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Gender-Specific Protection of Estrogen against Gastric Acid-Induced Duodenal Injury: Stimulation of Duodenal Mucosal Bicarbonate Secretion

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Because human duodenal mucosal bicarbonate secretion (DMBS) protects duodenum against acid-peptic injury, we hypothesize that estrogen stimulates DMBS, thereby attributing to the clinically observed lower incidence of duodenal ulcer in premenopausal women than the age-matched men. We found that basal and acid-stimulated DMBS responses were 1.5 and 2.4-fold higher in female than male mice *in vivo*, respectively. Acid-stimulated DMBS in both genders was abolished by ICI 182,780 and tamoxifen. Estradiol-17β (E_2) and the selective estrogen receptor (ER) agonists of ERα [1,3,5-*Tris*(4-hydroxyphenyl)-4-propyl-1H-pyrazole] and ERβ [2,3-*bis*(4-hydroxyphenyl) propionitrile], but not progesterone, rapidly stimulated ER-dependent murine DMBS *in vivo*. E_2 dose dependently stimulated murine DMBS, which was attenuated by a Cl^-/HCO_3^- anion exchanger inhibitor 4,4′-didsothio- cyanostilbene-2, 2′-disulfonic acid, removal of extracellular Cl^-, and in cystic fibrosis transmembrane conductance regulator knockout female mice. E_2 stimulated murine DMBS *in vitro* in both genders with significantly greater response in female than male mice (female to male ratio = 4.3). ERα and ERβ mRNAs and proteins were detected in murine duodenal epithelium of both genders; however, neither ERα nor ERβ mRNA and protein expression levels differed according to gender. E_2 rapidly mobilized intracellular calcium in a duodenal epithelial SCBN cell line that expresses ERα and ERβ, whereas BAPTA-AM abolished E_2-stimulated murine DMBS. Thus, our data show that E_2 stimulates DMBS via ER dependent mechanisms linked to intracellular calcium, cystic fibrosis transmembrane conductance regulator, and Cl^-/HCO_3^- anion exchanger. Gender-associated differences in basal, acid- and E_2-stimulated DMBS may have offered a reasonable explanation for the clinically observed lower incidence of duodenal ulcer in premenopausal women than age-matched men. (*Endocrinology* 149: 4554–4566, 2008)

PEPTIC DUODENAL AND gastric ulcers raise serious health problems and significant economic cost worldwide. There are approximately 500,000 new cases and 4.5 million people suffering from these diseases each year in the United States (1). Duodenal ulcer is three times more common than gastric ulcer. Available evidence suggests that duodenal ulcer most likely results from an imbalance between "aggressive" factors, such as infection of *Helicobacter pylori* (*H. pylori*), gastric acid and pepsin, and "defensive" factors, such as duodenal mucosal bicarbonate (HCO_3^-) secretion (DMBS) (1, 2). DMBS is critical to defend the vulnerable duodenal epithelium against the aggressive factors (3, 4). In general, the small intestine luminal pH remains near neutral, even when acidic gastric contents are delivered into the human proximal duodenum, except for a brief (<30 sec) decrease in pH related to gastric emptying (1, 2). In the proximal duodenum, surface epithelial HCO_3^- secretion plays a key role in the maintenance of the neutrality of luminal pH (3, 4). In addition to protecting duodenal mucosa from gastric acid-peptic injury, DMBS ensures a nonacidic environment for the proper functionality of intestinal enzymes. The importance of DMBS in protecting duodenal mucosa has been confirmed in patients with duodenal ulcer, whose acid-stimulated DMBS is only 41% of that in healthy subjects (5). DMBS is impaired in the duodenal tissues from patients with cystic fibrosis (6), the most common severe autosomal genetic disorder in the Caucasian population. These studies suggest a pivotal role of cystic fibrosis transmembrane conductance regulator (CFTR) in mediating DMBS (6–8).

