In vivo imaging reveals an essential role of vasoconstriction in rupture of the ovarian follicle at ovulation

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Rupture of the ovarian follicle releases the oocyte at ovulation, a timed event that is critical for fertilization. It is not understood how the protease activity required for rupture is directed with precise timing and localization to the outer surface, or apex, of the follicle. We hypothesized that vasoconstriction at the apex is essential for rupture. The diameter and blood flow of individual vessels and the thickness of the apical follicle wall were examined over time to expected ovulation using intravital multiphoton microscopy. Vasoconstriction of apical vessels occurred within hours preceding follicle rupture in wild-type mice, but vasoconstriction and rupture were absent in Amhr2−/− SmoM2 mice in which follicle vessels lack the normal association with vascular smooth muscle. Vasoconstriction is not simply a response to reduced thickness of the follicle wall; vasoconstriction persisted in wild-type mice when thinning of the follicle wall was prevented by infusion of protease inhibitors into the ovarian bursa. Ovulation was inhibited by preventing the periovulatory rise in the expression of the vasoconstrictor endothelin 2 by follicle cells of wild-type mice. In these mice, infusion of vasoconstrictors (either endothelin 2 or angiotensin 2) into the bursa restored the vasoconstriction of apical vessels and ovulation. Additionally, infusion of endothelin receptor antagonists into the bursa of wild-type mice prevented vasoconstriction and follicle rupture. Processing tissue to allow imaging at increased depth through the follicle and transabdominal ultrasonography in vivo showed that decreased blood flow is restricted to the apex. These results demonstrate that vasoconstriction at the apex of the follicle is essential for ovulation.

Significance

Release of the oocyte from the ovarian follicle at ovulation occurs with precise timing and spatial localization to the outer surface of the ovary to insure deposition of the oocyte in the oviduct for fertilization. The role that vasoconstriction of follicular vessels may play in follicle rupture was tested using multiphoton microscopy to measure repeatedly, at intervals, blood flow and the diameter of individual follicular vessels relative to follicle rupture in vivo. Blocking the acute vasoconstriction that occurred at the outer surface of the follicle before ovulation prevented follicle rupture, whereas restoring vasoconstriction induced rupture. These findings contribute to our understanding of the mechanics of ovulation, showing that vasoconstriction of follicular vessels is essential for follicle rupture.


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protease inhibitor activity in the follicle to allow precise rupture at the apex while protecting most of the follicle structure from protease activity are not understood (1, 6, 7).

We postulated that vasoconstriction of vessels within the theca at the apex of the follicle is required to promote follicle rupture. Our first approach was to examine mice with conditional expression of a dominant active allele of smoothened (SMO), the transmembrane protein that relays signaling by the hedgehog (HH) pathway. In these Amhr2Cre/+SmoM2 mice, preovulatory follicles develop normally in many respects, including changes in the expression of critical genes in response to the preovulatory LH surge (8, 9). However, follicles fail to rupture, and oocytes remain trapped as the follicles luteinize. The major ovarian phenotype in these mice is a pronounced deficiency of vascular smooth muscle (VSM) surrounding vessels in the theca cell layer, whereas other vessels that are present throughout the stroma of the ovary have normal maturation with VSM. Because VSM is required for vasoconstriction, the mice provided a model to test whether failure of vasoconstriction contributes to anovulation. In additional experiments with wild-type mice, we blocked the increase in the expression of endothelin 2 (Edn2) by granulosa cells that normally occurs within hours before follicle rupture (10, 11). Because EDN2 is a potent vasoconstrictor, this approach allowed us to test the effect on follicle rupture of inhibiting vasoconstriction versus treatment with exogenous compounds to restore vasoconstriction. In addition, treatment of wild-type mice with EDN2 receptor antagonists was used to test the role of EDN2 in vasoconstriction and rupture. Vasoconstriction and changes in the follicle wall were monitored repeatedly relative to the time of ovulation using intravital multiphoton microscopy.

