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Prostaglandin-Independent Anovulatory Mechanism of Indomethacin Action: Inhibition of Tumor Necrosis Factor α-Induced Sheep Ovarian Cell Apoptosis[1]

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ABSTRACT

Indomethacin, a nonsteroidal anti-inflammatory agent, is a potent inhibitor of ovulation in vertebrates. The presumptive obligate anovulatory mode of indomethacin action is via suppression of ovarian prostaglandin production. We report that a very high systemic dose of indomethacin (800 mg i.m.) is required to block ovulation in gonadotropin-treated anestrous ewes. A lower dose of indomethacin (200 mg), which negated the preovulatory rise in follicular prostaglandin ($PGF_{2\alpha}$) biosynthesis, did not prevent ovulation. Endothelial secretion of tumor necrosis factor (TNF)-α within the apical follicular wall (prospective site of rupture) was not altered by indomethacin; notwithstanding, the apoptosis (DNA-fragmentation)-inducing effect of TNF-α (a determinant of ovulatory stigma formation) was attenuated by 800 (but not 200) mg indomethacin. A suprapharmacological concentration of indomethacin also was necessary to protect ovarian surface epithelial cells from a (prostaglandin-independent) cytotoxic effect of TNF-α in vitro. It is concluded that indomethacin inhibits ovulation by anti-apoptotic mechanisms that can be dissociated from the paradigm of prostanoid down-regulation.

INTRODUCTION

Ovarian cells associated with the formative site of ovulation (stigma) undergo biochemical and morphological changes indicative of apoptosis [1]. Apoptosis is an active mode of programmed cellular death characterized by calcium influx, endonuclease activation, oligonucleosomal DNA fragmentation, and cytoplasmic/nuclear condensation. Pyknotic cells afflicted by apoptosis lose contact with their neighbors; those that line cavities are sloughed. Residual bodies of degenerative cells are resorbed (within a few hours) by phagocytes [2–5]. A localized phenomenon of apoptotic cellular deletion contributes to ovarian wall weakening that underscores follicular rupture.

Tumor necrosis factor (TNF)-α has recently emerged as a candidate mediator of stigma development and ovulation. Cells generate TNF-α as an integral transmembrane precursor protein [6] that is truncated from the cell surface by a metalloproteinase disintegrin [7] or serine protease [8]. Mature TNF-α is a soluble noncovalent homotrimer. Common cell types that secrete TNF-α include leukocytes, smooth muscle, fibroblasts, and endothelium. Virtually all nucleated cells display plasma membrane receptors for TNF-α [6, 9] that can evoke transcriptional and death effector signals [10–12]. Cleavage by plasmin of TNF-α exodomain from thecal endothelium along the apex of preovulatory ovine follicles (following urokinase release from contiguous LH-stimulated ovarian surface epithelium) was related to apoptosis within a limited diffusion radius [13, 14]. TNF-α also was secreted by preovulatory bovine [15], human [16, 17], and rat [18, 19] follicles. And the addition of TNF-α (with LH) to perfusates of rat ovaries enhanced ovulation rates [20]. Ovulation in ewes was inhibited by intrafollicular injection of TNF-α antibodies [13].

Indomethacin is a nonsteroidal anti-inflammatory inhibitor of ovulation [21]; in fact, it can protect ovarian cells from apoptosis [22]. It is generally believed that indomethacin prevents follicular rupture by negating the preovulatory increase in ovarian prostaglandin synthase activity [21]. Biosynthesis of prostaglandins were up-regulated by TNF-α in human luteinized granulosa cells [16], placenta [23], decidual macrophages [24], and rat preovulatory follicles [25]. Prostaglandins also are putative mediators of apoptosis [26, 27]. Thus, there is a potential interaction between TNF-α and prostaglandins in ovarian apoptosis and ovulation.

The initial objective of this investigation was to define in ewes the dose-response effects of indomethacin on ovulation relative to follicular-ovarian surface dynamics of TNF-α, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production, and apoptosis. Effects of indomethacin on TNF-α-induced apoptosis in ovarian surface epithelial cells were then evaluated in vitro.

MATERIALS AND METHODS

Experiments were performed with the approval of the University of Wyoming Animal Care and Use Committee. Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise.