It has long been observed that the ratio between men and women who develop duodenal ulcer is 1.9:1 in the United States, whereas in Europe and Asia, this ratio is 2.2:1 (9–12) and 3.1:1 (13), respectively. These clinical observations suggest a gender difference in the incidence and severity of duodenal ulcer; however, the underlying mechanism(s) is currently unknown. As far as gender differences are concerned, sex hormones have been often evaluated as the causative factors. For example, numerous studies have suggested a protective role of estrogen in the development of various diseases, including cardiovascular diseases (14–16), cerebral

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Abbreviations: AM, Acetoxymethyl ester; CFTR, cystic fibrosis transmembrane conductance regulator; DIDS, 4,4′-didsothio- cyanostilbene-2, 2′-disulfonic acid; DMBS, duodenal mucosal bicarbonate secretion; DMSO, dimethylsulfoxide; DPN, 2,3-*bis*(4-hydroxyphenyl) propionitrile; E_2, estradiol-17β; ER, estrogen receptor; $[Ca^{2+}]_{cyt}$, free cytoplasmic Ca^{2+}; I_{sc}, short-circuit current; PPT, 1,3,5-*Tris*(4-hydroxyphenyl)-4-propyl-1H-pyrazole; SERM, selective ER modulator; TER, transepithelial resistance.

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damage and mortality (17, 18), and osteoporosis (14–16, 19, 20). In contrast, information regarding the protective effects of sex hormones in the gastrointestinal tract is very limited (21, 22). In particular, it is completely unknown whether estrogen protects duodenum via stimulation of DMBS. A few studies have shown that putative estrogen receptors (ERs) are present in rat duodenal epithelial cells (23, 24), implicating the duodenum as possibly a direct target for estrogen action. Interestingly, pregnant women or women taking oral contraceptive pills (estrogen/progesterone compounds) exhibited a reduced frequency of duodenal ulcer (25, 26). Moreover, duodenal ulcer runs a more serious course in postmenopausal than premenopausal women (27). Thus, it is reasonable to infer that, similar to other organs, estrogen may have protective effects in the gastrointestinal tract.

Over 20 yr ago, Isenberg *et al*. (28, 29) first measured human DMBS in 1986. Surprisingly, in their landmark studies and other follow-up studies, none had systematically compared DMBS in women and man. Thus, no comment has been drawn on any gender differences in human DMBS. Alvaro *et al*. (30) found that 17α-ethinyl estradiol inhibited rat biliary HCO_3^- secretion; however, the underlying mechanisms are elusive because 17α-ethinyl estradiol stimulated Na^+/HCO_3^- cotransporters (HCO_3^- loading process) without altering activities of both Cl^-/HCO_3^- and Na^+/H^+ exchangers (HCO_3^- and H^+ extruding process) in the rat hepatocyte couplets. It is conflicting for 17α-ethinyl estradiol to stimulate HCO_3^- loading process but to inhibit biliary HCO_3^- secretion simultaneously. Moreover, Singh *et al*. (31) reported estradiol-17β (E_2) inhibition of CFTR channel, which is permeable to both Cl^- and HCO_3^- in human colonic crypt T84 cells at pharmacological concentrations. To date, whether estrogen plays a physiological role in regulating epithelial HCO_3^- secretion, needless to say, the underlying mechanisms are unknown. In the present study, we aimed at exploring whether there are gender differences in basal and acid-stimulated DMBS and, if any, what the underlying cellular mechanisms are. By performing physiological and biochemical studies at the levels of cell, tissue, and whole animal, we present evidence herein showing that: 1) substantial gender differences in DMBS exist in response to acid and E_2 stimulation; and 2) E_2 rapidly stimulates DMBS that is linked to specific ERs, free cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$), CFTR, and Cl^-/HCO_3^- anion exchanger. Therefore, our data support a novel concept that E_2 plays a protective role against the acid-peptic duodenal injury in females. This mechanism might have provided a reasonable explanation for the clinically observed gender difference in the prevalence of duodenal ulcer.

Materials and Methods

Reagents and solutions

Mouse anti-ERα monoclonal antibody (AER320) raised against amino acid 495–595 of calf uterus ERα was purchased from Lab Vision Corp. (Fremont, CA). Rabbit anti-ERβ polyclonal antibody against amino acids 55–70 of human ERβ was from Affinity BioReagents, Inc. (Golden, CO). Tamoxifen, E_2, 1,3,5-*Tris*(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), or 2,3-*bis*(4-hydroxyphenyl) propionitrile (DPN), 4,4′-didsothiocyanostilbene-2, 2′-disulfonic acid (DIDS), and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO). ICI 1 82,780 is from Tocris (Ellisville, MI). All other chemicals were obtained from Fisher Scientific (Santa Clara, CA). Fura-2 acetoxymethyl ester (AM) was from Molecular Probes, Inc. (Eugene, OR). The mucosal solution used in Ussing chamber experiments contained the following (in mM): 140 Na^+, 5.4 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 120 Cl^-, 25 gluconate, and 10 mannitol. The serosal solution contained the following (in mM): 140 Na^+, 5.4 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 120 Cl^-, 25 HCO_3^-, 2.4 HPO_4^{2-}, 2.4 $H_2PO_4^-$, 10 glucose, and 0.01 indomethacin. The physiological salt solution used in digital Ca^{2+} measurement contained the following (in mmol/liter): Na^+ 140, K^+ 5.0, Ca^{2+} 2, Cl^- 147, HEPES 10, and glucose 10. For the Ca^{2+}-free solution, Ca^{2+} was omitted, and 0.5 mM EGTA was added to prevent potential Ca^{2+} contamination. The osmolalities for all solutions were approximately 284 mOsm/kgH_2O.

