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Estrogens and Glucocorticoids Have Opposing Effects on the Amount and Latent Activity of Complement Proteins in the Rat Uterus

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ABSTRACT

The mammalian uterus faces unique immunological challenges. It must nurture and protect the semiallogenic fetus from attack by the maternal immune system while guarding against infection by pathogens that compromise fetal and maternal health. Complement has recently been implicated in the etiology of pregnancy loss, but its regulation by steroid hormones and its role in host defense in the uterus are not clearly defined. Here we use biochemical, functional, and physiological assays to elucidate the regulation of complement proteins in the rat uterus. We demonstrate that estrogens (17 beta-estradiol) and glucocorticoids (dexamethasone) have major, but opposing, effects on the amount and latent activity of complement effectors in the uterus. Treatment with 17 beta-estradiol induced vasodilation and an increase in vascular permeability, which resulted in extravasation of plasma and complement into the uterus, rather than de novo complement biosynthesis. In vitro assays revealed that 17 beta-estradiol induced a potent bactericidal activity in uterine luminal fluid and that the antibacterial component was complement. These proinflammatory and immunomodulatory effects were evident within 4 h of treatment and were blocked by coadministration of dexamethasone. We also found that estrogen effects on the vasculature were mediated in part by activation of the contact system and bradykinin B1 receptors. These results indicate that complement plays a central role in innate immunity in the female reproductive tract and suggest that estrogens or glucocorticoids might be used therapeutically to enhance or inhibit complement-dependent processes in the uterus.

complement, estradiol, female reproductive tract, immunology, inflammation, reproductive immunology, rodent, steroid hormones, uterus

INTRODUCTION

The complement system includes over 30 interacting proteins that play a major role in inflammatory responses, serve as a first line of defense against infection, and act to bridge the innate and adaptive immune systems [1, 2]. Recent studies, however, have shown that complement can have adverse effects during pregnancy [3]. Embryonic mice deficient in the complement regulatory protein CRRY die in utero due to uncontrolled activation and deposition of maternal complement, inflammation, and infiltration of granulocytes at the fetomaternal interface [4]. In another animal model, inhibition of indoleamine 2,3 dioxygenase leads to T cell mediated rejection of semiallogenic fetuses [5]. Rejection of fetuses in this paradigm is strongly associated with placental inflammation and T cell-dependent, antibody-independent activation of complement [6]. Complement is also the principal cause of pregnancy loss in a mouse model of antiphospholipid syndrome, which is characterized by recurrent abortion in humans [7]. In isolation, these studies show that successful gestation of the developing fetus requires strict control of the complement system.

On the other hand, complement is likely to be a key mediator of immunity to sexually transmitted infections and other infections of the reproductive tract. Estrogens induce de novo synthesis of complement component 3 (C3) and histocompatibility 2, complement component factor B (H2-Bf) in uterine epithelial cells in rodents while these proteins are expressed during the luteal phase in humans [8–11]. Susceptibility to infection by *Chlamydia trachomatis* or *Escherichia coli* is reduced in ovariectomized rats by estrogen treatment and is lowest in cycling female rats when levels of endogenous estrogens are at their peak (i.e., when C3 and H2-Bf expression is greatest) [12–15]. Although steroid hormones also modulate susceptibility to urogenital infection by many other pathogens in both humans and animal models [16, 17], there is currently no experimental evidence regarding the defensive role that complement may play in the uterus. Such information is particularly important because infection before and during pregnancy is a major cause of morbidity and mortality in females and their unborn offspring [18–21].

Together, these observations highlight the conflicting need for protection against infection versus the requirement for maternal tolerance of the semiallogenic fetus [22–24]. Although the complement system may play both beneficial and detrimental roles in the female reproductive tract, the major factors that regulate complement levels and function are unknown. Here we use a suite of biochemical, functional, and physiological assays to examine the regulation of the effector arm of complement in the uterus of immature female rats.

MATERIALS AND METHODS

Animals and Hormone Treatments

Immature female rats (21–23 d; strain CD; Charles River Laboratory) were treated according to a protocol approved by Institutional Animal Care and Use Committees at National Institute of Environmental Health Sciences and the University of North Dakota. Female rats were injected with saline vehicle, 1 μg of 17 beta-estradiol (E_2) dissolved in saline, 1 mg of the synthetic glucocorticoid dexamethasone (DEX) in saline, or 1 μg E_2 and 1 mg DEX as previously described [25]. This dose of E_2 produces serum levels of E_2 in the range observed on the evening of proestrus in intact, cycling female rats [26]. Intact, immature females respond to estrogens in the same way as mature, ovariectomized females and are a widely accepted model for studying estrogen action in the uterus [27]. Corticosterone, the endogenous rodent glucocorticoid, activates both glucocorticoid and mineralocorticoid receptors. In contrast, DEX is a highly specific glucocorticoid receptor agonist [28].

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Collection of Uterine Tissue, Histology, and Western Blots

Animals were killed by decapitation and their uteri dissected 4 h after treatment. Uteri were cleaned, weighed, fixed in 4% formalin-PBS solution, and stored in 70% ethanol. Uteri were embedded in paraffin, cut at 4 µm,, and adhered to slides. Cross sections were deparaffinized in xylene, 100% ethanol, 95% ethanol, rehydrated in 1× PBS (2× for 5 min in each solution), and stained with hematoxylin-eosin.

Vascular permeability in the uterus was assessed using Evans blue dye [29, 30]. Females were treated with saline vehicle, 1 µg of E_2, 1 mg DEX, or 1 µg of E_2 and 1 mg DEX. Females were injected 3 h later with sodium pentabarbitol (40 mg/kg). After animals were under full anesthesia (~3.5 h after hormone treatment), the jugular vein was exposed and Evans blue injected intravenously (20 mg/kg). Evans blue adsorbs strongly to albumin and accumulation of Evans blue/albumin in blood-free tissue is a direct measure of vascular permeability to large serum proteins [29]. Animals were perfused with PBS 30 min after injection of Evans blue to drain blood from all tissues. Animals were killed and uteri dissected approximately 4 h after hormone treatment. Uteri were cleaned of fat and connective tissue, weighed, and frozen. Uteri were subsequently homogenized in 600 µl of distilled water and incubated with two volumes of formamide at 60°C for 18 h. Samples were centrifuged at 5000 × g for 30 min and supernatant collected. Absorbance of the supernatant at 620 nm was determined and Evans blue concentration calculated using a standard curve. Total content of Evans blue in each uterus was then estimated.

Uteri for Western blots were cleaned of fat and connective tissue, weighed, and placed in ice-cold RIPA buffer containing protease inhibitors (Complete Mini, Roche Diagnostics, Germany). Total protein was isolated from each uterus. Thirty micrograms of total protein was separated under reducing and denaturing conditions on a polyacrylamide gel and transferred to a nitrocellulose membrane as described [25]. Blots were incubated with primary antibodies diluted in TBS-T and 5% nonfat milk for 1 h at room temperature.

Antibodies to human complement were tested for cross-reaction to rat complement using rat and human serum as positive controls. Antibody concentrations were optimized using rat and human serum: horseradish peroxidase-conjugated donkey antisera to rat IgG was diluted 1:10 000 (Jackson ImmunoResearch, West Grove, PA); goat antisera to human complement component 1, q subcomponent (alpha, beta, and gamma polypeptides; C1QA, C1QB, C1QC) was diluted 1:1000 (Quidel, San Diego, CA); goat antisera to human complement component 2 (C2) was diluted 1:500 (CalBiochem); goat antisera to human complement component 4 (C4) was diluted 1:500 (CalBiochem); goat antisera to rat complement component 3 (C3) was diluted 1:300 (ICN Pharmaceuticals, Aurora, OH); goat antisera to human complement component 6 (C6) was diluted 1:500 (CalBiochem); rabbit antisera to actin was diluted 1:200 (Sigma-Aldrich, St. Louis, MO). After washing in TBS-T (3× for 10 min each), blots were probed with peroxidase-conjugated secondary antibodies (donkey antisera to goat IgG, Jackson ImmunoResearch; donkey antisera to rabbit IgG, Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature. Secondary antibody concentrations were 1:10 000 for C1Q, 1:5000 for C2, 1:5000 for C4, 1:10 000 for C3, 1:5000 for C6. Blots were washed in TBS-T (3× for 10 min each) and bands visualized using ECL (Amersham Biosciences).