Results

Transgenic Amhr2Cre/+SmoM2 mice, in which thecal blood vessels are deficient in VSM (8, 9), and genotype-matched Amhr2−/−SmoM2 control mice were used to test the requirement for VSM in follicle rupture. Vessels of preovulatory follicles were imaged by multiphoton microscopy as described in detail in SI Materials and Methods and ref. 12. Prepubertal mice were treated with equine chorionic gonadotropin (eCG) to induce the growth of multiple preovulatory follicles followed 48 h later by injection with human CG (hCG), which causes ovulation within 12–13 h (8). Mice were anesthetized, and the ovary with attached bursa and reproductive tract were exteriorized and then were immobilized on a specialized dish for imaging and were perfused continuously with 37 °C lactated Ringer's solution. The vasculature was labeled by retroorbital injection of rhodamine-labeled dextran [molecular weight (MW) 2 × 10^6]. Multiple individual vessels within the theca layer at the outer surface of a selected preovulatory follicle were imaged by acquiring z-stacks every 60 min over 2-h time intervals between −2 h and 13 h after hCG injection (−2 to 0, 5−7, 8−10, and 11−13 h). Imaging protocols included repeated line scans to measure the velocity of red blood cells within a vessel and repeated z-series to measure the diameter and orientation of those vessels. These values were used to calculate blood flow (Fig. 1A–E and Fig. S1). Leakage of some of the fluorescent dextran from vessels into the antral cavity provided sufficient contrast to identify the antrum and the granulosa and theca layers of the follicle, allowing the thickness of the apical follicle wall to be measured (Fig. 1F). The base of a preovulatory follicle could not be imaged because the typical diameter of a follicle (>300 μm) exceeds the ~200 μm depth of imaging possible in the highly light-scattering ovarian tissue in vivo (12).

Blood flow and vessel diameter were constant in control and Amhr2Cre/+SmoM2 mutant mice during time intervals from 2 h before to 10 h after hCG injection; these measures decreased acutely within 11–13 h after hCG injection in control mice but decreased only slightly in mutant mice (Fig. 2). A decrease in both blood flow and vessel diameter is indicative of vasoconstriction. A similar pattern was observed in larger vessels located in close proximity to the theca layer but just external to it, in the interstitium at the side of the follicle (Fig. S2). The thickness of the follicle wall remained relatively constant in both control and mutant mice from 2 h before to 11 h after hCG injection. In control mice the thickness of the follicle wall decreased acutely between 11 and 13 h postinjection, and ovulation occurred just after 13 h postinjection, but in mutant mice the thickness of the follicle wall did not change, and follicle rupture did not occur (Fig. 2). These data demonstrate that in control mice vasoconstriction...
To test the requirement for vasoconstriction in follicle rupture, an anovulatory mouse model was used in which the rise in expression of the vasoconstrictor Edn2 by granulosa cells, which normally occurs several hours before rupture, was blocked. Previous studies showed that treatment of mice or rats with an inhibitor of the hypoxia-inducible factors (HIFs), echinomycin (Ech), reduced ovulation and suppressed the increase in Edn2 mRNA (14, 15). In our experiments, injection of Ech (1 mg/kg) at 6 h after hCG injection reduced the ovulation rate (Fig. S4A) and prevented the increase in Edn2 that normally occurred between 0 and 11 h after hCG injection (Fig. S4B). The follicles that failed to rupture in Ech-treated mice differed from normal follicles in that there was no reduction in blood flow or vessel diameter between 10 and 13 h after hCG injection, and the thickness of the follicle wall did not decrease (Fig. 4A). These results reinforced the positive association between vasoconstriction and follicle rupture. We then tested whether induction of vasoconstriction by bursal infusion of vasoconstrictors could overcome the block to ovulation in Ech-treated mice. A single bursal cavity of an Ech-treated mouse was first infused with 2% BSA-saline as a control beginning 11.25 h after hCG injection and continuing for 45 min; then the infusion was changed to 100 nM EDN2 until 15 h after hCG injection. During infusion with BSA-saline, the blood flow, vessel diameter, and thickness of the follicle wall were constant. Within 15 min after initiating infusion of EDN2, blood flow and vessel diameter were reduced; by 45 min the thickness of the follicle wall decreased (Fig. 4B); and follicles ovulated between 75 and 105 min after the initiation of perfusion. Collection of oocytes from the oviducts at 15 h after hCG injection resulted in recovery of 8.7 ± 1.1 oocytes from the oviduct ipsilateral to EDN2 infusion and 0.7 ± 0.7 oocytes from the contralateral oviduct (P < 0.001). A similar protocol in which the bursa of Ech-treated mice were infused with BSA-saline followed by the vasoconstrictor angiotensin 2 (ANG2) also resulted in vasoconstriction and thinning of the follicle wall (Fig. 4B) and induced ovulation from the infused ovary but not from the contralateral ovary (9.3 ± 0.4 and 0.7 ± 0.7 oocytes recovered, respectively).
infused vessels and follicle walls imaged repeatedly at 30-min intervals (by continuous infusion of vasoconstrictors (arrows) until 15 h after hCG injection with continuous infusion of 2% BSA-saline for 45 m followed mice restored the changes in thecal vessels and the thinning of the follicle was blocked. Data are mean ± SEM from hourly repeated measurements of 17 vessels and of the wall thickness of single follicles from three mice. (B) Infusion of EDN2 (Upper) or ANG2 (Lower) into the bursa of Ech-treated mice restored the changes in thecal vessels and the thinning of the follicle wall that normally occur before rupture. Treatment began at 11.25 h after hCG injection with continuous infusion of 2% BSA-saline for 45 m followed by continuous infusion of vasoconstrictors (arrows) until 15 h after hCG injection. Data are mean ± SEM from 13 EDN2-infused vessels or 15 ANG2-infused vessels and follicle walls imaged repeatedly at 30-min intervals (n = 3 mice for each vasoconstrictor). Data points without a common superscript are different (P < 0.05).

