

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^{cre/+}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.

ovary | ovulation | endothelin | in vivo imaging | vasculature

During ovulation in typically mono-ovulatory species such as humans, as well as in poly-ovulatory species such as rodents, the oocyte is released from the preovulatory follicle by extrusion through a rupture site on the outer surface, or apex, of the follicle, which protrudes from the surface of the ovary (1). Precise timing and accurate spatial localization of rupture at the apex are essential to allow capture of the oocyte by a hormonally primed oviduct where fertilization occurs, but the mechanisms involved are not yet understood. The rupture site breaches multiple layers of cells and their associated extracellular matrix and basement membranes (2). These include the single layer of epithelial cells that covers the surface of the ovary, the basement membrane that supports it, and the multiple cell layers comprising the wall of the preovulatory follicle. The outer wall of the ovarian follicle contains androgen-secreting theca cells and extensive vasculature. This vasculature consists of an inner and an outer plexus of capillaries with associated arterioles and venules that supply nutrients to the entire follicle (3–5). Underlying the theca and separated from it by a basement membrane is the avascular granulosa cell layer that serves as the major source of estrogen. The oocyte resides in the center of the follicle surrounded by multiple layers of specialized granulosa cells known as “cumulus cells.” In a mature preovulatory follicle, formation of a fluid-

filled antral cavity separates the oocyte–cumulus complex from the mural granulosa cells that form the wall of the follicle except at a region known as the “stalk,” which connects the oocyte–cumulus complex to the antral granulosa cells of the follicle wall. At ovulation the oocyte is released from the follicle in association with attached cumulus cells.

The preovulatory release of surge levels of luteinizing hormone (LH) from the anterior pituitary acts on receptors in the follicle to trigger events critical for the rupture and remodeling of the follicle and differentiation of granulosa and theca cells into progesterone-producing cells of the corpus luteum. The cumulus cells are induced to secrete a mucoelastic extracellular matrix which causes loosening of contacts between granulosa cells and between granulosa cells and the oocyte, a process known as “cumulus expansion,” which is essential for ovulation (1). Expression of proteases belonging to several major families, including the matrix metalloproteinase, plasminogen activator/plasmin, and ADAMTS (a disintegrin and metalloproteinase with thrombospondin-like motifs) families, increases. Simultaneously, follicle cells express protease inhibitors such as tissue inhibitors of metalloproteinases (TIMPs 1–4) and plasminogen activator inhibitors (PAI 1–3) (6, 7). The increase in protease activity is essential for rupture of the follicle and for the breakdown of the basement membrane separating theca and granulosa cells to allow the ingrowth of blood vessels to establish the corpus luteum. The mechanisms that regulate the balance of protease and

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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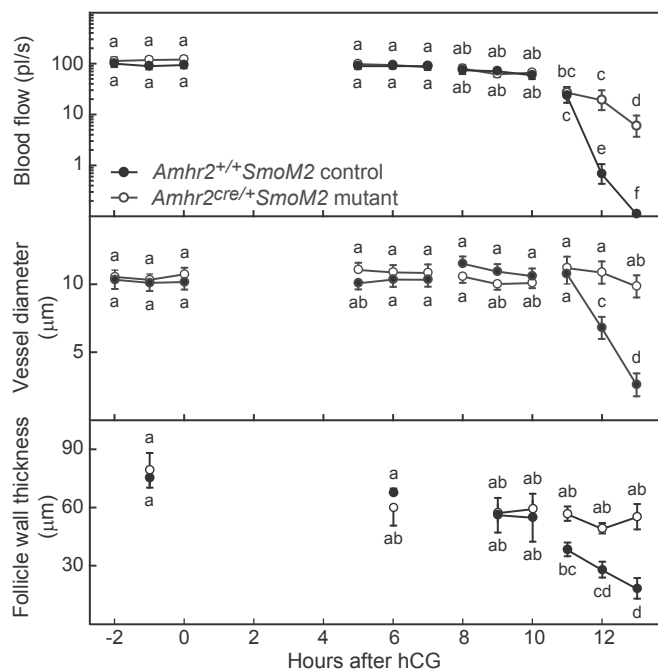