Real-Time Quantitative PCR

Uteri were cleaned, placed in RNAlater, and stored at 4°C until RNA was extracted (Ambion, Austin, TX). Total RNA was isolated from individual uteri of eight animals per treatment group using the Qiagen RNeasy kit (Qiagen, Valencia, CA). Total RNA had 260/280 absorbance ratios of 1.8–1.9 in pure water and displayed discrete 18S and 28S ribosomal RNA bands on formaldehyde-agarose gels. Real-time PCR for complement *C1qa*, *C1qb*, *C1qc*, *C2*, *C3*, *C4*, *C6*, and complement component 1, q subcomponent, receptor 1 (*C1qr1*) mRNA and 18s rRNA was performed using the TaqMan Gold RT-PCR kit and primers and probes designed using Beacon Designer software (Table 1; Premier Biosoft, Palo Alto, CA). Total RNA (1 µg) was reverse transcribed in a 50-µL reaction mixture containing 1× TaqMan RT buffer, 500 µM dNTPs, 5.5 mM MgCl2, 200 nM of each sequence specific reverse primer, 2.5 µM random hexamers (for 18S rRNA), 1.25 U/µl MultiScribe reverse transcriptase, and 0.4 U/µl Rnase inhibitor. Primers were allowed to hybridize at 25°C for 10 min. Reverse transcription was carried out at 48°C for 30 min. Samples were heated to 95°C for 5 min to inactivate reverse transcriptase and then were cooled to 4°C. A 2-µl aliquot of cDNA was added to separate PCR reactions for each gene. Each 50-µL reaction contained 1× TaqMan buffer A, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP, 5.5 mM MgCl2, 200 nM forward primer, 200 nM reverse primer, 100 nM probe, and 0.025 U/µl of Taq polymerase. Two-step PCR was performed with 40 cycles of 15 sec at 95°C and 1 min at 60°C. Reverse transcription and PCR reactions were run on a MyiQ Single color Real-Time PCR Detection System (Bio-Rad, Hercules, CA).

Pooled total RNA from uteri of vehicle-treated females was used as a control and to generate a standard curve for each gene. This RNA was added to a tube containing no reverse transcriptase. No signal was detected after 40 PCR cycles in the absence of reverse transcriptase, indicating that samples were free of DNA. In addition, no signal was detected when reverse transcriptase was added but RNA template was not, indicating that there was no contamination from exogenous RNA. Control RNA from uteri of vehicle-treated females was diluted and added to reaction mixtures in the following quantities to generate a standard curve for each gene: 1 µg, 0.5 µg, 0.25 µg, 0.125 µg, 0.0625 µg, and 0.03125 µg. The standard curve for all genes displayed an increase of one threshold cycle for each halving of template concentration. Threshold cycle, or C_T, was recorded for all 32 samples. The C_T value for each sample was subtracted from the mean C_T value for the vehicle-treated group for each gene. This results in a mean of zero for the vehicle-treated group. In addition, a change of ± 1 C_T between treatment groups and the control group equals a 2-fold change in mRNA levels.

Biochemical and Functional Assays for Complement in Uterine Luminal Fluid

In vitro assays were used to test whether complement in luminal fluid was functional. Animals were treated with E_2 and/or DEX and sacrificed 4 h later. Uteri were removed, cleaned, weighed, and placed on plastic six-well cell culture plates. Each uterine horn was isolated and flushed with ice-cold gelatin veronal buffer (40 µl of GVB per horn; Sigma) containing 0.15 mM $CaCl_2$ and

TABLE 1. Primer and probe sets for real-time PCR analysis of indicated genes.

Gene	Primers (sense and antisense)	Probe
C1qa	5′-CAG GAA CAT CAT GGA GAC CTC TC-3′ 5′-ACA CAG TGG AGT GAG ATC TTG AC-3′	5′-AGC ACA CAA GCC ACC AGC CAT CCC-3′
C1qb	5′-ACA CAG TGG AGT GAG ATC TTG AC-3′ 5′-TTT GCC ATC AGA GCC AGG GA-3′	5′-TGC TGC TGC TCC TGG GTT TGC TCC-3′
C1qc	5′-TAG AAT GGC AGG CTG GGT CC-3′ 5′-AAG GAA GAG GTC TGA GTG AGG AT-3′	5′-ACG CCC GCC TCG CTC CCT CTG-3′
C2	5′-CTA CCT GGA CAT CTA TGC GAT TGG-3′ 5′-CAC AGC TTT TGC GTC CTG CA-3′	5′-CGT GCC TCT CGC CGT CCT TCT TGG-3′
C3	5′-GAA GAT CCT GAG TGC GCC AAG-3′ 5′-CTT TGT CCA TCC TCC TTT CCA TCA-3′	5′-CCG CCG CCG TCG CTC AGT GC-3′
C4	5′-GTC CTG TTG CAA GTT TGC TGA G-3′ 5′-CGC ACG AGA ATG TCA TCT TCA TC-3′	5′-CCG CAG GAA CCA GAC CAG GAG CCA-3′
C6	5′-CAG ACA GAA CCT GGA GGC AAG-3′ 5′-GGA CAT CCT GAA CGA CTG GTT T-3′	5′-CGA ATG CCA ACG GAC CGA GTG CCT-3′
C1qr1	5′-ACT GGG CAA TAT TCA CTA GAA GAC-3′ 5′-CCC AAG CCC TCA GTA AGA ACA T-3′	5′-ACT CCT GCC TGA ACA GCC CAC AGT-3′