The fact that two different vasoconstrictors, EDN2 and ANG2, overcome the inhibitory effect of Ech on ovulation indicates that Ech-induced suppression of ovulation most likely was mediated by the inhibition of vasoconstriction, an effect that may have been caused by lack of Edn2 expression.

To test more directly the requirement for EDN2-induced vasoconstriction in follicle rupture, antagonists to the EDN2 receptors (EDNR) type A and B (JKC-301 for EDNRA and BQ-788 for EDNRB) were infused into the bursal cavity of wild-type mice. Beginning at 10 h after hCG injection, a single bursa was infused with 2% BSA-saline for 30 min; then the infusion was changed to 10 μm JKC-301 plus 10 μm BQ-788 in 2% BSA-saline, which was continued until 15 h after hCG injection. The decrease in blood flow and in the diameter of apical vessels that normally occur between 11 and 13 h after hCG injection were absent in follicles of the EDNR antagonist-infused ovaries, and ovulation was dramatically reduced in the infused ovary but not in the contralateral ovary (Fig. 5). These results show that inhibiting the action of endogenous EDN2 prevents vasoconstriction and suppresses ovulation.

Although multiphoton microscopy was effective in imaging events at the follicle apex in vivo, light scattering prevented imaging deep enough into the follicle to analyze the base. Therefore additional methods were used to analyze vascular changes throughout the wall of the follicle. First, power Doppler ultrasonography was used to assess differences in blood flow between the apex and the base of preovulatory follicles as the time to ovulation approached. In the images generated, blood flow appears as a yellow signal surrounding dark circular areas which are the antral cavities of preovulatory follicles (Fig. 6). At 0 and 9 h after hCG injection in wild-type mice, most follicles had blood flow surrounding their circumference including the apex (89.2% and 88.9%, respectively), but at 12 h in mice that had not yet ovulated, few follicles (13.9%) had blood flow at the apex (Fig. 6). The results show that the decreased blood flow before follicle rupture in wild-type mice occurs at the apex of the follicle, as is consistent with the demonstration of vasoconstriction at the apex, and shows that blood flow to the base does not change. In mice that had been treated with Ech to inhibit ovulation (as described above), 80% of follicles had blood flow surrounding the circumference at 12 h after hCG injection, similar to the observations in wild-type mice at 0 h after hCG injection, indicating that the inhibition of ovulation was associated with a lack of vascular changes at the apex. As a second approach to assess vascular changes throughout the follicle, ovaries of anesthetized wild-type mice at 0 and 12 h after hCG injection (n = 3 mice per time period) were ligated and were removed 5 min after retroorbital injection of rhodamine dextran. Ovaries were fixed and subjected to a clearing procedure to remove optically dense components that cause light scattering (16). This procedure allowed multiphoton imaging of rhodamine dextran-filled vessels throughout the wall of the follicle (Fig. S5). Projections of z-stacks (two follicles per ovary) were used to calculate the percent of the follicle area at the apex and the base covered by vessels (representative images are shown in Fig. S6). At 0 h after hCG injection, the percent of the area covered by vessels was similar at the apex and base of the follicle (26.7 ± 1.2% and 26.7 ± 4.9%, respectively). However, at 12 h after hCG injection, in mice that had not yet ovulated, the percent of the area covered by vessels was dramatically reduced at the apex (8.0 ± 2.9%, P < 0.05 vs. other values) compared with the base (20.0 ± 5.0%). The decrease in vessel coverage at the apex is consistent with the finding that vasoconstriction occurs at the apex before follicle rupture and indicates that similar vasoconstriction does not occur at the base.