Ovulatory Model/Effects of Indomethacin

Mature Western-range ewes were fitted for 10 days during the anestrous season with an intravaginal progesterone-releasing device (CIDR; InterAg, Hamilton, New Zealand). Follicular development was enhanced by injection of eCG (500 IU i.m.) at implant removal. Ovaries were examined by laparoscopy at 32 h after eCG, and the ovarian location of the dominant follicle was recorded. A preovulatory surge of gonadotropins was induced by administration of an agonistic analogue of GnRH (5 μg i.m. des-Gly^{10}-Ala^{6}-ethylamide) at 36 h after eCG. At 10 h after GnRH injection, animals were treated with 200 or 800 mg indomethacin (6 ml PBS i.m. suspension) or injection vehicle (control). Six ewes were included in each group. The fate of the noted follicle (sustained dominance and ovulation) was assessed by laparoscopy at 18 and 28 h after GnRH (follicular rupture normally occurs at approximately 24 h post-GnRH [28]).

Ovarian/Follicular Effects of In Vivo Indomethacin Administration

The dominant ovarian follicle of control ewes was isolated at 0 and 18 h after GnRH injection. The dominant follicle of ewes treated with 200 or 800 mg indomethacin was obtained at 18 h after GnRH injection. Four animals were included in each group. Tissue collections were made

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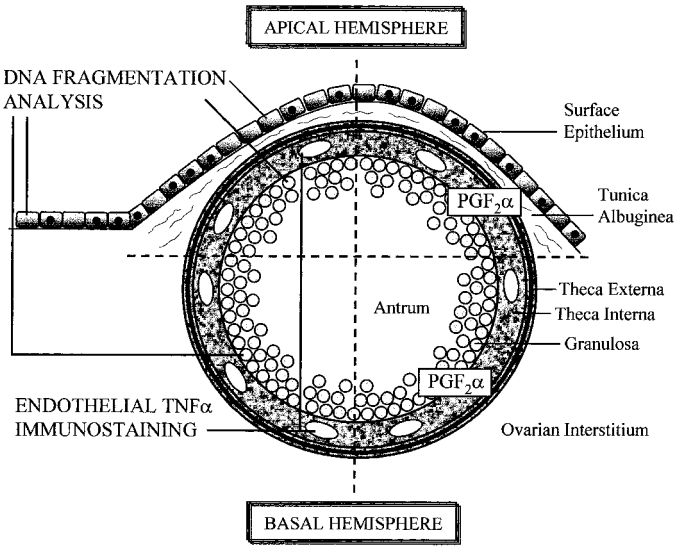

FIG. 1. Schematic of partitioning of ovarian/follicular components for morphometric and biochemical analyses. Surface epithelial cells for in situ apoptotic measurements were isolated from one half of the area overlying the preovulatory follicle and a peripheral (follicle-unassociated) site using a Teflon scraper (Becton Dickinson, Lincoln Park, NJ) designed to dislodge adherent cells from culture flasks. Follicles were dissected, and extraneous ovarian stroma was removed using precision microsurgical tenotomy scissors (Accurate Surgical & Scientific Instruments, San Diego, CA). A single-edged razor blade was used to section tissues into quadrants (hashed lines). Granulosa cells for analyses of apoptosis were removed (as described for ovarian epithelium) from the designated apical and basal surfaces lining the follicular antrum; corresponding theca were assessed by histology for TNF-α immunoreactivity. Ipsilateral apical and basal follicular walls were assayed for $PGF_{2\alpha}$.

at the time the animals were killed by i.v. injection of Beuthanasia-D (Schering-Plough Animal Health, Kenilworth, NJ). Follicles and ovarian surface cells were allotted for TNF-α, $PGF_{2\alpha}$, and DNA fragmentation analyses as depicted in Figure 1.

In Vitro Effects of TNF-α and Indomethacin on $PGF_{2\alpha}$ and Apoptosis in Ovarian Surface Epithelium

Ovarian surface epithelial cells of ewes express prostaglandin synthase [29]. Surface cells were removed from ovaries of ewe lambs at slaughter (University of Wyoming Meat Science Abattoir), pooled, and aliquoted (75 000/0.1 ml Medium-199) for $PGF_{2\alpha}$ (95 μl) and DNA fragmentation (5 μl) measurements after incubation (0 or 4 h; 37°C; 0.5-ml microfuge tubes) with TNF-α (0, 2 ng) and(or) indomethacin (0, 0.05, 0.2 ng). Each within-experiment treatment was replicated five times. The selected dose of TNF-α caused apoptosis in a previous study [14]. Doses of indomethacin were calculated to represent unbound circulatory maxima after 200- or 800-mg injections [30]. Cells and incubation media for $PGF_{2\alpha}$ assay were separated by centrifugation (8000 × *g*, 10 min).