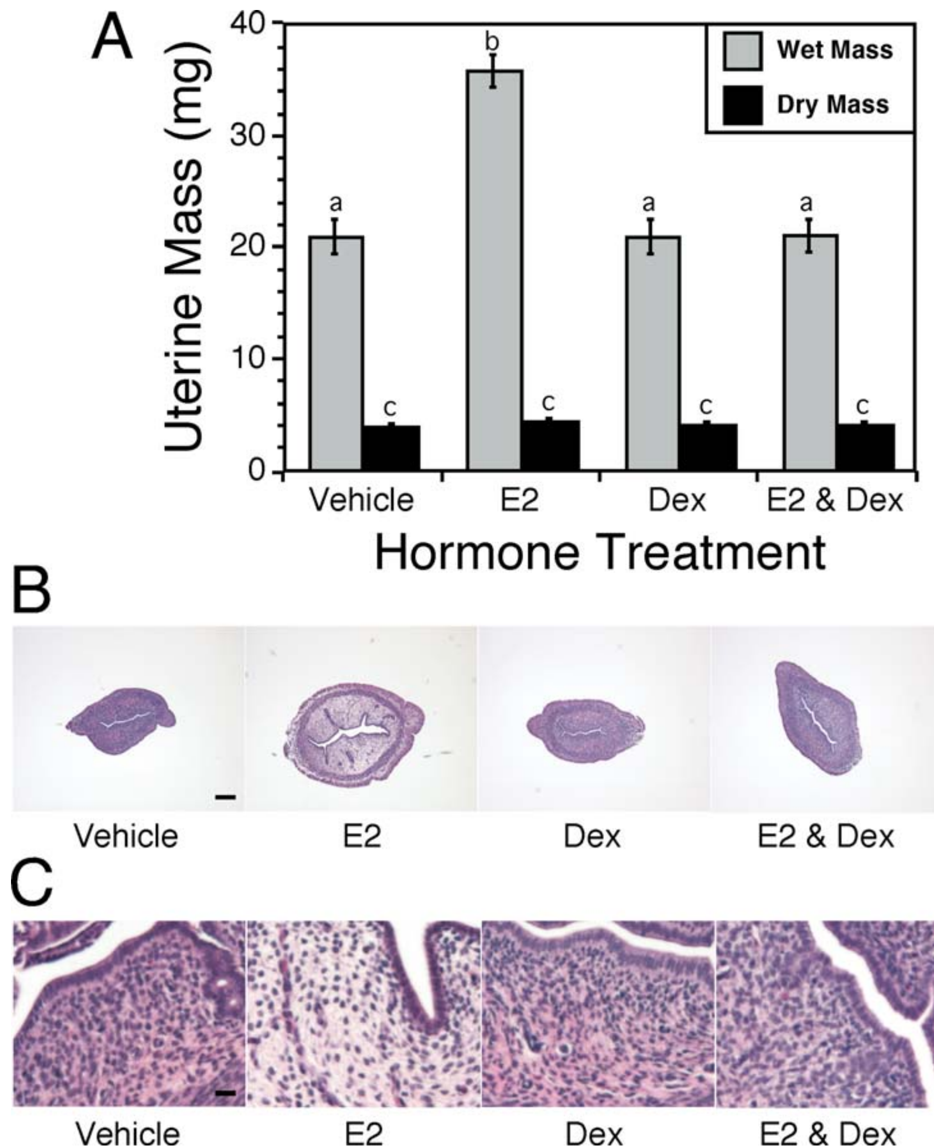

FIG. 1. **A**) Influence of 1 μg of E_2, 1 mg DEX, or 1 μg E_2 and 1 mg DEX on wet and dry mass of rat uteri 4 h after in vivo hormone administration. Masses are means ± 1 SEM. Groups with different letters are significantly different using Tukey honestly significant difference test. **B**) Cross sections of uteri from vehicle-, E_2-, DEX-, and E_2 and DEX-treated females. **C**) Higher magnification of uterine stroma and luminal epithelium from vehicle-, E_2-, DEX-, and E_2 and DEX-treated females. Bar = 200 μM for B and 20 μM for C

0.5 mM $MgCl_2$. The uterine luminal fluid and GVB mixture (hereafter uterine luminal fluid) from each animal (~80 μl total) was collected and kept on ice for later use.

Serial dilutions of the serum-sensitive J5 strain of *E. coli* (ATCC, Manassas, VA) were made for bactericidal assays. After dilution, 20 μl of the bacterial solution was added to 80 μl of uterine luminal fluid from vehicle- and hormone-treated animals. The solution was thoroughly mixed with a pipette tip and gentle flicking of the tube. A 10-μl aliquot of this mixture was immediately plated on LB plates and incubated at 37°C. The remainder of the solution was incubated at 37°C in a shaking incubator for 1 h. A second 10-μl aliquot was then plated on LB plates and incubated at 37°C. The number of colony-forming units (CFUs) per 10 μl-aliquot was recorded at the start and after 1 h of incubation with luminal fluid.

In another study, cobra venom factor (CVF; CalBiochem) was added to uterine luminal fluid to determine whether it would form C3 convertase in vitro. In brief, 0.5 μg of CVF in 1 μl of distilled water was added to ~100 μl of uterine luminal fluid from vehicle- and E_2-treated rats (each horn was flushed with 50 μl of GVB in this study). After thorough mixing, a 10-μl aliquot was immediately removed, added to a second tube containing 4 μl of loading buffer, and heat inactivated at 95°C for 4 min. The remainder of the uterine luminal fluid was incubated at 37°C in a shaking incubator and successive 10-μl aliquots removed after 5, 10, and 20 min. These aliquots were added to 4 μl of loading buffer and heat inactivated at 95°C for 4 min. As a negative control, 100 μl of uterine luminal fluid from vehicle- and E_2-treated rats was heat inactivated at 56°C for 10 min before addition of 0.5 μg CVF. As an additional negative control, 2 μl of 1 M EDTA was added to 100 μl of uterine luminal fluid (final concentration ~20 mM EDTA) from vehicle- and E_2-treated rats before addition of 0.5 μg CVF. Aliquots from negative controls were processed immediately after addition of CVF and again after incubation at 37°C for 5, 10, and 20 min. All aliquots were separated under reducing and denaturing conditions on Tris-glycine gels for analysis of C3 hydrolysis by Western blot.

Experiments were conducted to test the hypothesis that E_2-induced bactericidal activity in uterine luminal fluid was complement dependent. The procedures described above were used to inactivate complement in luminal fluid from vehicle- and E_2-treated females, i.e., uterine luminal fluid was heat inactivated, EDTA was added to luminal fluid, or CVF was added to luminal fluid. Serial dilutions of the J5 strain of *E. coli* were made and 20 μl of the solution added to 80 μl of uterine luminal fluid from control samples and samples in which complement had been inactivated. Bactericidal activity of luminal fluid was assessed as previously described.

Physiological Assays for Complement Activation in the Uterus

We treated immature, pathogen-free rats with E_2 and various inhibitors of complement to determine whether complement activation plays a role in E_2-induced inflammation in the uterus. Females were injected with saline or human serine peptidase inhibitor, clade G, member 1 (SERPING1; formerly known as complement C1-inhibitor) in saline (i.e., 5, 20, 40, or 80 mg SERPING1/kg, CalBiochem). SERPING1 is a serine protease inhibitor that blocks the enzymatic activity of complement component 1, r and s subcomponents (C1R and C1S) of the classical pathway of complement [31].

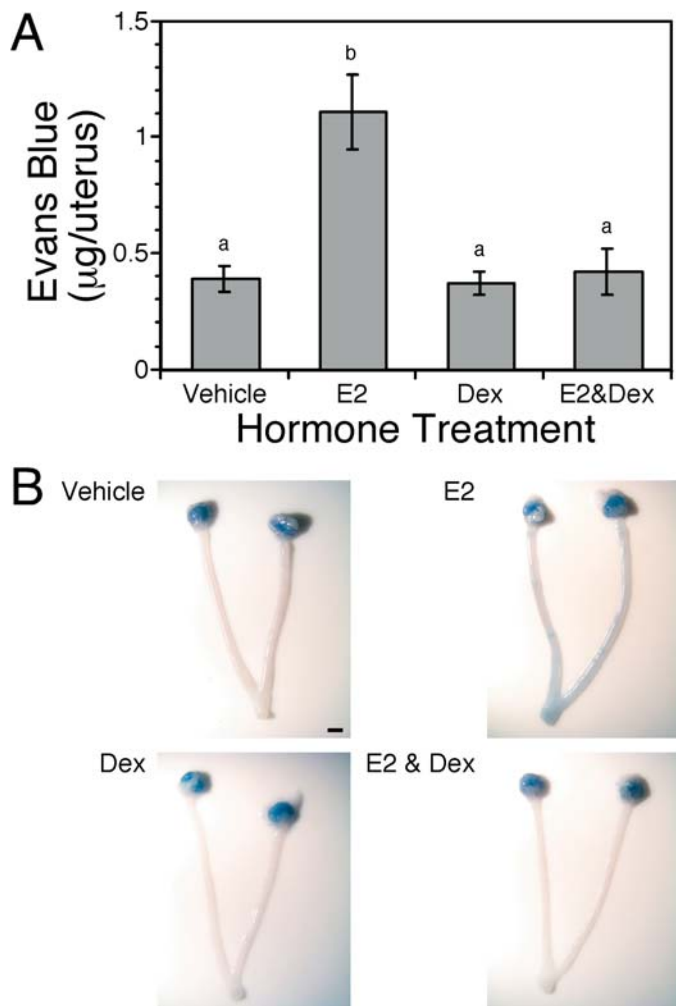

FIG. 2. **A**) Influence of 1 µg of E_2, 1 mg DEX, or 1 µg E_2 and 1 mg DEX on the extravasation of Evans blue/albumin from the vascular system into the uterus 4 h after hormone administration and 30 min after intravenous dye injection. The total content of Evans blue per uterus is expressed as the mean ± 1 SEM. Groups with different letters are significantly different using Tukey honestly significant difference test. **B**) Photographs of representative ovaries and uteri from vehicle-, E_2-, DEX-, and E_2 and DEX-treated females. Bar = 2 mm.