Discussion

Intravital multiphoton microscopy was used to demonstrate, for the first time to our knowledge, that vasoconstriction of thecal

![Fig. 4. Vascular changes within the theca at the apex of preovulatory follicles associated with the suppression of ovulation in Ech-treated wild-type mice and the restoration of ovulation by bursal infusion of vasoconstrictors in Ech-treated mice.](image)

![Fig. 5. Bursal infusion of EDN2 receptor antagonists in wild-type mice prevents vasoconstriction of vessels at the apex of the preovulatory follicle and suppresses ovulation. Beginning at 10 h after hCG, the bursa was infused with 2% BSA-saline for 30 min, followed by infusion of antagonists of EDN2 receptors type A and B (10 μm JKC-301 plus 10 μm BQ-788; arrows) until 15 h after hCG injection. Multiphoton microscopy imaging to determine blood flow (Left) and vessel diameter (Center) was performed on 15 vessels from three mice. (Right) The ovulation rate was determined by counting oocytes in the ampulla 15 h after hCG injection. Data points without a common superscript are different (P < 0.05).](image)
vessels at the apex of the preovulatory follicle is necessary for follicle rupture at ovulation. The acute decrease in the thickness of the apical follicle wall within hours of rupture occurred simultaneously with a decrease in vessel diameter and blood flow indicative of vasoconstriction. The fact that vasoconstriction, acute thinning of the follicle wall, and rupture failed to occur in \textit{Amhr2}^{+/-}SmoM2 mice, in which thecal vessels are deficient in VSM, supports a positive association between vasoconstriction and rupture. Previous studies using corrosion casts of the vasculature in a number of species reported a clearing of vessels at the apex of the preovulatory follicle before rupture (5, 17, 18), and light microscopy visualization in vivo revealed decreased blood flow to the apex before ovulation in rat and rabbit (19, 20). Multiphoton microscopy of fluorescently labeled vessels in the current study allowed direct testing of the relationship between vasoconstriction and follicle rupture in vivo. An initial step was to demonstrate that vasoconstriction is not simply a compensatory response to the thinning of the follicle wall; when thinning of the follicle wall and ovulation were blocked experimentally by bursal infusion of serum as a source of protease inhibitors, vasoconstriction occurred with normal timing after hCG injection. This result allowed further testing of the alternative possibility that vasoconstriction contributes to follicle rupture. A dramatic increase in the expression of \textit{Edn2} by granulosa cells occurs within several hours before ovulation, and ovulation was suppressed by treatment of mice with EDN2 antagonists (10, 11) and by global knockout of \textit{Edn2} (21). These reports suggested an important role for EDN2 in ovulation, but the mechanism had not been determined. Treatment of mice with the HIF inhibitor Ech was previously shown to block the increase in the expression of \textit{Edn2} by the preovulatory follicle and to suppress ovulation (14). Here we showed that both vasoconstriction at follicle rupture were inhibited in mice in which the preovulatory rise in \textit{Edn2} was suppressed by treatment with Ech. Our finding that bursal infusion of a vasoconstrictor, either EDN2 or ANG2, restored vasoconstriction and ovulation in Ech-treated mice indicates that vasoconstriction is critical for follicle rupture. Furthermore, inhibiting the action of endogenous EDN2 by using EDN2 receptor antagonists into the bursa prevented vasoconstriction and ovulation. The results suggest that the major role of EDN2 in ovulation is likely to be the induction of vasoconstriction to allow follicle rupture. The light-scattering properties of ovarian tissue in vivo prevented the use of multiphoton microscopy to image deeply enough into the preovulatory follicle to determine whether vasoconstriction before ovulation is restricted to the apex. However, optical clearing of ovarian tissue ex vivo allowed multiphoton imaging of rhodamine dextran-filled vessels throughout the follicle. This procedure revealed decreased coverage by rhodamine dextran-filled vessels at the apex of the follicle close to the time of rupture but no change in vessel coverage at the base. Additional analysis of ovaries in vivo using Doppler ultrasound showed that blood flow changed as ovulation approached, from complete surrounding of the follicle to being absent at the apex immediately before follicle rupture, a pattern that is consistent with vasoconstriction occurring only at the apex. Doppler ultrasound of ovaries in women and cattle also showed that blood flow to the apex of the follicle decreases around the time of ovulation, but blood flow to the base of the follicle remains strong (22, 23).