TNF-α Immunohistochemistry

Thecal specimens for endothelial TNF-α immunostaining were fixed by immersion in 10% buffered formalin, washed in PBS, dehydrated in a graded series of ethanol, cleared in xylene, embedded with paraffin, sectioned at 5-μm thickness, and transferred onto microscope slides treated with subbing solution (0.025% chromium potassium sulfate, 0.25% gelatin; to enhance tissue adherence). Tissue sections were deparaffinized, rehydrated, incubated with 10% normal goat serum (10 min), washed (2 times with PBS), incubated with rabbit anti-sheep TNF-α serum (1:200, 30 min; Chemicon International, Temecula, CA), and washed (3 times with PBS). Antibody-ligand complexes were detected with a secondary goat anti-rabbit immunoglobulin G-fluorescein isothiocyanate (FITC) conjugate (F 9887; 1:160, 10 min). Negative controls were carried out in the absence of primary antibody and with antiserum preabsorbed with recombinant human TNF-α (1 μg/ml; R & D Systems, Minneapolis, MN). Slides were examined using an Olympus BH-2 microscope (Tokyo, Japan) equipped with a reflected light fluorescence attachment. Images of thecal areas (six/sample chosen at random) were captured (×1000), and endothelial cells therein were categorized (Optimas, Bothell, WA) as stained (luminance intensity > 2 × background) or unreactive. Data are expressed as the percentages of endothelial cells that are labeled.

$PGF_{2\alpha}$ Immunoassay

$PGF_{2\alpha}$ was quantified in tissue, cellular, and incubation medium extracts (ethyl acetate) by enzyme immunoassay using a commercial kit according to the instructions of the manufacturer (Assay Designs, Ann Arbor, MI). Parallelism existed between inhibition curves obtained with standards and different dilutions of a pooled follicular extract. The assay was sensitive to 0.3 pg. Coefficients of variation were < 8%.

In Situ DNA Fragmentation Analysis

End-labeling of fragmented DNA was used as an index of progressive (nuclear) apoptosis in permeabilized epithelial cells [1, 31]. Briefly, 3′-OH ends of DNA were linked with digoxigenin-11-d uridine triphosphate by terminal deoxynucleotidyl transferase (TdT) catalysis. Incorporated nucleotide heteropolymers were localized with antidigoxigenin Fab-FITC (Oncor ApopTag Kit S7111; Gaithersburg, MD). Conjugate or TdT were omitted in negative control reactions. Cells (20/sample selected at random; ×400) were classified by computer analysis as immunostained or not.

Statistics

Subsample data were averaged. Percentage data were transformed (arc sine) for the purpose of analyses. Mean comparisons were made by Student's *t*-test or ANOVA and protected least-significant difference. Contrasts were considered significantly different at $P < 0.05$.

RESULTS

In Vivo Studies

Dominance of the ovarian follicle identified at 32 h after eCG prevailed at 18 h after GnRH injection irrespective of interim treatments. Follicular rupture had occurred by 28 h after GnRH in all ewes treated with injection vehicle or 200 mg indomethacin. Ovulation was blocked by 800 mg indomethacin.

Endothelial TNF-α immunostaining along the apex of follicles was diminished (release into surrounding interstitium) after GnRH administration regardless of exposure to indomethacin. Intensities of TNF-α immunoreactions within the basal follicular wall were not affected by time or treatments (Fig. 2).

Concentrations of $PGF_{2\alpha}$ were elevated in gonadotropin-stimulated control follicles. There were no regionalized (apical