However, SERPING1 also inhibits plasma kallikrein of the contact system [31]. Accordingly, we used specific inhibitors that act downstream of SERPING1 in the complement or contact cascades.

In one study, rats were injected with vehicle or soluble complement receptor 1 at a dose (24 mg/Kg; AVANT Immunotherapeutics, Needham, MA) known to inhibit complement-mediated ischemia-reperfusion injury in rats [32, 33]. Soluble complement receptor 1 (sCR1) acts as a cofactor in complement component factor I (CFI) mediated cleavage and inactivation of C3b. In a second study, rats were pretreated with saline or 25 µg of CVF in saline for 24 h before being treated with saline or E_2. Intraperitoneal injection of CVF depletes circulating C3 within 6 h and serum C3 remains depleted for 4–6 days.

Specific antagonists of the bradykinin receptor, beta 1 or BDKRB1 (des-Arg^9-[Leu^8]-bradykinin and des-Arg^{10}-HOE 140; Sigma) and bradykinin receptor, beta 2 or BDKRB2 (HOE 140, Sigma) were used to evaluate the potential role of kinins in mediating E_2-induced inflammation in the uterus. Bradykinin (a specific BDKRB2 agonist) is produced when activated plasma kallikrein cleaves high molecular weight kininogen. Further metabolism of bradykinin to des-Arg^9-bradykinin is essential for activation of BDKRB1. Thus, we treated rats with 0.1185 µg of Plummer inhibitor (CalBiochem). Plummer inhibitor is a carboxypeptidase N inhibitor that blocks cleavage of the terminal arginine from bradykinin. Rats were killed and their uteri dissected 4 h after treatment. Uteri were cleaned of fat and connective tissue and weighed to assess edema.

Statistical Analysis

All statistics were done using JMP 5.0.1.2 software (SAS Institute). Two-way ANOVA was used to test for main effects and interactions between compounds. Tukey honestly significant difference test was then used for multiple comparisons among treatment groups.

RESULTS

Injection of 1 µg E_2 into immature female rats induced a 1.7-fold increase in uterine wet mass 4 h after treatment (Fig. 1A). Treatment with E_2 caused vasodilation, edema in the stroma and myometrium, and accumulation of fluid in the lumen (Fig. 1B). Coadministration of 1 mg DEX blocked these E_2-induced changes. Closer examination of the stroma shows accumulation of interstitial fluid in the E_2-treated group and that this effect was antagonized by DEX (Fig. 1C). Edema and the increase in luminal fluid were entirely responsible for the E_2-induced increase in uterine wet mass because there was no change in uterine dry mass (Fig. 1A).

Treatment with E_2 caused a large increase in vascular permeability in the uterus, as assessed by injection of Evans blue dye into the jugular vein 30 min before rats were killed. Females treated with E_2 for 4 h had nearly 3-fold more Evans blue/albumin in their uteri than vehicle-treated females (Fig. 2A). Coadministration of DEX blocked the E_2-induced increase in vascular permeability (Fig. 2A). The opposing effects of E_2 and DEX on Evans blue/albumin can easily be seen in hormone-treated uteri (Fig. 2B). Interestingly, the baseline level of vascular permeability was much greater in the ovaries than in the uterus and was not affected by hormone treatments (Fig. 2B).

Western blots were used to determine if E_2 and DEX regulate levels of IgGs and complement proteins in the uterus. Whereas treatment with E_2 increased heavy and light chains of IgG, coadministration of DEX blocked this effect (Fig. 3A). Treatment with E_2 also increased levels of C1QC and coadministration of DEX blocked this effect (Fig. 3B). The C1QA, C1QB, and C1QC proteins were present in equimolar amounts in serum, but there was proportionately less of the C1QA and C1QB subunits in uteri from E_2-treated animals. We suspect that the C1QA and C1QB subunits may be interacting with other proteins and running at a higher molecular weight, although we have yet to test this hypothesis. E_2 and DEX also had opposing effects on levels of C2a and C2b fragments in the uterus (Fig. 3C). In contrast, levels of actin were not affected by hormone treatment (Fig. 3D). Levels of the alpha and gamma subunits of C4 were increased by E_2 and blocked by DEX (Fig. 3E). Levels of the alpha and beta subunits of complement C3 and iC3b were elevated by E_2 and antagonized by DEX (Fig. 3F). Although procomplement C3 was not detected in the uterus, it was found in the liver, which is the primary source of circulating C3 (Fig. 3F). Hormones had no detectable effect on the level of procomplement C3 or mature C3 in the liver (data not shown). Finally, levels of C6 in the uterus were increased by E_2 and blocked by DEX cotreatment (Fig. 3G).

Real-time PCR was used to measure complement mRNA in the uterus. Treatment with E_2 and/or DEX for 4 h had no effect on levels of *C1qa*, *C1qb*, or *C1qc* mRNA (Fig. 4A). Hormones did not have any effect on levels of *C2* or *C4* mRNA (Fig. 4B). Treatment with E_2 induced a 4-fold increase in *C3* mRNA (Fig. 4B). Nevertheless, coadministration of DEX did not block E_2 induction of *C3* mRNA (Fig. 4B). These results are in accord with previous studies of *C3* transcriptional regulation by estrogens and glucocorticoids [34, 35]. Hormones did not affect levels of *C6* mRNA (Fig. 4C). We measured *C1qr1*

