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Editor's Summary

Urothelial Defense Tug-of-War

It is well known that urinary tract infections (UTIs) are common in women, but which women are at more risk of UTIs is still far from certain, with some studies suggesting that it's younger women, and others showing evidence of increased risk after menopause. Now, a study by Lüthje and coauthors suggests that both of those views may be partially correct and demonstrates the mechanisms for each.

To understand how estrogen contributes to UTI pathogenesis, the authors examined cells from the urothelium (bladder lining) of menstruating women and older, postmenopausal subjects, studying the postmenopausal women before and after a 2-week period of estrogen supplementation. Then, to determine the mechanistic basis for their observations, they studied urothelial cells in an estrogen-depleted mouse model and in vitro. Thus, they demonstrated that estrogen has a protective effect on the urothelium, stimulating production of antimicrobial peptides and strengthening the attachment between urothelial cells. At the same time, the authors found that estrogen also increases the amount of bacteria taken up inside the urothelial cells.

On the basis of the findings of Lüthje *et al.*, one can conclude that young women may experience a greater incidence of UTIs because the high-estrogen environment increases the risk of bacterial invasion of the urothelium. Conversely, postmenopausal women may have more difficulty fighting off the infections because of their decreased production of antimicrobial peptides and diminished integrity of the urothelial lining. Additional studies of human and animal subjects will be needed to learn more about the molecular mechanism of estrogen's effects on the urothelium and understand what determines the balance between its pro- and anti-UTI effects. However, this line of research does advance us closer to understanding and eventually helping prevent the problem of recurrent UTIs in women.

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URINARY TRACT INFECTION

Estrogen Supports Urothelial Defense Mechanisms

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Epidemiological data imply a role of estrogen in the pathogenesis of urinary tract infections (UTIs), although the underlying mechanisms are not well understood. However, it is thought that estrogen supplementation after menopause decreases the risk of recurrent infections. We sought to investigate the influence of estrogen on host-pathogen interactions and the consequences for UTI pathogenesis. We analyzed urothelial cells from menstruating and postmenopausal women before and after a 2-week period of estrogen supplementation, and also studied the influence of estradiol during *Escherichia coli* UTI in a mouse infection model. Important findings were confirmed in two human urothelial cell lines. We identified two epithelial defense mechanisms modulated by estrogen. Estrogen induced the expression of antimicrobial peptides, thereby enhancing the antimicrobial capacity of the urothelium and restricting bacterial multiplication. In addition, estrogen promoted the expression and redistribution of cell-cell contact–associated proteins, thereby strengthening the epithelial integrity and preventing excessive loss of superficial cells during infection. These two effects together may prevent bacteria from reaching deeper layers of the urinary tract epithelium and developing reservoirs that can serve as a source for recurrent infections. Thus, this study presents some underlying mechanisms for the beneficial effect of estradiol after menopause and supports the application of estrogen in postmenopausal women suffering from recurrent UTI.

INTRODUCTION

Urinary tract infections (UTIs) are among the most common infectious diseases worldwide and cause substantial costs to the health care system (*1*). About half of all women experience at least one UTI during their lifetime. Moreover, in more than 25% of these women, the infection recurs within 6 months, and in a still substantial number of patients, even more often (*2*). Although estrogen may constitute a risk factor for infections in young women, the low estradiol levels after menopause have been related to recurrent infections (*3*–*7*). This contradiction points toward a dual mechanism in the effect of estrogen on the urinary tract. It is, however, generally thought that estrogen supplementation in postmenopausal women reduces the incidence of UTI (*8*–*10*), although the underlying mechanisms are not fully understood.

Most UTIs are caused by uropathogenic *Escherichia coli*. After adhering to the bladder epithelium, bacteria rapidly invade into the superficial umbrella cells lining the bladder lumen. Within these cells, *E. coli* multiply and form intracellular bacterial aggregates (*11*–*13*), referred to as intracellular bacterial communities (IBCs), which are a hallmark of the acute infection (*14*). Bacteria released from these IBCs may then invade another cell and become dormant. These intracellular collections of dormant bacteria, known as quiescent intracellular reservoirs (QIRs), are regarded as a potential source for recurrent infections (*12*, *13*).

This process is highly dependent on the differentiated urothelium. Uroplakins, which serve as the major receptors for *E. coli* adherence to the host cell, are localized within plaques on the apical membranes of the mature umbrella cells (*15*). The organization of the cytoskeleton in the urothelial cells changes during the differentiation process (*15*, *16*) and influences the ability of bacteria to grow within the cells. For example, the development of IBCs is possible only in the terminally differentiated umbrella cells, which contain peripheral actin filaments. In contrast, bacterial replication in less differentiated cell layers is inhibited by the denser actin network (*14*, *17*), which may explain the formation of QIRs.

Because estrogen is an important modulator of cell growth and differentiation, we expected to find an interconnection between the estrogen status of the host and bacterial infection at the level of the urothelium. To assess this, we investigated the influence of estrogen on key receptors involved in bacterial infection and two defense mechanisms against *E. coli*, namely, the production of antimicrobial peptides and the exfoliation of infected cells. Epithelial cells of all organs express antimicrobial peptides to kill invading pathogens. We have previously demonstrated the importance of the human cathelicidin LL-37/hCAP-18 for antibacterial defense in the urinary tract (*18*), and recently, the ribonuclease (RNase) 7 was identified to play a major role in this context (*19*, *20*). In contrast, little is known about the relevance of human β defensins (hBDs) and psoriasin during UTI, although expression of these peptides is well established in the urogenital tract (*21*–*24*). Another defense strategy of the host is the shedding of infected cells by apoptosis-induced exfoliation (*25*–*27*). As a consequence, however, underlying cells become directly exposed to bacteria.