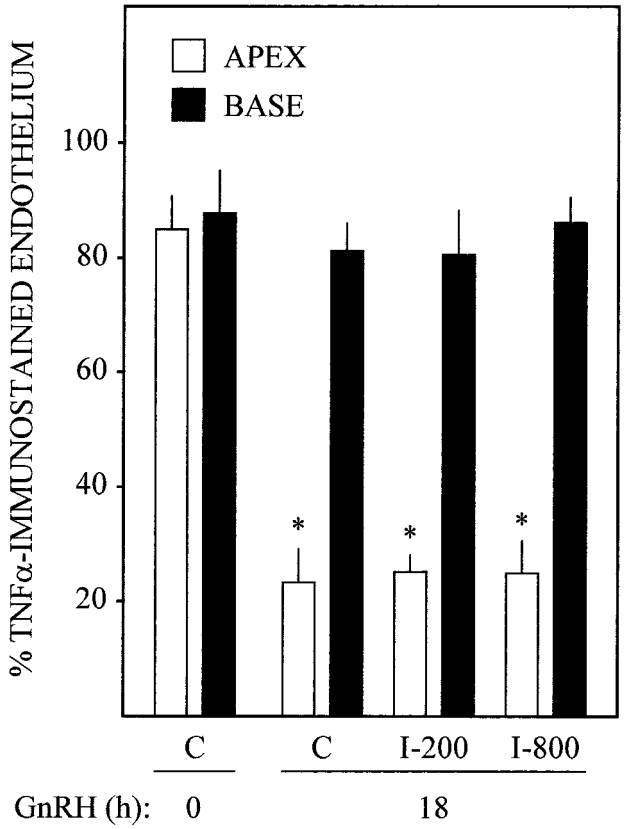

FIG. 2. Alterations in TNF-α immunostaining of thecal endothelial cells of preovulatory follicles as affected by spatial orientation (apex versus base), time after administration of GnRH, and treatments with indomethacin. C, Control; I-200, 200 mg indomethacin; I-800, 800 mg indomethacin. Data are expressed as the percentages of endothelial cells that are labeled. Means + standard errors are plotted. *Apical differences ($P < 0.01$) from 0-h control.

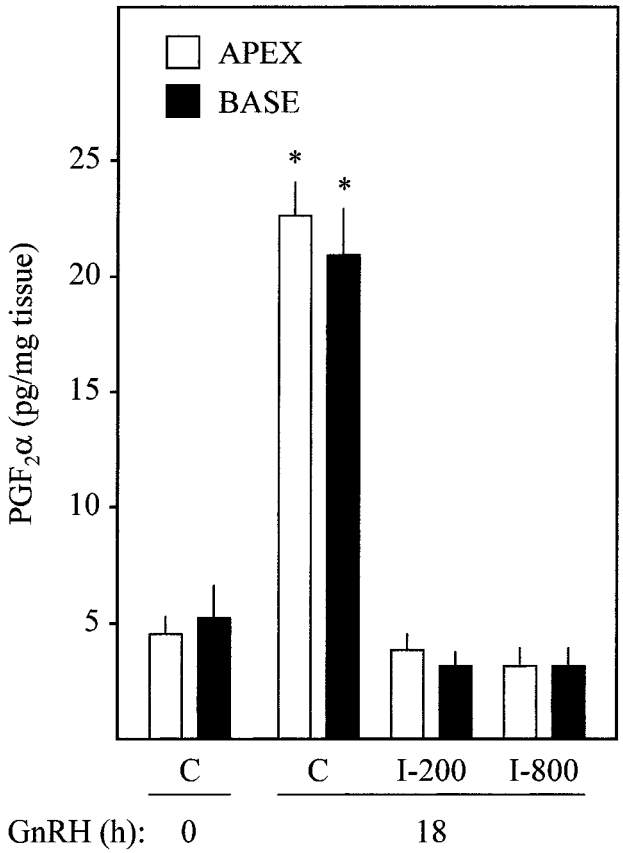

FIG. 3. Alterations in $PGF_{2\alpha}$ concentrations within the follicular wall after treatments with GnRH and indomethacin. *Significant ($P < 0.01$) elevations.

compared to basal) distinctions in follicular $PGF_{2\alpha}$ production due to time of tissue collections or indomethacin treatments. To the same extent, both doses of indomethacin negated the innate preovulatory rise in tissue $PGF_{2\alpha}$ (Fig. 3).

Ovarian surface epithelial cells associated with the apical dome of preovulatory follicles exhibited extensive evidence of apoptosis (in situ DNA fragmentation); this reaction was suppressed only by the high (anovulatory) indomethacin dosage. Very few cells of the peripheral ovarian epithelium (i.e., not associated with the formative ovulatory site) were immunostained, and there were no significant variations due to time after GnRH injection or indomethacin exposure. Although the degree of response was not as marked as that of ovarian surface epithelium, there also was an increase in apical granulosa cells immunostained positively for DNA fragments within preovulatory follicles—which, again, was attenuated exclusively by 800 mg indomethacin. Low incidences of apoptosis within the basal granulosa were not influenced by time of collection or indomethacin (Fig. 4).

In Vitro Experiment

Mean contents of $PGF_{2\alpha}$ in ovarian surface epithelial cells were not affected by treatments with TNF-α and(or) indomethacin (0.7–0.85 ± 0.25 pg). Prostaglandin $F_{2\alpha}$ was not detectable in conditioned incubation medium.