Fig. 2. Blood flow (Top) and the diameter of vessels within the theca at the apex of the preovulatory follicle (Center) decreased acutely within hours of follicle rupture in *Amhr2^{+/+}SmoM2* control mice but remained relatively constant in anovulatory *Amhr2^{cre/+}SmoM2* mutant mice in which follicular vessels are deficient in VSM. (Bottom) The thickness of the apical follicle wall decreased simultaneously with decreased blood flow in control mice but not in mutant mice. Individual vessels and the thickness of the follicle wall were measured hourly in groups of anesthetized mice studied during 2-h intervals between -2 h to 13 h after injection of hCG. Ovulation occurred in control mice between 13 and 13.5 h after hCG injection. Data on blood flow and vessel diameter at each time point are from 22–36 vessels from individual follicles of five mice (mean \pm SEM). Data on the thickness of the follicle wall are from single follicles measured at the times shown in three or four mice. Data points without a common super- or subscript are different ($P < 0.05$).

of thecal vessels at the apex of the follicle occurs coincident with the acute thinning of the follicle wall before rupture. Acute thinning of the follicle wall and rupture fail to occur in *Amhr2^{cre/+}SmoM2* mice in which thecal vessels lack the normal association with VSM and do not constrict. Vasoconstriction before rupture was further supported by measurements of the area at the outer surface of follicles covered by rhodamine dextran-filled vessels. Vessel coverage declined between 6 h after hCG and prior to ovulation at 12 h after hCG in control mice but remained constant in mutants (Fig. S3).

It was important to address the possibility that vasoconstriction at the apex of the follicle before rupture might simply be a response to the thinning of the follicle wall and a compensatory decrease in blood supply. If so, experimentally blocking thinning should prevent vasoconstriction. However, if vasoconstriction is independent of thinning, it should occur even when thinning is blocked. This question was tested by inhibiting the protease activity that leads to thinning of the follicle wall. Because serum is a rich source of protease inhibitors, a number of which are present at milligram-per-milliliter concentrations [e.g., alpha 2 macroglobulin and alpha 1 anti-trypsin (13)], we infused serum into the bursal cavity to inhibit protease activity and observed the effect on vasoconstriction. Thinning of the follicle wall was blocked in mice in which the bursa was infused with serum between 10 and 15 h after hCG injection; at 15 h only 1.3 ± 0.8 oocytes were recovered from the oviduct ipsilateral to infusion, and 16 ± 1.9 oocytes were recovered from the contralateral oviduct (Fig. 3). In contrast, in mice in which the bursa was infused with BSA as a control, the acute thinning of the follicle wall between 10 and

13 h after hCG injection occurred normally, and oocytes were recovered from oviducts ipsilateral and contralateral to infusion (15.7 ± 2.3 and 15.7 ± 1.1 oocytes, respectively). Despite this difference in the thickness of the follicle wall in response to BSA or serum infusion, the decrease in blood flow and vessel diameter occurred similarly (Fig. 3), indicating that vasoconstriction before follicle rupture is not initiated by or dependent on thinning of the follicle wall. This result allowed testing of the alternative possibility: that acute thinning of the follicle wall before rupture is dependent on 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,

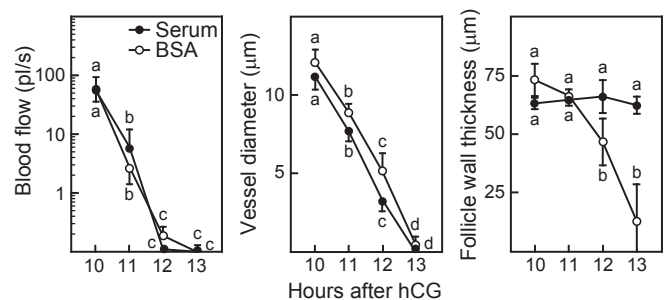


Fig. 3. Infusion of serum into the bursal cavity, as a means to deliver protease inhibitors, blocked the thinning of the follicle wall that normally occurs before ovulation (Right) but did not prevent the decrease in blood flow (Left) or in the diameter of thecal blood vessels (Center). Serum (1:1 in saline) or 2% BSA in saline as control was infused into the bursa 10–15 h after hCG injection. Data are mean \pm SEM from hourly repeated measurements of 14 or 15 vessels and of the wall thickness of single follicles from three mice at each time point. Data points without a common super- or subscript are different ($P < 0.05$).