Here, we sought to investigate the role of estradiol in the pathogenesis of *E. coli* UTI, with emphasis on its influence on the urothelium and the endogenous defense strategies in the urinary bladder.

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RESULTS

Estrogen supplementation enhances the production of antimicrobial peptides

The human antimicrobial peptide cathelicidin LL-37/hCAP-18 is expressed by various epithelia but also by neutrophils and other immune

cells and is thus easily measurable in serum. Therefore, we initially chose to determine the concentration of this peptide in the serum of healthy postmenopausal and menstruating women (median serum estradiol levels, 40 and 245 pM, respectively). Serum cathelicidin was significantly lower in postmenopausal women (fig. S1; $P = 0.007$). Within this group of postmenopausal women, peptide levels correlated directly to estradiol levels (Spearman $r = 0.57$, $P = 0.01$), implying that estrogen has an influence on the expression of this and possibly other antimicrobial peptides.

On the basis of these observations, we were interested in whether estrogen also affects the epithelial expression of antimicrobial peptides in the urinary tract. Therefore, we collected exfoliated cells from the urine of healthy postmenopausal women and measured mRNA for five different antimicrobial peptides before and after a 2-week period of estradiol supplementation. For comparison, urinary cells from menstruating women were analyzed. In 75% of the tested postmenopausal women (12 of 16), estradiol supplementation enhanced the expression of at least three of the five antimicrobial peptides. In half of the women, four or all five of the investigated peptides were elevated after estradiol treatment. Performing pre- and posttreatment comparisons on an individual basis, the most pronounced (and statistically significant) effect was observed for hBD3, followed by hBD2 and psoriasin, whereas no change could be detected for hBD1 or RNase 7 (Fig. 1, A to E). The expression of cathelicidin in the urinary cells was low, and thus, statistical analysis for this peptide was not possible.

To confirm that estradiol could induce these changes in cells of urothelial origin, we used the human bladder epithelial cell line 5637 and telomerase-immortalized normal human urothelial cells (TERT-NHUC). Expression of hBD1, hBD2, psoriasin, RNase 7, and cathelicidin was enhanced in the presence of estradiol (Fig. 1, F to J, and fig. S2), demonstrating that estradiol can indeed act on the urothelium. The use of receptor-specific estradiol analogs revealed that estrogen receptor β (ERβ) was more important than ERα in this context (table S1). Expression of hBD3 could not be detected in either cell line, and cathelicidin expression was detectable only in TERT-NHUC.

Estradiol strengthens intercellular junctions in the urothelium

Exfoliation of infected umbrella cells is an efficient strategy to shed invading pathogens. However, the loss of the superficial cells facilitates bacterial invasion of underlying less-differentiated cells and establishment of persistent reservoirs. We sought to investigate whether this process was influenced by estrogen. For this purpose, we collected urine samples from patients with *E. coli* UTI as well as from persons with a negative urine culture. We speculated that the size of the cells found in the urine should decrease during *E. coli* cystitis, as a consequence of bacterial contact with smaller cells in less-differentiated layers of the urothelium. We used flow cytometry to evaluate the sizes of exfoliated cells and found that during infection, the relative number of small cells of epithelial origin increased (Fig. 2A). Among the women with UTI, the urine from postmenopausal women contained a higher proportion of small cells than that from menstruating patients (Fig. 2B).

Estradiol has been previously described to promote epithelial barrier function by enhancing intercellular contacts (*28*, *29*). In urothelial cells, we detected a significant up-regulation of relevant proteins after treatment with estradiol, with increased mRNA for zona occludens protein 1 (ZO-1) and occludin ex vivo and in vitro (Fig. 2, C to F), as well as increased E-cadherin protein (Fig. 2G). This up-regulation was accompanied by a redistribution and concentration of these proteins at cell-cell contact points (Fig. 2H). Cells exposed to estradiol also formed more pronounced focal adhesions and more stress fibers, predominantly at the cell periphery (Fig. 2I).