Incubation and the presence of indomethacin did not alter the frequency (< 15%) of cells with fragmented DNA in the absence of TNF-α. TNF-α caused DNA fragmentation in the majority of isolated ovarian surface epithelial cells. There was a tendency for the low dose of indomethacin to protect cells from the apoptotic effect of TNF-α. The efficacy of indomethacin to nullify apoptosis was attained at the 4-fold dose (Fig. 5).

DISCUSSION

Results of the in vivo indomethacin study support previous evidence in cyclic ewes indicating that inhibition of the preovulatory increase in follicular prostaglandin production is not a requisite of apical ovarian DNA fragmentation, apoptosis, and rupture [22, 32, 33]. The ovulatory process has been compared to an acute inflammatory reaction [34]. In fact, higher therapeutic doses of nonsteroidal anti-inflammatory agents are often required to suppress inflammation than are needed to blunt prostaglandin biosynthesis [35].

Novel in vivo findings of this study demonstrate that indomethacin does not alter TNF-α release from follicular endothelium, but can (at an anovulatory dose) subsequently interfere with the induction of apoptosis. We also have established a predictable model for examining preovulatory follicular dynamics in gonadotropin-stimulated anestrous animals. In vitro results confirm that very high concentrations of indomethacin are necessary to circumvent the apoptosis-evoking effects of TNF-α. There was no evidence that $PGF_{2\alpha}$ accumulation within the follicular wall was regionalized or that TNF-α was a stimulus of $PGF_{2\alpha}$ production (at least by ovarian epithelium).

Cell-killing properties of TNF-α are mediated by a trans-

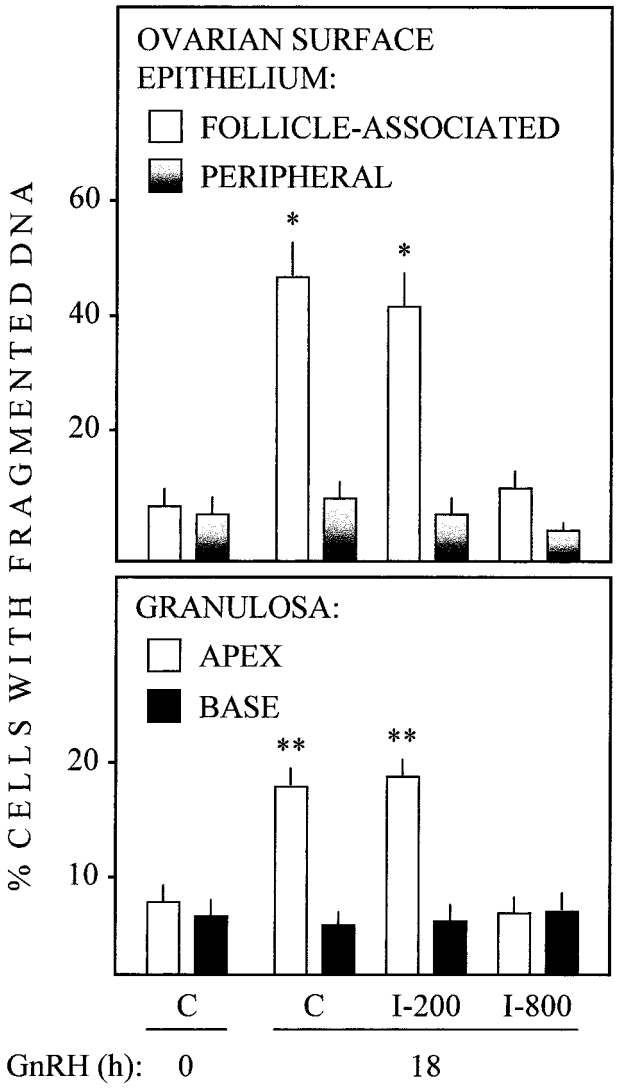

FIG. 4. Inhibitory effects of the anovulatory dose of indomethacin on apical ovarian/follicular epithelial apoptosis. Data are expressed as percentages of ovarian surface epithelial and granulosa cells that displayed fragmented DNA. Follicular-associated and peripheral regions of the ovarian surface were examined. Asterisks indicate increases (*$P < 0.01$; **$P < 0.05$).