Animals and genotyping of CFTR mutant mice

Animal use protocol in this study was approved by the University of California San Diego, Committee on Investigations Involving Animal Subjects. All experiments were performed with adult Harlan C-57 black mice, CFTR wild-type ($CFTR^{+/+}$) and homozygous ($CFTR^{-/-}$) mice. All the mice were at the age of 70–90 d, housed in a standard animal care room with a 12-h light, 12-h dark cycle, and were allowed free access to food and water. Male and female mice were kept separately for at least 1 wk before experimental use. Four female mice were caged together. Thus, all females used were believed to be in anestrous with low endogenous levels of estrogen, although confirmatory vaginal smears or E_2 measurements were not performed. Before each experiment, all mice were deprived of food and water for at least 1 h.

A murine CF colony, *cftr*^{m1UNC}, was established by mating animals heterozygous for the CFTR gene disruption ($CFTR^{+/-}$; Jackson Laboratories, Bar Harbor, ME). $CFTR^{+/+}$ mice were produced by mating heterozygous ($CFTR^{+/-}$) mice or $CFTR^{-/-}$ mice. Because $CFTR^{-/-}$ mice often die prematurely due to intestinal obstruction and perforation (32), an electrolyte solution containing polyethylene glycol 3350 (GoLYTELY; Braintree Laboratories, Braintree, MA) was administered in drinking water to all mice *ad libitum*. This solution has increased survival significantly in CF mice, without altering the histomorphological integrity of the intestine (33). This solution was also made available to littermates to maintain experimental consistency. Genotyping of CFTR mutant mouse progeny was analyzed by PCR according to a protocol from Jackson Laboratories. Briefly, approximately 1 cm tail clipped from each pup at weaning was digested with lysis buffer and proteinase K (DNeasy kit; QIAGEN, Inc., Valencia, CA) at 55 C overnight. DNA was extracted using DNeasy Tissue kit according to the manufacturer's instructions. PCR was performed in mixture of 1× PCR buffer, 2.5 mM MgCl, 1 μM 2-deoxynucleotide 5′-triphosphate, 5 U/μl *Taq* DNA polymerase, oligonucleotide primers, and 100 ng DNA as the template. PCR was executed in a DNA thermal cycler (Hybaid; Thermo Fisher Scientific Inc., Waltham, MA) for 35 cycles of 94 C, 50″, 56 C, 45″, and 72 C 45″. The PCR products were analyzed on 1.5% agarose gel and visualized under UV light. Wild-type genotype was reflected by a single band at 526 bp, heterozygous by two bands at 526 and 357 bp, and knockout by a single band at 357 bp. Only $CFTR^{+/+}$ and $CFTR^{-/-}$ female mice were used in the present study.

Ussing chamber experiments in vitro. Ussing chamber experiments were performed as previously described (34). After C57 black mice were anesthetized by halothane, the abdomen was opened with a midline incision. The proximal duodenum was removed and immediately placed in ice-cold mannitol and indomethacin (10 μM) solution to suppress trauma-induced prostaglandin release. The duodenal tissue from each animal was stripped of seromuscular layers, divided, and examined in three chambers (window area 0.1 cm^2). Experiments were performed under continuous short-circuited conditions (voltage-current clamp, VCC 600; Physiologic Instruments, San Diego, CA), and luminal pH was maintained at 7.40 by the continuous infusion of 5 mM HCl under the automatic control of a pH-stat system (ETS 822; Radiometer America, Westlake, OH). The volume of the titrant infused per unit time was used to quantitate HCO_3^- secretion. Measurements were recorded at 5-min intervals, and mean values for consecutive 5- or 10-min periods were averaged. The rate of luminal HCO_3^- secretion is expressed as micromoles per square centimeter per hour. The short-circuit current (I_{sc}) was measured in microamperes and converted into microequivalents per square centimeter per hour. After a 30-min period when basal

parameters were measured, inhibitor or control vehicle was added for another 30 min, as dictated by the figures, followed by addition of E_2 to both sides of the tissue. Electrophysiological parameters and HCO_3^- secretion were then recorded for 60 min. As shown in our previous studies (34) and in control experiments, addition of 10 μl vehicle [dimethylsulfoxide (DMSO) or distilled water] to both sides of the duodenal tissue in 3-ml chambers did not change I_{sc} and HCO_3^- secretion, which were sustained during the 90-min experimental period (data not shown).