The mechanism whereby vasoconstriction promotes follicle rupture at ovulation must be determined. We postulate that vasoconstriction at the apex causes the localized depletion of components derived from serum that prevent the breakdown of the follicle wall; their absence thus promotes rupture. Protease inhibitors are present in serum at high concentrations (13), and their role in contributing to homeostasis in a number of tissues has been recognized. The potency of these inhibitors in the ovary is supported by our finding that thinning of the follicle wall and rupture were blocked by bursal infusion of diluted serum. The preovulatory LH surge causes increased expression of proteases in cells throughout the follicle, and these proteases are believed to contribute to the tissue remodeling critical for follicle rupture and the formation of the corpus luteum. The expression of tissue inhibitors of proteases by follicular cells also increases in response to the LH surge (1, 6, 7). Furthermore, the LH surge increases the permeability of follicular vessels (24, 25), which would promote the influx of serum protease inhibitors, some of which have relatively high MW but are present in follicular fluid (26–30). The presence of protease inhibitors may balance the
activity of proteases to protect follicular tissue while allowing remodeling to form the corpus luteum. Localized, acute vasoconstriction at the apex of the preovulatory follicle may prevent access of protease inhibitors in serum, shifting the balance toward destruction of a small region at the apex and rupture to release the oocyte. Although the cause of the selective vasoconstriction at the apex of the follicle is not understood, it might be generated by the actions of vasoconstrictors on the unique vascular structure of the follicle (4, 5). Precise timing of vasoconstriction within the preovulatory follicle may be essential to release the oocyte from the apex before it becomes trapped by the luteinization process.

Knowing that vasoconstriction is essential for follicle rupture contributes to a more complete understanding of the mechanics of ovulation. This information has the potential to contribute to future developments of methods for regulating fertility. For example, the causes of a condition in women in which follicles fail to rupture and the oocyte becomes trapped in the luteinized follicle (31) could be investigated with an appreciation for the importance of vasoconstriction.

Materials and Methods

Mouse strains, surgical preparation of mice, and the multiphoton microscopy and Doppler ultrasonography methods used to image preovulatory follicles are detailed in SI Materials and Methods. Protocols for superovulation, cannulation, infusions into the ovarian bursa, collection of oocytes from the ovudix, and RT-PCR are described in SI Materials and Methods.


Mice. Amhr2cre/+ mice, provided by Richard Behringer, M.D. Anderson Cancer Center, University of Texas, Houston (32), and GT(ROSA26)Sortm1(smox2 Cre/+) mice (33), purchased from The Jackson Laboratory, were mated to obtain Amhr2cre/+;SmoM2 mice (mutants) and Amhr2+/+;SmoM2 controls. Mice were genotyped from tail DNA using protocols from The Jackson Laboratory. B6/129 mice were obtained from The Jackson Laboratory. Mice were maintained according to the NIH Guide for the Care and Use of Laboratory Animals (34), and studies were approved by the Cornell University Institutional Animal Care and Use Committee.

Statistical Analyses. General linear mixed models were used to analyze blood flow (log 10 scale) and vessel diameter and thickness of the follicle wall (arithmetic scale) with JMP Pro 10 software. The models included fixed effects for type (mouse genotype or treatment), time of sampling, and their interaction and random effects contributed by repeated measurements of multiple vessels. Tukey’s honest significant difference test was used for pairwise post hoc tests. Gene-expression data were log transformed and analyzed by one-way ANOVA followed by post hoc comparisons with the Student–Newman–Keuls procedure. Ovation rates were compared by Student’s t test or ANOVA. Ultrasound data were analyzed by the χ2 test.

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34. Committee on Care and Use of Laboratory Animals (1996) Guide for the Care and Use of Laboratory Animals (Nati Inst Health, Bethesda), DHHS Publ No (NIH) 85-23.