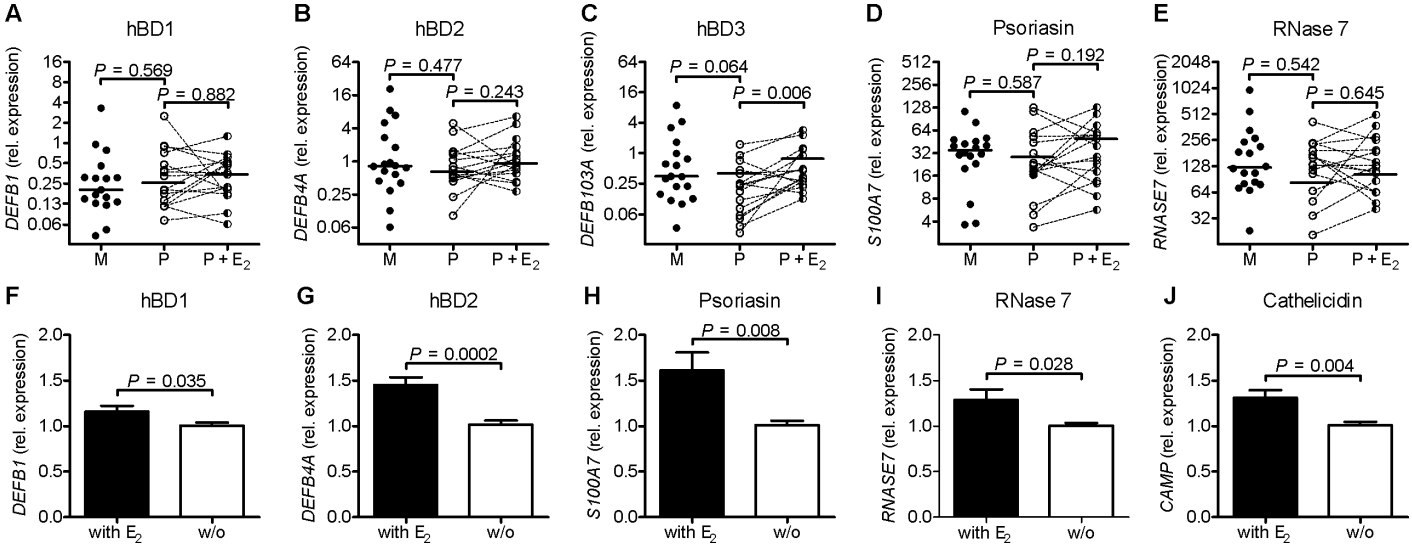

Fig. 1. Estradiol increases the production of antimicrobial peptides in bladder epithelial cells. (**A** to **E**) Exfoliated urinary cells were collected from menstruating (M, $n = 18$) and postmenopausal women before (P, $n = 16$) and after (P+E_2, $n = 16$) estradiol supplementation. Expression of mRNA for hBD1 (A), hBD2 (B), hBD3 (C), psoriasin (D), and RNase 7 (E) was determined by real-time reverse transcription polymerase chain reaction (RT-PCR) and is expressed in relation to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Individual values and median are shown. (**F** to **J**) The expression of mRNA for antimicrobial peptides in bladder epithelial cell line 5637 (F to I) and normal human telomerase-immortalized urothelial cells (J) grown with (With E_2) or without estradiol (w/o) for 48 hours was quantified in relation to GAPDH mRNA by real-time RT-PCR; cells without estradiol were set to 1. The data are shown as means and SEM from five experiments in triplicate.