Acid-stimulated duodenal HCO_3^- secretion in vivo. *In vivo* experiments were performed with a well-validated technique, as described previously (7), using C57 black male and female mice. Mice were anesthetized by ip injection of hypnorm-midazolam cocktail (25% Hypnorm, 25% midazolam, and 50% distilled water). After anesthetization the abdomen was opened, and the duodenum was accessed through two small incisions: one just below the rib cage on the left side and the other just below the sternum. Through the first incision, the stomach was located; a tiny incision was made just proximal to the pyloric sphincter. Through this incision a soft polyethylene catheter was inserted into the stomach, gently pushed through the pyloric sphincter, and tied firmly into position with silk suture thread around the outside of the pyloric sphincter, isolating the proximal duodenum (5–10 mm) from the stomach. Through the incision below the sternum, the junction of the pancreatic duct and the duodenum was located. A tiny incision was made in the duodenum, and a second polyethylene catheter was inserted and tied into place just proximal to the junction with the pancreatic duct but distal to the duodenal blood supply. Thus, the pancreatic contributions were occluded from the isolated duodenal segment, while the blood supply remained intact. Throughout the duration of the experiment, the duodenum maintained a healthy pink color and was kept moist within the abdominal cavity.

After surgery, the proximal duodenum was perfused with isotonic saline for 20 min. After this initial washout and recovery period, basal HCO_3^- secretion was measured for 20 min. The mouse was then given an ip injection of either tamoxifen (10 mg/kg) or control vehicle (DMSO), and then HCO_3^- secretion was measured for 6 min. The duodenal segment was then perfused with 10 mM HCl in isotonic saline for 5 min. After acidification, the segment was gently flushed to remove any residual acid and allowed a 5-min washout period. HCO_3^- secretion was then measured for an additional 42 min. In some experiments the mouse was given an ip injection of either tamoxifen (10 mg/kg) or control vehicle (DMSO), and HCO_3^- secretion was measured for 10 min. Afterwards, the duodenal segment was perfused with 100 nM E_2 in isotonic saline (140 mM NaCl) or Cl^--free solution (NaCl replaced with Na-glucose), or the mouse was given an ip injection of E_2, a selective ERα agonist PPT, or a selective ERβ agonist DPN, and then HCO_3^- secretion was measured for an additional 50 min. After each experiment the length of the duodenal segment tested was measured *in situ* to the nearest millimeter. As shown previously (8), animals could be sustained under these experimental conditions for more than 2 h. Sample volumes were measured by weight to the nearest 0.01 mg. The amount of HCO_3^- in the effluents was quantitated through the use of a CO_2^- sensitive electrode (Thermo Orion, Beverly, MA). The electrode was calibrated before each day's use by constructing a semilogarithmic standard curve using known HCO_3^- concentrations. HCO_3^- outputs were determined for 6-min periods and expressed as micromoles per square centimeter per hour. Stimulated HCO_3^- outputs are presented as HCO_3^- output over time and as net HCO_3^- output (peak minus average basal output). A total of 122 C57 black mice and 15 $CFTR^{+/+}$ and $CFTR^{-/-}$ mice was used for this study.

SCBN cell culture. SCBN is a duodenal epithelial cell line of canine origin. It grows as polarized confluent monolayers and expresses calcium-dependent chloride secretion (35, 36). SCBN cells were grown as previously described (35, 36). Briefly, cells of passages 23–33 were grown to confluence (~5 d) in 75 cm^2 flasks. Cells were fed with fresh DMEM supplemented with 10% fetal bovine serum, L-glutamine, and streptomycin every 2–3 d. After the cells had grown to confluence, they were replated onto 12-mm round coverslips (Warner Instruments Inc., Hamden, CT) and incubated for at least 24 h before use.