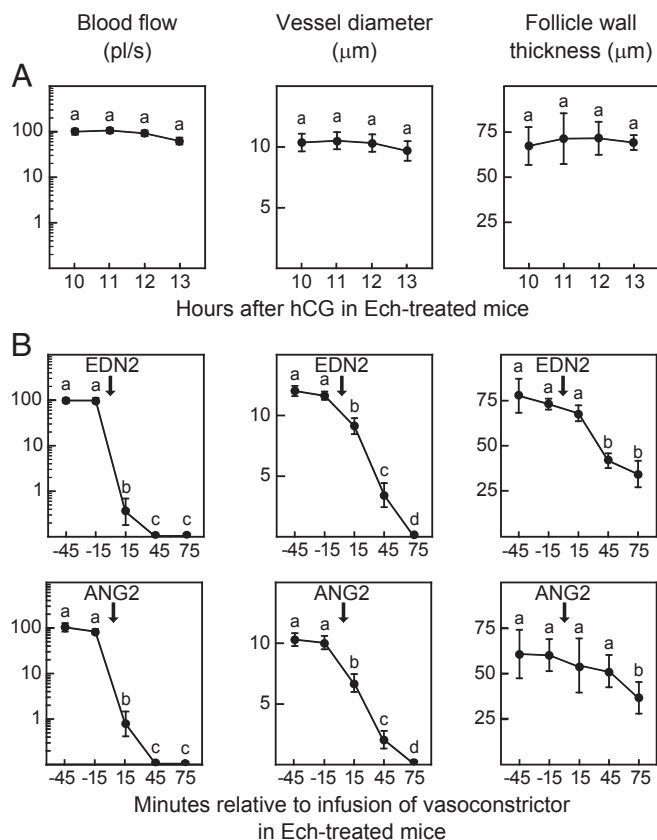


Fig. 4. Vascular changes within the theca at the apex of preovulatory follicles associated with the suppression of ovulation in Ech-treated wild-type mice and the restoration of ovulation by bursal infusion of vasoconstrictors in Ech-treated mice. (A) Blood flow (Left), the diameter of thecal vessels (Center), and the thickness of the apical follicle wall (Right) were constant from 10 to 13 h after hCG injection in Ech-treated mice in which ovulation was blocked. Data are mean \pm 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 min followed by continuous infusion of vasoconstrictors (arrows) until 15 h after hCG injection. Data are mean \pm 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 ($P < 0.05$).

respectively). The fact that two different vasoconstrictors, EDN2 and ANG2, overcame 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 μ M JKC-301 plus 10 μ M 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 retro-orbital 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 ($26.7 \pm 1.2\%$ and $26.7 \pm 4.9\%$, 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 ($8.0 \pm 2.9\%$, $P < 0.05$ vs. other values) compared with the base ($20.0 \pm 5.0\%$). 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

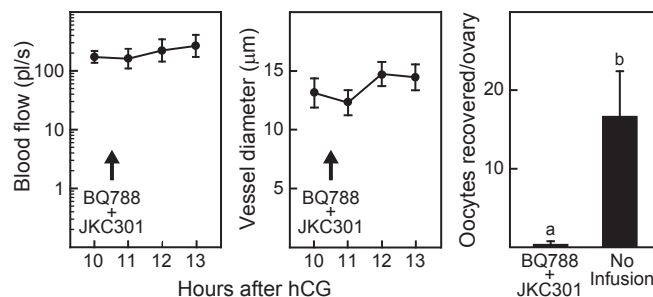


Fig. 5. Bursal infusion of EDN2 receptor antagonists in wild-type mice prevents vasoconstriction of vessels at the apex of the preovulatory follicle and suppresses ovulation. Beginning at 10 h after hCG, the bursa was infused with 2% BSA-saline for 30 min, followed by infusion of antagonists of EDN2 receptors type A and B (10 μ M JKC-301 plus 10 μ M 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 ($P < 0.05$).