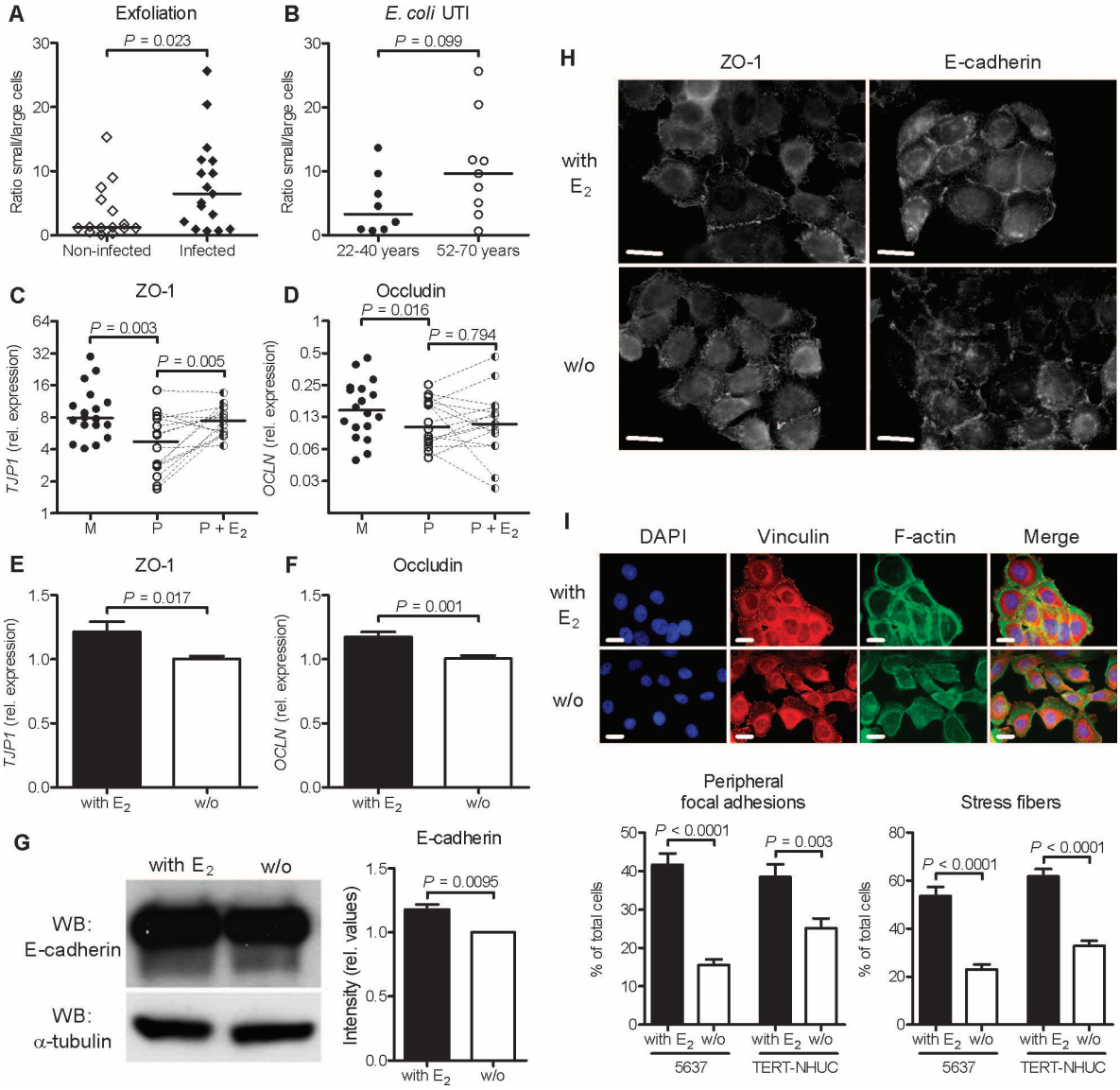

Fig. 2. Estradiol strengthens cell-cell contacts and reduces exfoliation. (**A** and **B**) Exfoliated cells from urine were separated by size with flow cytometry and presented as the ratio between small and large cells. (A) Cells from individuals with negative culture results (n = 14) and from patients with *E. coli* infection (n = 17) were compared. (B) Samples from *E. coli*–infected patients were grouped according to the age of the women (22 to 40 years, n = 8; 52 to 70 years, n = 9). (**C** and **D**) Expression of ZO-1 (C) and occludin (D) mRNA was analyzed by real-time RT-PCR in relation to GAPDH mRNA in exfoliated cells from menstruating (M, n = 18) and postmenopausal women before (P, n = 16) and after (P+E_2, n = 16) estradiol supplementation. (**E** and **F**) Expression of ZO-1 (E) and occludin (F) mRNA in relation to GAPDH mRNA was determined in bladder epithelial cells 5637 cultured with (With E_2) or without estradiol (w/o) for 48 hours; cells without estradiol were set to 1. Data are derived from four to five experiments in triplicate. (**G**) E-cadherin was detected in lysates from 5637 cells cultured with (With E_2) or without estradiol (w/o) by Western blotting (left). Intensity of the bands from three independent experiments performed in duplicate was determined and expressed in relation to α-tubulin (right). (**H** and **I**) 5637 cells cultured with (With E_2) or without estradiol (w/o) were stained for ZO-1 or E-cadherin (H), or vinculin (red) and F-actin (green), with 4′,6-diamidino-2-phenylindole (DAPI)–stained nuclei shown in blue (I). Scale bars, 20 μm. (I) Peripheral focal adhesions (left) and stress fibers (right) were quantified in 20 random view fields of one representative experiment. (A to D) Individual values with median (A to D) or mean (E to G and I) and SEM are shown.

Estrogen restricts *E. coli* proliferation during the acute infection and reduces residual bacteria

To study the consequences of estrogen depletion in vivo, we used a mouse model of surgically induced menopause. During the acute phase of urinary *E. coli* infection, the bacterial load was significantly higher in ovariectomized mice compared to sham-operated control animals (Fig. 3A). In most of the mice, bacteria were primarily found intracellularly at 24 hours, organized into a few well-defined IBCs (Fig. 3B, left). In contrast, the epithelium of three animals, all of which were in the ovariectomized group, harbored numerous IBCs, with epithelial regions completely occupied by fused bacterial inclusions and a highly colonized epithelial surface (Fig. 3B, right). Seven days after infection,

the bacterial load in bladders dropped to titers previously related to the presence of QIRs (*13*). The amount of these residual bacteria was higher in ovariectomized mice (Fig. 3C), indicating a greater risk for reemergence of the infection.

Estrogen facilitates bacterial uptake by urothelial cells

Because the host cell actively participates in bacterial uptake, we assumed that estradiol-induced cellular changes might also affect this early step during infection. The major receptors mediating *E. coli* adhesion and invasion into urothelial cells are uroplakin Ia (UPIa) and β_1 integrin, respectively. The expression of UPIa was higher in menstruating women (Fig. 4A), indicating the influence of estrogen; a similar tendency was seen in β_1 integrin (Fig. 4B), although the results were not statistically significant. The low expression of UPIa in postmenopausal women could be reversed by estrogen supplementation (Fig. 4A). Immunofluorescence analysis of mouse bladder tissue confirmed the positive correlation between UPIa and estrogen on the protein level (Fig. 4C). However, despite the higher levels of UPIa, there was no difference in the total bacterial load in mouse bladders at this early time point after infection (Fig. 4D). This might be due to the high bacterial inoculum used for artificial infections. In contrast, the number of intracellular bacteria appeared increased in sham-operated mice (Fig. 4D), indicating that the differences in receptor expression are of biological relevance. This finding was reproducible in vitro in two different human cell lines treated with estradiol (Fig. 4E).

DISCUSSION

Here, we demonstrate the effects of estrogen on the pathogenesis of UTI. We show that estradiol improves the antimicrobial capacity of the urothelium and prevents excessive exfoliation upon infection. At the same time, estradiol also facilitates bacterial invasion into host cells. These findings together provide an explanation for the clinical effect of estrogen supplementation after menopause to prevent recurrent infections as well as the high susceptibility of young women to UTI.

Divergent results from clinical studies and animal experiments indicate complex estradiol-associated effects on the urinary tract (*10*, *30*–*33*). To address the question experimentally in vivo, we chose a mouse model of surgically induced menopause and delivered bacteria directly into the bladder. This model allowed us to study the impact of endogenous pre- and postmenopausal estrogen levels in age-matched mice. Thus, factors related to aging other than estrogen levels, for example, body weight and composition, were diminished or excluded. It has also been argued that rising vaginal pH and changes in vaginal microflora enhance susceptibility toward UTI after menopause (*34*). For example, in fertile female mice, estrogen supplementation modulated *E. coli* colonization of the bladder, not only directly but also indirectly by affecting the commensal flora of the vaginal epithelium (*32*). By instillation of bacteria directly into the bladder, we bypassed this complex interaction and were able to focus our investigation on urothelial factors and estradiol alone.