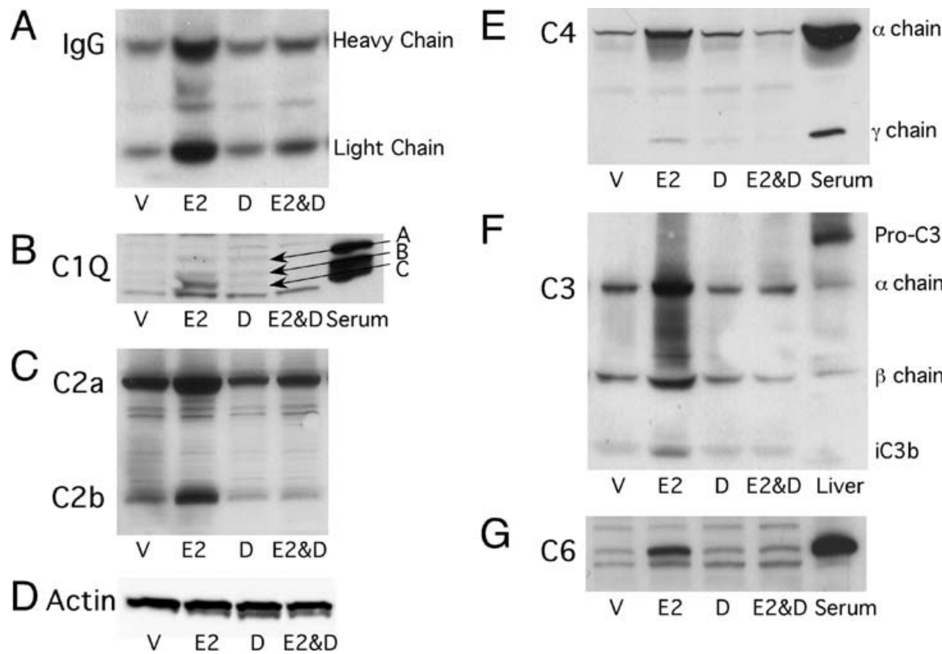

FIG. 3. Western blots for IgG and complement proteins using extracts from uteri of rats treated with vehicle, 1 μg of E_2, 1 mg DEX, or 1 μg E_2 and 1 mg DEX for 4 h. Thirty micrograms of total protein was loaded in each lane: Anti-rat IgG (**A**), anti-human C1Q (**B**), anti-human C2 (**C**), anti-actin (**D**), anti-human C4 (**E**), anti-rat C3 (**F**), anti-human C6 (**G**).

mRNA and 18S rRNA as positive and negative controls, respectively. Levels of *C1qr1* mRNA were increased by E_2 and blocked by DEX (Fig. 4C). Antagonistic regulation of *C1qr1* mRNA by E_2 and DEX is in accord with previous studies [25]. Finally, levels of 18s rRNA were not influenced by E_2 or DEX (Fig. 4C). These results suggest that C1QA, C1QB, C1QC, C2, C4, C3, and C6 proteins were not synthesized de novo in the uterus but were preexisting serum proteins exuded into the uterus (i.e., like Evans blue/albumin). Supporting this idea is the observation that full-length procomplement C3 protein was not detected in E_2-treated uteri. In addition, coadministration of the translation inhibitor puromycin did not block E_2-induced inflammation or the accumulation of IgG, C1qC, C2, C3, C4, or C6 proteins in the uterus (data not shown).

Uterine luminal fluid from vehicle-, E_2-, DEX-, and E_2 and DEX-treated rats was collected and inoculated with *E. coli* to study the bactericidal activity of uterine secretions. The number of CFUs per 10-μl aliquot of luminal fluid did not differ among groups immediately after inoculation, demonstrating that equal numbers of bacteria were initially added to luminal fluid from each group (Fig. 5A; representative LB plates shown in Fig. 5B). There was, however, a significant decrease in the number of CFUs in luminal fluid from E_2-treated females after incubation for 1 h. This E_2-induced bactericidal activity was blocked by DEX, thus implicating complement as the antibacterial component of luminal fluid (Fig. 5A; representative plates shown in Fig. 5B).

Conditions were established for in vitro inactivation (or inhibition) of complement in uterine luminal fluid to test the hypothesis that E_2-induced bactericidal activity was mediated by complement. A low level of spontaneous C3 activation (and inactivation) was observed in luminal fluid from E_2-treated females, as evidenced by a slow increase in the level of the 63 kDa iC3b fragment with time incubated at 37°C (Fig. 6A). Addition of 0.5 μg of CVF, which interacts with H2-Bf and complement component factor D to form C3 convertase, resulted in rapid cleavage of the C3α subunit to form activated C3α′ in uterine luminal fluid from both vehicle- and E_2-treated females (Fig. 6B). Much of the activated C3α′ fragment was cleaved within 5 min to form inactive iC3b in luminal fluid from E_2-treated females, but not in luminal fluid from vehicle-treated females (Fig. 6B). Heat inactivation of luminal fluid at 56°C for 10 min prevented the formation of CVF-dependent C3 convertase in luminal fluid from vehicle- and E_2-treated females (Fig. 6C). Heat inactivation also blocked the subsequent formation of iC3b in luminal fluid from E_2-treated females (Fig. 6C). Addition of EDTA (~20 mM final concentration) prevented CVF-dependent cleavage of C3 in luminal fluid from vehicle and E_2-treated females as well as the ensuing formation of iC3b in luminal fluid from E_2-treated females (Fig. 6D). Heat inactivation, addition of EDTA, and preincubation with 0.5 μg of CVF were used to block the complement cascade in the following studies.

Uterine luminal fluid from vehicle- and E2-treated rats was either kept on ice or heat inactivated at 56°C for 10 min before inoculation with *E. coli*. The number of CFUs per 10-μl aliquot of luminal fluid did not differ among treatment groups immediately after inoculation (Table 2). The number of CFUs in luminal fluid from E_2-treated females decreased dramatically after incubation at 37°C for 1 h (Table 2). Heat inactivation eliminated this E2-induced bactericidal activity (Table 2). In other studies, we added vehicle or EDTA (~20 mM final concentration) to uterine luminal fluid from vehicle- and E_2-treated rats. The number of CFUs per 10-μl aliquot of luminal fluid did not differ among treatment groups after inoculation with *E. coli* (Table 2). Addition of EDTA to uterine luminal fluid from vehicle-treated females reduced the number of CFUs, but not to the same degree as luminal fluid from E_2-treated females (Table 2). More importantly, addition of EDTA blocked bactericidal activity in luminal fluid from E_2-treated females, bringing the viability of *E. coli* up to the level observed when EDTA was added to luminal fluid from vehicle-treated females (Table 2). Finally, vehicle or 0.5 μg CVF were added to luminal fluid from vehicle- and E_2-treated rats. Uterine luminal fluid was then incubated at 37°C for 5 min before inoculation with *E. coli*. The number of CFUs per 10-μl aliquot of luminal fluid did not differ among treatment groups after inoculation, indicating that equal numbers of bacteria were added to luminal fluid from each group (Table 2). The number of CFUs per 10-μl aliquot of luminal fluid from E_2-treated females decreased dramatically after incubation at 37°C for 1 h (Table 2). Preincubation with CVF blocked the