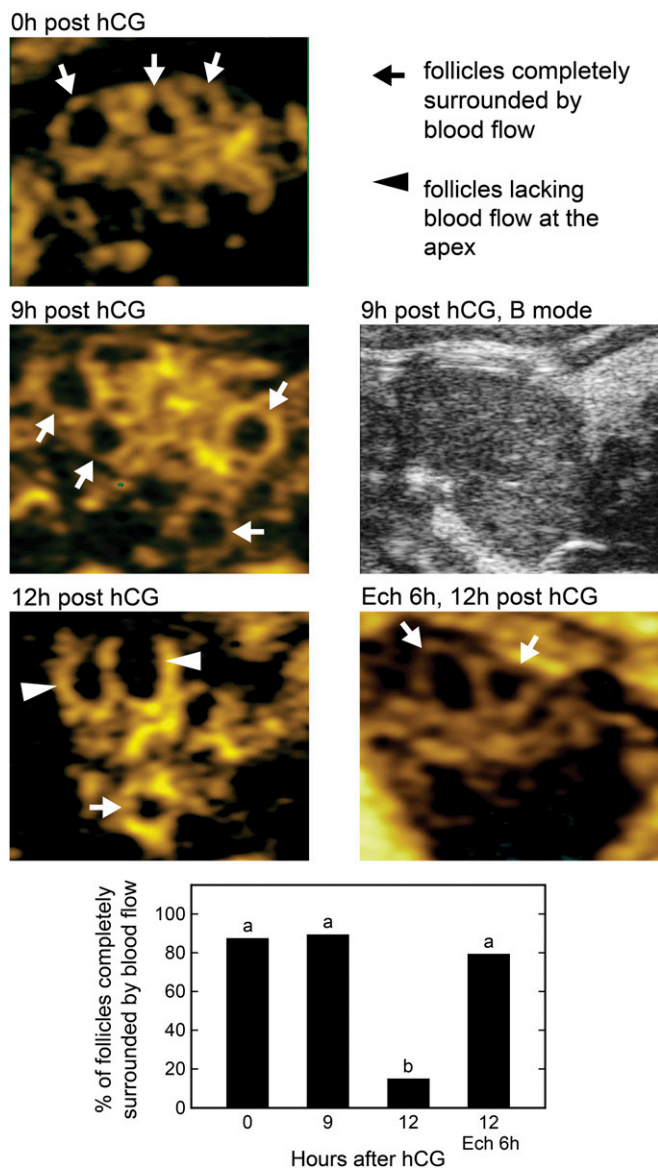


Fig. 6. Power Doppler ultrasound imaging of ovaries reveals changes in the pattern of blood flow around follicles at ovulation. Areas of blood flow appear yellow. Follicles are identified by a dark antral cavity associated with areas of blood flow and by their large size (>200 μ m). There was continuous blood flow around the circumference of preovulatory follicles at 0 h and 9 h after hCG injection (arrows) and a lack of blood flow to the apex of follicles at 12 h after hCG injection (arrowheads), just before the expected time of follicle rupture. Ovaries from mice treated with Ech at 6 h and examined at 12 h after hCG injection also showed continuous blood flow around the circumference of follicles. A B-mode ultrasound image of a mouse ovary at 9 h after hCG injection, taken at the same depth as the power Doppler image, is shown. The percent of follicles completely surrounded by blood flow is shown in the bar graph. A total of 19–46 follicles from three to five mice were analyzed at each time point. Data points without a common superscript are different ($P < 0.05$).

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 *Amhr2^{cre/+}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 *Edn2* by granulosa cells occurs within several hours before ovulation, and ovulation was suppressed by treatment of mice with EDNR antagonists (10, 11) and by global knockout of *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 *Edn2* by the preovulatory follicle and to suppress ovulation (14). Here we showed that both vasoconstriction and follicle rupture were inhibited in mice in which the preovulatory rise in *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 infusing 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 completely surrounding 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 oviduct, and RT-PCR are described in *SI Materials and Methods*.

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