All antimicrobial peptides investigated here were elevated in the presence of estradiol. Although the estradiol-mediated increase for each single peptide was relatively small, the simultaneous elevation of several peptides may produce a biologically relevant effect. In agreement with this finding, the lower bacterial loads in the bladders of sham-operated mice at 24 hours of *E. coli* infection may indicate that these animals are better able to restrict bacterial multiplication and distribution, although additional factors beside the production of antimicrobial peptides in the urothelium may contribute. Estrogen-

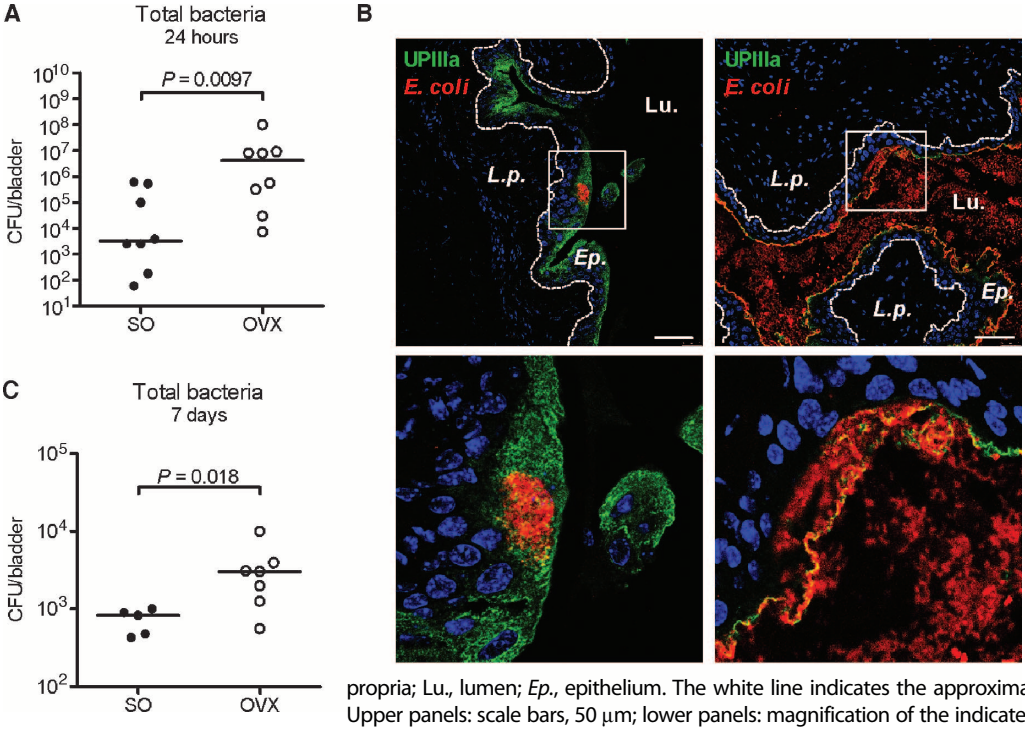

Fig. 3. The bacterial burden is higher in mice with low estrogen levels. (**A** to **C**) Sham-operated control mice (SO, ●) and ovariectomized mice (OVX, ○) were infected with *E. coli* for 24 hours (A and B) or 7 days (C). (A) The total number of bacteria was higher in the bladders of ovariectomized compared to sham-operated mice 24 hours after infection ($n = 8$ per group). Individual values and median are shown. (B) Representative sections from mouse bladders 24 hours after infection were stained for UPIIIa (marker of terminally differentiated umbrella cells, green) and *E. coli* (red), with DAPI to show nuclei (blue). Left: Most of the mice (total $n = 8$ per group) harbored few extracellular bacteria and at most four characteristic IBCs per section. Right: Among the ovariectomized mice, three animals showed a highly colonized urothelium with a high number of IBCs. *L.p.*, lamina propria; Lu., lumen; *Ep.*, epithelium. The white line indicates the approximate location of the basement membrane. Upper panels: scale bars, 50 μm; lower panels: magnification of the indicated area in the upper panels. (C) One week after infection, the total number of bacteria was higher in ovariectomized compared to sham-operated mice ($n = 8$ per group). Individual values and median are shown. CFU, colony-forming units.