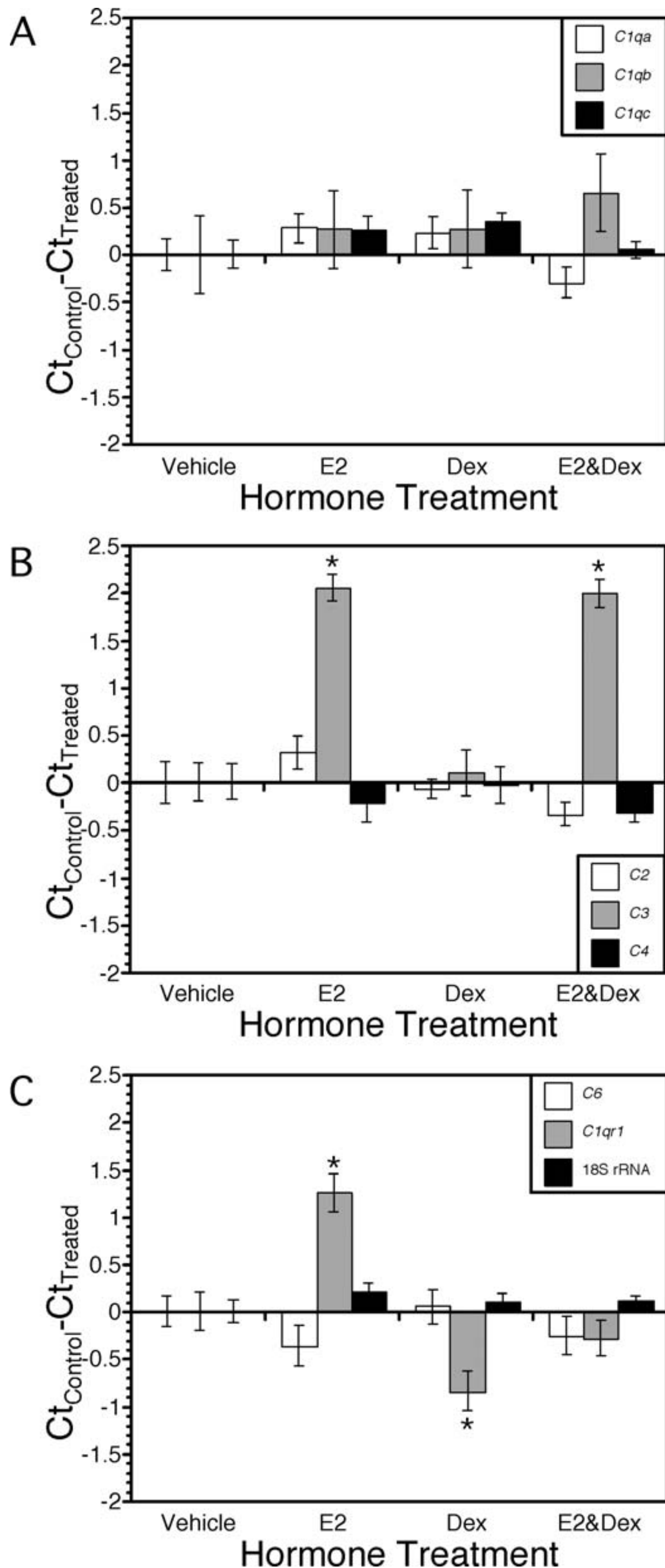

FIG. 4. Influence of 1 μg of E_2, 1 mg DEX, or 1 μg E_2 and 1 mg DEX on expression of complement mRNA in uteri 4 h after in vivo hormone administration. Relative gene expression levels were determined by real-time RT-PCR analysis and graphed as the difference between the threshold cycle of the vehicle- and the hormone-treated groups. Groups with an asterisk are significantly different from the vehicle control (* $P < 0.05$). Expression values are means ± 1 SEM. **A**) Treatment with E_2 and DEX had no influence on levels of *C1qa, C1qb,* or *C1qc* mRNA. **B**) Treatment with E_2 significantly increased levels of *C3* mRNA but did not influence levels of *C2* or *C4* mRNA. **C**) Treatment with E_2 and DEX had no influence on levels of *C6* mRNA or 18S rRNA (the negative control). However, E_2 and DEX had opposing effects on expression of *C1qr1* mRNA (the positive control).

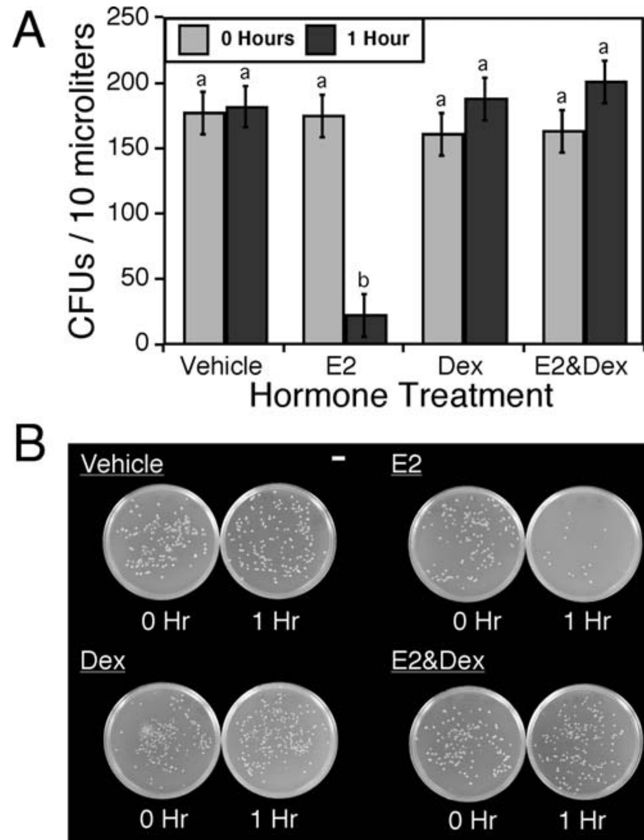

FIG. 5. **A**) Influence of 1 μg of E_2, 1 mg DEX, or 1 μg E_2 and 1 mg DEX on in vitro antibacterial activity of uterine luminal fluid collected 4 h after in vivo hormone administration. Aliquots of uterine luminal fluid were plated immediately (0 h) after inoculation with *E. coli* and after incubation at 37°C for 1 h (1 h). The number of CFUs/10-μL aliquot of uterine luminal fluid are means ± 1 SEM. Groups with different letters are significantly different using Tukey honestly significant difference test. **B**) Representative plates from the study summarized above. Bar = 1 cm.

bactericidal activity of luminal fluid from E_2-treated females (Table 2).

Given the strong correlation between the proinflammatory effects of E_2 and the accretion of complement, we tested the idea that complement plays a role in mediating the proinflammatory effects of E_2 in the pathogen-free uterus. Treatment of immature female rats with human SERPING1 (complement C1 inhibitor), the only known physiological inhibitor of the classical pathway of complement, partially blocked the E_2-induced increase in uterine wet mass (i.e., edema) (Fig. 7A). The E_2-induced edema was inhibited by approximately 40% with various doses of human SERPING1. Western blots showed that human SERPING1 was only present in the uteri of rats treated with human SERPING1 (Fig. 7A).

This result suggests that activation of the classical pathway may mediate the proinflammatory effects of E_2. However, SERPING1 also inhibits plasma kallikrein of the contact system. Exogenous human SERPING1 may therefore inhibit E_2-induced uterine inflammation by blocking production of bradykinin (or its vasoactive metabolites). To test these competing hypotheses, we used two specific complement inhibitors that act downstream at the level of C3 as well as specific antagonists of bradykinin B1 and B2 receptors. Depletion of complement C3 via CVF pretreatment did not inhibit E_2-induced edema (Fig. 7B). Coadministration of sCR1, which acts as a cofactor in the cleavage and inactivation of

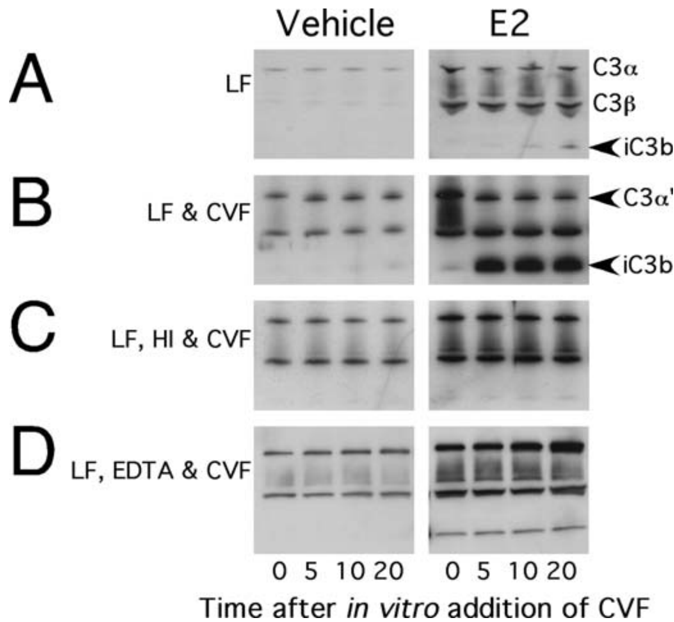

FIG. 6. **A**) Western blots showing spontaneous hydrolysis of complement C3α to produce iC3b in uterine luminal fluid from E_2-treated females. No hydrolysis was detected in luminal fluid from vehicle-treated females. **B**) Western blots showing CVF-dependent hydrolysis of complement C3α to produce C3α′ in uterine luminal fluid from vehicle-and E_2-treated females. Complement C3α′ was further hydrolyzed to produce iC3b in luminal fluid from E_2-treated females but not vehicle-treated females. **C**) Western blots showing inhibition of CVF-dependent hydrolysis of complement C3α by heat inactivation of uterine luminal fluid. **D**) Western blots showing inhibition of CVF-dependent hydrolysis of complement C3α by addition of EDTA to uterine luminal fluid.

activated C3b, also had no effect on E_2-induced edema (data not shown).