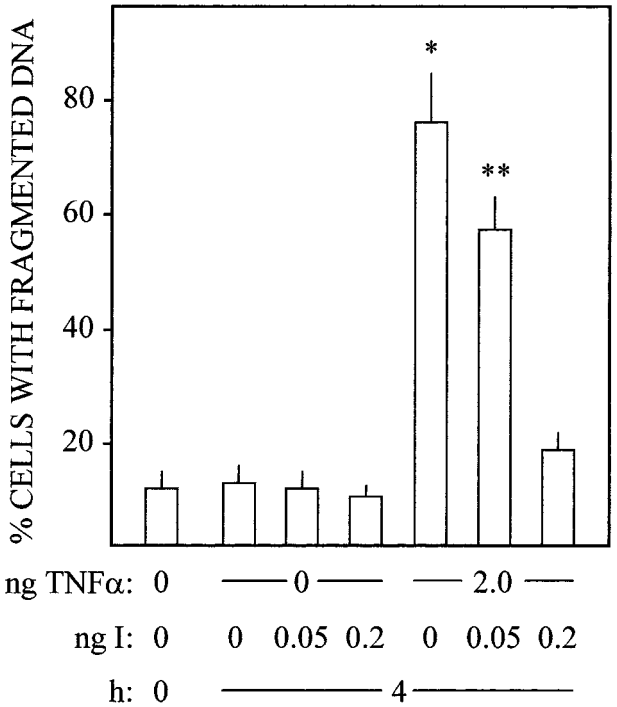

FIG. 5. In vitro inhibitory effects of indomethacin (I) on TNF-α-induced apoptosis in ovarian surface epithelial cells. Data are expressed as percentages of cells with fragmented DNA. Asterisks indicate increases ($P < 0.01$). **$P < 0.1$ versus TNF-α without indomethacin.

membrane glycoprotein receptor of 55 kDa (TNFRI) that binds trimeric ligand through an extracellular N-terminal motif. The cytoplasmic segment of TNFRI contains a death domain (DD) that can trigger apoptosis. Aggregation of receptors orients the death domains in a conformation that recruits the adapter proteins TRADD (TNFR-associated protein with DD) and FADD (Fas-associated protein with a DD). The death effector region of FADD assembles the zymogen forms of certain cysteine proteases (caspases), which upon activation evoke a proteolytic cascade leading to perturbations within the plasma membrane, DNA fragmentation, and cytoplasmic condensation [36, 37]. The net capacity of TNF-α to initiate apoptosis is contingent upon the extent of soluble cytokine accumulation, patterns of receptor expression, intracellular transduction attributes, and relative concentrations of endogenous apoptotic inhibitors [38]. Granulosa cells are apparently more resistant to the lethal effects of TNF-α than is ovarian surface epithelium.

A primary protective mode of indomethacin action on the physiochemical properties of the plasmalemma is suggested. Nonsteroidal anti-inflammatory drugs are planar, anionic, and lipophilic; they can readily enter lipid bilayers and thereby alter ion fluxes and signal generation mechanisms [35]. Diminution of calcium entry into cells could be of particular importance. An elevation in intracellular calcium is an early and major event that precedes apoptosis. Furthermore, calcium has been implicated in distal processes (e.g., endonuclease activation) that are affected by TNF-α [39]. Moreover, indomethacin (800 mg in vivo) inhibited calcium accretion in preovulatory sheep ovarian cells [22]. Others have reported that nonsteroidal anti-inflammatory drugs reduce epithelial lipid peroxidation, TNF-α cytotoxicity, and apoptosis [40–42].

It has become dogma that cyclooxygenase inhibitors, most notably indomethacin, interrupt ovulation by precluding the gonadotropin surge-activated increase in ovarian prostanoid production. Nonetheless, follicular prostaglandin up-regulation can be disassociated from apical ovarian cellular death and ovulation. That follicular rupture can occur in spite of anti-inflammatory treatments that abrogate the preovulatory increase in ovarian prostaglandin biosynthesis also has been noted in rabbits, rats, and hens [43–45]. An exacting role for eicosanoids in the process of mammalian ovulation should be carefully reevaluated.

Finally, it appears that ovarian surface cells located along the margins of ruptured follicles that survive the (sublethal) insult of ovulation (i.e., contain damaged DNA but are not committed to apoptosis) are the progenitors of common epithelial ovarian cancer [46]. Indeed, the risk of ovarian cancer of surface epithelial origin is decreased by factors that suppress ovulation [47, 48]. Our observations indicate that drugs that inhibit ovulation avert the degenerative DNA alterations in follicle-associated ovarian surface epithelial cells that are predisposing to malignant transformation.

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