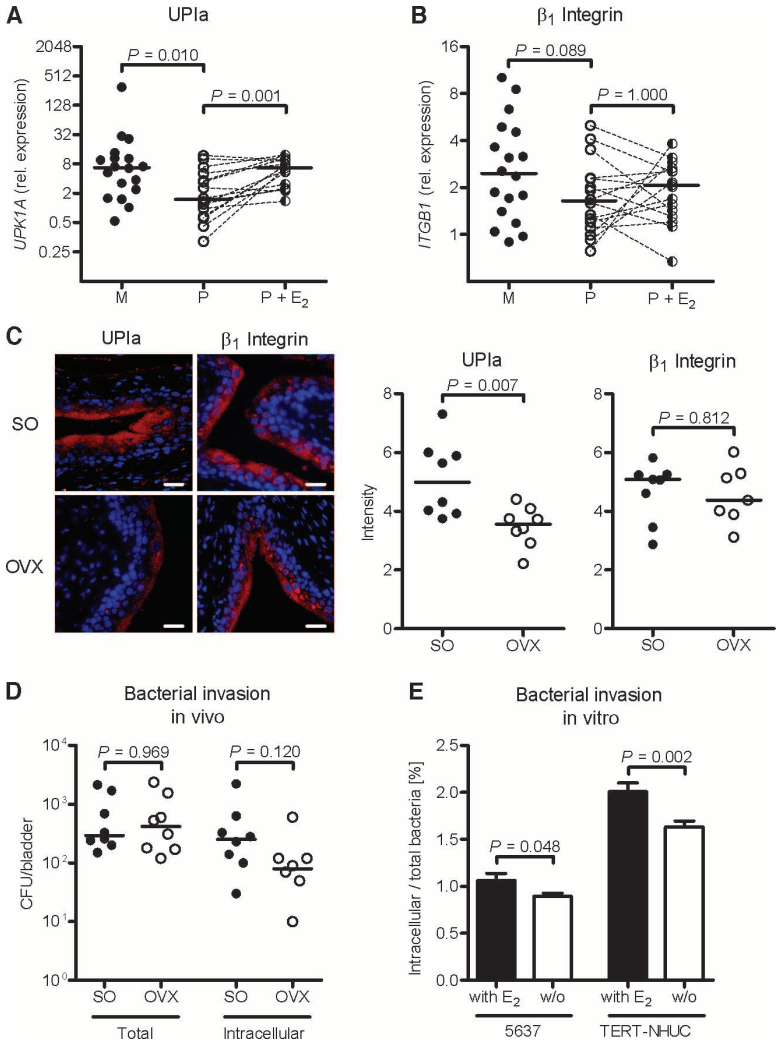

Fig. 4. Estradiol facilitates early bacterial invasion into urothelial cells. (**A** and **B**) Exfoliated urinary cells from menstruating (M, n = 18) and postmenopausal women before (P, n = 16) and after (P+E_2, n = 16) estradiol supplementation were analyzed by real-time RT-PCR for expression of UPIa (A) and β_1 integrin (B) mRNAs in relation to GAPDH mRNA. (**C**) Bladder sections from sham-operated (SO) and ovariectomized (OVX) mice were stained for UPIa and β_1 integrin (red) 2 hours after infection; nuclei were shown with DAPI (blue). Scale bars, 20 μm. Expression of UPIa and β_1 integrin was quantified by mean fluorescence intensity in relation to the number of epithelial cells (n = 8 per group; two or more images per animal). (**D**) Sham-operated control mice (●) and ovariectomized mice (○) were infected with *E. coli* for 2 hours. In half of the mice, the number of total bacteria in the bladder was enumerated. In the other half of the mice, the number of intracellular bacteria was determined after incubation with the cell-impermeable antibiotic gentamicin to kill any extracellular bacteria. (A to D) Data are shown as individual values and median. (**E**) Bladder epithelial cells 5637 and TERT-NHUC were cultured with (with E_2) or without estradiol (w/o) for 48 hours and infected with *E. coli*. Bacterial invasion is expressed as the relation between intracellular and total bacteria, determined in a gentamicin protection assay. Means and SEM are shown from at least four experiments with three to four replicates.

dependent antimicrobial activity of epithelia has been recognized in the uterus (*35*, *36*) and vaginal epithelium (*37*) and has been ascribed to estradiol-induced expression of hBD1 and hBD2 (*38*, *39*). In addition to β defensins, we also detected significant transcription of psoriasin, RNase 7, and cathelicidin in the bladder epithelial cells. The presence of psoriasin in the tissue of healthy women suggests an impact on urothelial immunity besides its already established association with a subtype of bladder carcinoma (*22*). Responsiveness of tissues to estrogen is mediated by two ERs, ERβ and ERα, and by the estrogen-responsive elements located in the promoter regions of target genes. We detected a primarily ERβ-mediated induction of hBD1, hBD2, and psoriasin; RNase 7 was induced by both an ERα and ERβ agonist. We could not identify such a connection for cathelicidin expression. Assessable databases (*40*) report estrogen-responsive elements in the promoters for hBD1, hBD2, and psoriasin, but not for hBD3, RNase 7, or cathelicidin. Therefore, we conclude that estradiol may influence the expression of these latter genes indirectly.

The exfoliation of infected cells is an effective defense mechanism to remove pathogens from the urinary tract. However, this also creates the opportunity for bacteria to invade and establish reservoirs in underlying less-differentiated cells. Thus, excessive exfoliation might eventually turn into an infection-promoting effect. In addition, delayed and incomplete differentiation of the basal cell layers in the absence of estrogen was recently demonstrated in the mouse urinary tract during *E. coli* infection (*41*). We furthermore speculated that the strength of cell-cell contacts influences the disposition of urothelial cells to exfoliation after pathogen-induced apoptosis in single umbrella cells. To support our assumption, we demonstrated that expression of cell junction–associated proteins was promoted by estradiol and that intercellular contacts were more pronounced in the presence of the hormone. Similar to our observations in the urothelium, several proteins participating in the formation of intercellular junctions in the intestinal epithelium were reported to be up-regulated by estradiol both in vitro and in vivo (*28*, *29*), and the dependency of this effect on ER signaling was demonstrated (*28*, *29*, *42*).

In addition to their antibacterial activity, antimicrobial peptides play an important role in tissue repair and maintenance (*43*–*45*). In particular, cathelicidin and hBD2 mediate an interdependent linkage between permeability and antimicrobial barrier of the epidermis (*46*, *47*). A similar observation has been reported for the lung epithelium (*48*). In light of the data presented here, we suggest that estradiol supports epithelial integrity through the combined action of antimicrobial peptides and intercellular contact formation.

Clinical data (*6*, *49*) and ex vivo experiments (*50*, *51*) indicate a positive correlation between susceptibility to *E. coli* UTI and estrogen levels in young women. Our data provide an explanation for this observation, because estrogen increased the expression of infection-promoting receptors UPIa and β_1 integrin, thereby facilitating bacterial invasion of the bladder epithelium early during infection. Moreover, focal adhesions and actin stress fibers, cellular components exploited by *E. coli* to invade the host cell, were relocated to the cell periphery under the influence of estradiol, which may furthermore assist the invasion process in vivo and may be the prime mechanism underlying reduced invasion in vitro. We could not confirm the more progressive UTI pathogenesis in mice with high estrogen levels that

has been reported in another animal-based study (*31*). However, it should be noted that those mice were supplemented with a supraphysiological concentration of estradiol, which is likely to cause different effects from the endogenous physiological concentrations present in the fertile females investigated in the current study. We showed that estrogen promotes the binding of *E. coli* to the host cells, and hypothesize that this early direct contact provides an advantage for the host: The cell can mobilize defense strategies more quickly and thus fight the invading pathogen more efficiently, before *E. coli* start multiplying and overwhelm the cell's antibacterial capacity. This strategy is supported by the estrogen-mediated increase of urothelial antimicrobial peptides.