In contrast, des-Arg[9]-[Leu[8]]-bradykinin (a BDKRB1 antagonist) had an inhibitory effect that mimicked the effect of human SERPING1 (Fig. 7C). Another specific BDKRB1 antagonist, des-Arg[10]-HOE 140, had the same effect as des-Arg[9]-[Leu[8]]-bradykinin and human SERPING1 (data not shown). Treatment with E_2 and/or des-Arg[9]-[Leu[8]]-bradykinin had no effect on expression of BDKRB1 in the uterus (Fig. 7C). The specific BDKRB2 antagonist HOE 140 had no effect on E_2-induced edema (results not shown). To test whether metabolism of bradykinin to des-Arg[9]-bradykinin was necessary for activation of BDKRB1, we treated immature female rats with a carboxypeptidase N inhibitor (Plummer inhibitor) that prevents cleavage of the terminal arginine from bradykinin. Plummer inhibitor blocked E_2-induced edema to the same extent as human SERPING1 and BDKRB1 antagonists (Fig. 7D). In concert, these findings demonstrate that activation of the contact system, and not complement, was partially responsible for the proinflammatory effects of E_2 in the pathogen-free uterus.

DISCUSSION

Major evolutionary changes in reproductive biology have placed conflicting demands on the female reproductive tract and immune system. The evolution of internal fertilization provided a novel route for transmission of microbial and viral pathogens to the female reproductive tract, which presumably favored mechanisms for rapid mobilization and activation of the immune system. In contrast, the evolution of viviparity required tolerance of the semiallogenic fetus. Elaboration of the placenta for transfer of nutrients to (and removal of waste from) the embryo introduced additional constraints on the maternal immune system. As a result, female mammals must balance the need for protection against infection versus tolerance of the embryo. Given the central position of the complement system in host defense and the potential for complement-mediated termination of pregnancy, it is important to identify the factors that regulate complement proteins in the uterus.

TABLE 2. The number of colony forming units/10 μL aliquot of luminal fluid from vehicle and E_2-treated females immediately after inoculation with *E. coli* and after incubation for 1 h at 37°C.

Treatment	Hours after inoculation[a]	
	0	1
Experiment 1		
Vehicle	214 ± 16[b]	238 ± 20[b]
E_2	205 ± 4[b]	5 ± 3[c]
Heat inactivation	232 ± 9[b]	243 ± 14[b]
E_2 & heat inactivation	215 ± 8[b]	246 ± 25[b]
Experiment 2		
Vehicle	245 ± 17[b]	294 ± 11[b]
E_2	252 ± 10[b]	3 ± 1[c]
EDTA	274 ± 12[b]	102 ± 13[d]
E_2 & EDTA	243 ± 19[b]	142 ± 11[d]
Experiment 3		
Vehicle	230 ± 10[b]	268 ± 33[b,d]
E_2	232 ± 7[b]	8 ± 3[c]
Cobra venom factor	228 ± 11[b]	291 ± 16[b,d]
E_2 & cobra venom factor	236 ± 8[b]	327 ± 25[b,d]

[a] Data are means ± 1 SEM.
[b-d] Groups with different superscripts within each experiment are significantly different using Tukey HSD test.

Using biochemical and functional assays, we have shown that E_2 increased the level of numerous complement proteins and the latent activity of the effector arm of complement in the uterus. To be precise, we detected elevated levels of IgG, C1Q, C2, C3, C4, and C6 and show that E_2 induced a potent bactericidal activity in uterine luminal fluid. Coadministration of DEX completely blocked the E_2-induced increase in uterine complement proteins and the bactericidal activity of luminal fluid. These results were consistent with the hypothesis that complement was the antibacterial component in uterine luminal fluid from E_2-treated females.

To test this premise, we established conditions for in vitro inactivation and inhibition of complement in uterine luminal fluid. Spontaneous activation of C3 was slow in luminal fluid from E_2-treated females. In contrast, addition of CVF resulted in rapid and complete cleavage of C3 to form C3α′, indicating that H2-Bf and factor D were also present in luminal fluid and available for in vitro formation of CVF-dependent C3 convertase [36]. Activated C3α′ was then cleaved to produce inactive iC3b. In accord with these results, preincubation with CVF blocked the bactericidal activity of luminal fluid from E_2-treated females. Heat inactivation and addition of EDTA inhibited in vitro formation of CVF-dependent C3 convertase and blocked E_2-induced bactericidal activity. Together, these results demonstrate that complement was the antibacterial component in luminal fluid from E_2-treated females, i.e., it was heat labile, Mg^{2+} dependent, and required intact complement C3. Our cell-free preparation of luminal fluid further suggests that killing of *E. coli* resulted from opsonization of bacteria and formation of the membrane attack complex.

These findings could have important implications for our understanding of immunity to sexually transmitted diseases, including chlamydia, gonorrhea, herpes simplex virus type 2, human immunodeficiency virus (HIV), and human papillomavirus. While each microbe and virus has its specific ecology

