%; and estradiol, 4.6%–9.3%. The intra-assay variability for the proAMH assay