Our findings demonstrate that estradiol acts as a two-edged sword in the context of UTI. On the basis of the data presented here, estradiol supplementation appears to be a beneficial modulator of urothelial immune defense in postmenopausal women suffering from recurrent cystitis. The mouse model applied in this study enabled us to specifically identify the action of estrogen on the urothelium. However, it was not possible to consider all physiological changes occurring during menopause. In addition, to ensure the infection of a reasonable number of mice, high bacterial concentrations in the inoculum were required (*52*); as such, this experimental setup may not accurately represent natural infection. In our study, we demonstrated estradiol-induced changes in healthy women on a cellular level and used mice to provide evidence for the biological consequences in the face of a bacterial challenge. Monitoring these parameters in postmenopausal women suffering from recurrent UTI before and during estrogen supplementation would further add to the evaluation of the mechanism for this process in human patients.

MATERIALS AND METHODS

Bacteria

For all experiments, we used the uropathogenic *E. coli* strain CFT073 grown to logarithmic phase in LB broth with shaking at 37°C. The bacterial concentration was adjusted in phosphate-buffered saline (PBS) spectrophotometrically and confirmed by viability count. Expression of type 1 fimbriae was confirmed by mannose-sensitive agglutination of yeast cells.

Mouse model of UTI

Mouse experiments were approved by the Northern Stockholm Animal Ethics Committee, and experiments were carried out according to the guidelines of the Federation of Laboratory Animal Science Association and in compliance with the Committee's requirements. In female C57BL/6 mice, bilateral ovariectomy or sham operation was performed at Harlan Laboratories, the Netherlands, following standard procedures. The mice were shipped 1 week after surgery. Mice were fed a phytoestrogen-free diet (Teklad Global 19% Protein Extruded Rodent Diet, Harlan Teklad) for 1 week before and throughout the experiment. At least 2 weeks after surgery, transurethral infection was induced as described before (*53*). Mice were anaesthetized and transurethrally infected with 0.5×10^8 colony-forming units of *E. coli* in 50 μl of PBS. At the indicated time points, the mice were sacrificed, and their bladders were aseptically removed, cut open, and washed in ice-cold PBS three times to remove urine and nonadherent bacteria. To determine the total bacterial load, we homogenized the bladders in 1 ml of PBS and plated serial dilutions of the homogenate on blood agar plates. For microscopy, bladders were fixed in 4% paraformaldehyde (PFA) overnight at 4°C and processed for sectioning. To count intracellular bacteria, bladders were incubated for 60 min at 37°C in PBS containing gentamicin (100 μg/ml), then washed three times in PBS, and homogenized.

Cell lines and culture conditions

Urothelial cell line 5637 (HTB-9, American Type Culture Collection) and telomerase-immortalized urothelial cells TERT-NHUC (provided by M. A. Knowles, Leeds, UK) were cultured as previously described (*54*). For experiments with estradiol, phenol red–free medium and 5% charcoal–treated fetal bovine serum were used. Stock solutions of 17β-estradiol (Sigma) and PPT and DPN (Tocris Biochemicals) were prepared in absolute ethanol and used at a final concentration of 10 nM for estradiol or 1 nM for PPT and DPN. Control cells were exposed to the vector alone (absolute ethanol, 0.001% in culture medium). Cells were treated for 48 hours, with medium exchanged after 24 hours.

Total RNA extraction and real-time RT-PCR

Total RNA extraction was performed as previously described (*54*), and up to 1 μg of RNA was transcribed to complementary DNA (cDNA) with the DyNAmo cDNA Synthesis Kit (Finnzymes) or the High Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturers' instructions. Gene expression was analyzed with TaqMan gene expression assays for antimicrobial peptides (human β-defensin 1, *DEFB1*, Hs00608345_m1; human β-defensin 2, *DEFB4A*, Hs00175474_m1; human β-defensin 3, *DEFB103A*, Hs00218678_m1; psoriasin, *S100A7*, Hs00161488_m1; human cathelicidin LL-37/hCAP-18, *CAMP*, Hs00189038_m1), tight junction proteins (occludin, *OCLN*, Hs00170162_m1; zona occludens protein 1, *TJP1*, Hs01551876_m1), β_1 integrin (*ITGB1*, Hs00559595_m1), and UPIa (*UPK1A*, Hs00199638_m1); GAPDH was used as an internal control to calculate relative gene expression. Expression of RNase 7 was determined with a SYBR Green–based assay (Qiagen) with previously described primers (*55*). After initial denaturation at 95°C for 15 min, each cycle consisted of 15 s at 95°C, 30 s at 60°C (touchdown of 1°C per cycle from 66° to 60°C), and 30 s at 72°C. Melting curves were produced at the end of the run to ensure specificity of the amplification; a plasmid containing the amplification product served as control (*55*).

Immunofluorescence staining of bladder sections and cells

Sections of paraffin-embedded tissue were deparaffinized and rehydrated, pretreated with 0.3% Triton X-100/PBS at room temperature (for UPIIIa and *E. coli* staining), or boiled in tris-EDTA buffer [1 mM EDTA, 10 mM tris, 0.05% Tween 20 (pH 9); for β_1 integrin and UPIa staining]. Cells were fixed in 4% PFA for 15 min at room temperature and permeabilized with 0.3% Triton X-100 in PBS. Thereafter, sections were blocked for 30 min with FX Signal Enhancer (Invitrogen), and both cells and sections were blocked for an additional 60 min with the sera from the species in which the secondary antibodies were raised. Incubation with primary antibodies was carried out for 60 min at room temperature (cells) or overnight at 4°C (tissue). Primary antibodies used were goat anti–*E. coli* (1:200; OBT0986, AbD Serotec), rabbit anti-UPIIIa (1:200; H-180, Santa Cruz Biotechnology), rabbit anti–β_1 integrin (1:250; LS-C49761, LifeSpan BioSciences), goat anti-UPIa (1:200; C-18, Santa Cruz Biotechnology), mouse anti-vinculin (1:200; hVIN-I, Sigma), mouse anti–E-cadherin (1:1000; 610181, BD Transduction), or mouse anti–ZO-1 (1:100; 33-9100, Zymed). Sections were then incubated