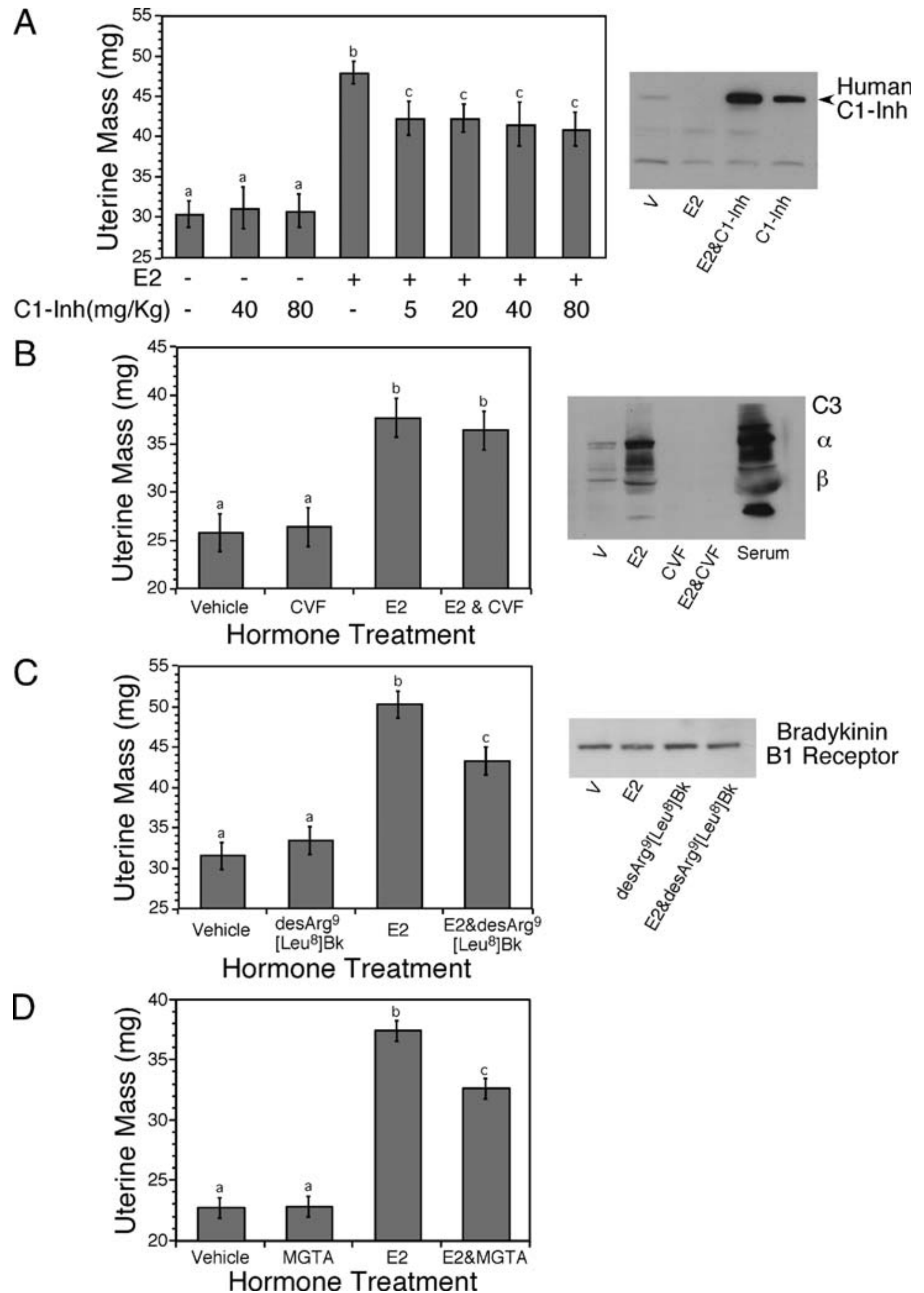

FIG. 7. Influence of various complement and contact system inhibitors on the E_2-induced increase in uterine wet mass (i.e., edema) 4 h after in vivo hormone administration. Masses are means ± 1 SEM. Groups with different letters are significantly different using Tukey honestly significant difference test. **A**) Wet mass of uteri from females treated with vehicle, the indicated doses of human C1-Inh, 1 μg of E_2, or 1 μg of E_2 and the indicated doses of C1-Inh. Representative western blot for human C1-Inh using extracts from uteri of rats treated with vehicle, E_2, C1-Inh, or E_2 and C1-Inh. **B**) Wet mass of uteri from females treated with vehicle, 25 μg of CVF, 1 μg of E_2, or 1 μg of E_2 and 25 μg of CVF. Representative western blot for C3 using extracts from uteri of rats treated with vehicle, E_2, CVF, or E_2 and CVF. **C**) Wet mass of uteri from females treated with vehicle, desArg^9[Leu^8] bradykinin, 1 μg of E_2, or 1 μg of E_2 and desArg^9[Leu^8] bradykinin. Representative western blot for bradykinin B1 receptor using extracts from uteri of rats treated with vehicle, E_2, desArg^9[Leu^8] bradykinin, or E_2 and desArg^9[Leu^8] bradykinin. **D**) Wet mass of uteri from females treated with vehicle, 0.1185 μg of Plummer inhibitor (MGTA), 1 μg of E_2, or 1 μg of E_2 and 0.1185 μg of MGTA.

and pathology, all contact the immune system for the first time in the reproductive tract. We, therefore, posit that hormonal regulation of complement may have a profound influence on uterine immunity [37–47]. Indeed, complement regulatory proteins (CR2, MCP, DAF1, and CD59A) are expressed on endometrial cells in the uterus, thus protecting host cells from autologous attack [48–50].

Our studies also provide insight into the source of uterine complement. Estrogen effects on the uterus occur in two phases. The early phase is reminiscent of an acute inflammatory response; E_2 causes vasodilation, an increase in vascular permeability with concommitant edema in the stroma and myometrium, accumulation of fluid in the lumen, and migration of leukocytes into the uterus. These changes are evident within 4 h but persist as long as estrogen levels are elevated. Estrogens induce cell proliferation and differentiation in the late phase, which begins 10–12 h after hormone administration. Although these phases are reflected in groups of genes whose expression changes early or late after estrogen treatment [51], the early proinflammatory changes can be dissociated from early genomic responses. Inhibitors of transcription like actinomycin D do not block E_2-induced edema in the uterus [52], but glucocorticoids have long been known to do so [53, 54]. In fact, we have recently shown that the genomic effects of E_2 are not widely inhibited by DEX under conditions where the proinflammatory effects of E_2 are completely blocked by DEX [25]. These studies suggest that the early effects of E_2 in the uterus, including the accumulation of complement, are mediated by nongenomic mechanisms rather than changes in gene expression. Our finding that DEX blocked the E_2-induced increase in several complement proteins and the bactericidal activity of uterine fluid is consistent with previous work on the regulation of complement C3 [55].

Several lines of evidence support the idea that complement comes from plasma exuded into the uterus and not de novo protein synthesis. First, E_2 caused the accumulation of fluid and large serum proteins like albumin in the uterus. Treatment with E_2 also increased levels of C1Q, C2, C3, C4, and C6 protein in the uterus, but levels of mRNA for these genes were not regulated, with the exception of *C3* mRNA. Nevertheless, C3 protein appears to be derived from plasma in the early phase. Full-length procomplement C3 protein was not detected in the E_2-treated uterus. Moreover, DEX antagonized the E_2-induced increase in C3 protein but not the increase in *C3* mRNA. We also found that the proinflammatory effects of E_2 and the accumulation of complement proteins in the uterus were insensitive to the translation inhibitor puromycin. In summary, treatment with E_2 caused vasodilation and an increase in vascular permeability, which resulted in extravasation of plasma and complement into the uterus.

While estrogens (and glucocorticoids) have a significant influence on vascular function [56–58], the mechanism(s) underlying these effects remain largely unknown. We found that E_2 effects in the uterus were partially mediated by activation of the contact system. The E_2-induced edema was reduced by 40% by SERPING1, Plummer inhibitor, and two BDKRB1 antagonists. Though the initial mechanism of activation is currently unclear, the data suggest that E_2 activates plasma kallikrein, which in turn cleaves high molecular weight kininogen to produce bradykinin. Carboxypeptidase N then cleaves bradykinin to produce des-Arg^9-bradykinin, which specifically activates BDKRB1. Involvement of des-Arg^9-bradykinin and BDKRB1 in uterine inflammation is a novel finding because this receptor is expressed at low levels in most tissues and is usually upregulated by inflammatory stimuli like lippolysaccharide [59].

In conclusion, we have shown that E_2 increased the amount and latent activity of complement proteins in the rat uterus and that coadministration of DEX completely blocked this increase. While estrogen induction of C3 and H2-Bf in the uterus has been known for some time [8–11], our study provides the first direct evidence that the complement system ($\sim$14 proteins in the effector arm) is a key mediator of innate immunity in the female reproductive tract. We suggest that estrogens (or glucocorticoids) might be used to enhance (or inhibit) complement-dependent processes in the uterus. Selective estrogen receptor modulators that only activate (or inhibit) nongenomic mechanisms may be especially useful in this regard [60]. Estrogen-induced elevation of uterine complement, for example, may aid in the clearance of particular sexually transmitted infections. Conversely, reduction of complement proteins in the uterus by antiestrogens (or glucocorticoids) might help prevent complement-mediated pregnancy loss [3, 4, 6, 7] or other reproductive disorders associated with complement activation (i.e., preeclampsia) [61]. Indeed, glucocorticoids are very effective in treating a severe form of preeclampsia called HELLP syndrome [62].

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