with secondary Alexa Fluor–conjugated antibodies or Alexa Fluor–labeled phalloidin (Invitrogen) for 30 min at room temperature and mounted in ProLong Gold Antifade mounting medium including DAPI (Invitrogen). Tissue was analyzed with a Leica SP5 confocal microscope or a Leitz-Leica DMRE microscope. Fluorescence intensity was quantified with the ImageJ software and expressed in relation to epithelial cells as determined by the number of nuclei. Cells were examined under a Zeiss AxioVert 40 CFL microscope with a Zeiss AxioCam MRm digital camera and the AxioVision software. Peripheral focal adhesions (indicated by vinculin) and stress fibers were counted manually in 20 random view fields.

Western blot analysis

Cells were lysed in a solution of 20 mM Hepes (pH 7.5), 0.1 M NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, and 1% aprotinin; the lysates were centrifuged for 15 min at 4°C, separated on SDS–polyacrylamide gel electrophoresis, and transferred onto nitrocellulose (Hybond C, GE Healthcare). Western blotting analyses were performed with mouse anti–E-cadherin (1:1000; 610181, BD Transduction) and mouse anti–α-tubulin (1:1000; T9026, Sigma-Aldrich) followed by horseradish peroxidase–conjugated secondary antibodies (1:5000; NA931V, GE Healthcare). Signals were detected by Luminol immunoblotting reagent (Santa Cruz Biotechnology).

Cell infection assays

Confluent cell layers were infected with 10^6 *E. coli* in 100 μl of PBS per well (1.9 cm^2), and the plates were centrifuged for 5 min at 600*g*. After 30 min at 37°C in a humidified incubator with 5% CO_2, cells were washed three times with prewarmed PBS to remove nonadherent bacteria. For total bacterial count, cells were lysed with 0.5% trypsin and 0.1% Triton X-100 in PBS. To measure bacterial invasion, we further incubated the cells with fresh antibiotic-free medium for another 30 min and then for an additional 30 min with medium containing gentamicin (100 μg/ml). Cells were lysed, and bacteria were enumerated by viability count. The invasion rate was calculated as the number of intracellular bacteria divided by the total number of bacteria from the same experiment.

Collection of urine, exfoliated urothelial cells, and serum

The collection of human material has been approved by the local ethics committee in Stockholm and the Swedish Medical Products Agency. Written informed consent was obtained from all participants. Inclusion criteria for the group of menstruating women were 18 to 40 years of age, no pregnancy, and no usage of hormonal contraceptives; for the group of postmenopausal women, 45 to 65 years of age, no menstruation for at least 1 year, and not receiving any hormonal preparations. Women with ongoing UTI or any antimicrobial treatment within the last month were excluded. The postmenopausal women were studied before and after 2 weeks of local daily supplementation with 25 μg of estradiol (Vagifem, Novo Nordisk Pharma). Midstream urine and venous blood samples were collected from all women. Serum was stored at −20°C; urinary cells were immediately harvested for total RNA extraction with the RNeasy Micro kit according to the manufacturer's instructions (Qiagen). Aliquots of the cells were examined by light microscopy to ensure the epithelial origin of the cells.

Analysis of urinary cells by flow cytometry

Urine samples were collected from women ≤40 years (regarded as premenopausal) and women >50 years (regarded as postmenopausal) and sent for microbiological diagnostics at Karolinska Universitetslaboratoriet, Stockholm. Women with known pregnancy, abnormalities of the urinary tract, cancer, or other severe general health issues were excluded. Urinary cells were washed and analyzed on a Becton Dickinson FACSCalibur flow cytometer with the CellQuest software (BD Biosciences Immunocytometry Systems). Similarly prepared cell pellets were fixed with Carnoy I fixative and stained by the Papanicolaou technique as described before (*56*). Most of the cells within the preparations were of epithelial origin.

Measurement of cathelicidin in serum

Serum samples from menstruating and postmenopausal women were analyzed by enzyme-linked immunosorbent assay specific for cathelicidin according to the manufacturer's protocol (Hycult Biotech).

Measurement of serum estradiol

Serum concentrations of 17β-estradiol were determined by a radioimmunoassay (lower detection limit, 5 pM; Spectria, Orion Diagnostica AB) in postmenopausal women or by a chemiluminescent enzyme immunometric assay (lower detection limit, 73 pM; Access Immunoassay System DXi Estradiol, Beckman-Coulter Inc.) in menstruating women.

Statistical analysis

Data from in vitro experiments were normally distributed and are presented as means and SEM; treated and control cells were compared by unpaired two-tailed *t* test. Data derived from animal experiments and clinical material are presented as individual values with median. Nonnormally distributed data were log-transformed to achieve normal distribution; comparisons on normally distributed data were performed by unpaired two-tailed *t* test or by paired *t* test as appropriate; otherwise, nonparametric tests were used. Differences with $P < 0.05$ were regarded as statistically significant. All statistical tests were performed with GraphPad Prism version 5.02.

SUPPLEMENTARY MATERIALS

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Fig. S1. Serum concentration of cathelicidin is higher in menstruating than in postmenopausal women.

Fig. S2. Estradiol induces expression of antimicrobial peptides in TERT-NHUC.

Table S1. The induction of antimicrobial peptides by estradiol involves ERs.

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