Measurement of $[Ca^{2+}]_{cyt}$ in SCBN cells by digital Ca^{2+} imaging. $[Ca^{2+}]_{cyt}$ levels in SCBN cells were measured by Fura-2 fluorescence ratio digital imaging as described previously (37). Briefly, SCBN cells, grown on coverslips, were loaded with 5 μM Fura 2-AM, dissolved in 0.01% Pluronic F-127 plus 0.1% DMSO in physiological salt solution described above, in the dark at room temperature (22–24 C) for 50 min, then washed in a physiological salt solution for at least 30 min to remove extracellular dye and allow intracellular esterase to cleave cytoplasmic Fura 2-AM into active Fura 2. Thereafter, the coverslips with SCBN cells were mounted in a perfusion chamber on a Nikon microscope stage (Nikon Corp., Tokyo, Japan). Cells were initially perfused with a physiological salt solution for 5 min, then perfused with the same solution with or without Ca^{2+} containing E_2 or E_2-BSA (10 nM). The ratio of Fura-2 fluorescence with excitation at 340 or 380 nm ($F_{340/380}$) was followed over time and captured using an intensified CCD camera (ICCD200) and a MetaFluor Imaging System (Universal Imaging, Corp., Downingtown, PA).

RT-PCR analysis. Total RNA from C57 black mouse duodenal mucosae of both genders was isolated with TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Total RNA samples were resuspended in water and quantified by $OD_{260/280}$. Five micrograms of total RNA were reverse transcribed into cDNA. After inactivation at 70 C for 10 min, 1 μl of the reverse transcribed reaction mixture (20 μl) was used as the template for PCR containing 0.2 mM 2-deoxynucleotide 5′-triphosphate, 3 mM $MgCl_2$, 500 mM KCl, 20 mM Tris·HCl (pH 8.0), 0.2 μM oligonucleotide primers as shown below, and 1 U *Taq* polymerase (Invitrogen). Primers were synthesized by Integrated DNA Technologies (Coralville, IA). Specific sense and antisense primers for mouse ERα (GenBank accession no. NM007956) were 5′-AAGGGCAGTCACAATGAACC-3′ and 5′-GCCAGGTCATTCTCCACATT-3′. The predicted size of the PCR-amplified product for ERα was 155 bp. Specific sense and antisense primers for mouse ERβ (GenBank accession no. NM207707) were 5′-GAAGCTGGCTGACAAG GAAC-3′ and 5′-AACGAGGTCTGGAGCAAAGA-3′. The predicted size of the PCR-amplified product for ERβ was 187 bp. Mouse glyceraldehyde-3-phosphate dehydrogenase sense and antisense primers were 5′-ACCACAGTCCATGCC ATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ (38). The samples were amplified in an automated thermal cycler (GeneAmp 2400; Applied Biosystems, Foster City, CA). DNA amplification conditions included an initial 3-min denaturation step at 94 C, 35 cycles of 30 sec at 94 C, 30 sec at 57 C, 40 sec at 72 C, and a final elongation step of 10 min at 72 C. The products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and then photographed under UV light. To confirm band identity, the RT-PCR products were also subjected to restriction enzyme analysis.

Western blot analysis. Duodenal tissues from C57 black mice were isolated and stripped to obtain mucosal layers. The mucosae, SCBN cells were immediately homogenized (PowerGen 125; Fisher Scientific) in lysis buffer [10 mM Tris·HCl (pH 7.8), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM sodium orthovanadate, 0.1% sodium dodecyl sulfate, 1 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonylfluoride] at 30,000 rpm on ice. The lysates were then centrifuged at 12,000 rpm for 10 min at 4 C to remove insoluble materials. Protein content of the supernatants was measured by a Bio-Rad procedure (Bio-Rad Laboratories, Inc., Hercules, CA) using BSA as the standard. The protein extracts were mixed with sodium dodecyl sulfate sample buffer and boiled for 5 min. The protein extracts were separated by SDS-PAGE (10%) and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). ERα and ERβ protein expression was detected by immunoblotting with polyclonal antibodies specific against ERα or ERβ as described previously (39). Protein extracts of uterine artery endothelial cells (39, 40) and recombinant human ERα and ERβ proteins (Affinity BioReagents) were used as positive controls.

Statistical analysis. Each experiment was performed at least five times using different animals. Results are expressed as means ± SE. Differences between means were considered to be statistically significant at $P < 0.05$, using the Student's *t* test for paired or unpaired values or ANOVA, as appropriate.

Results

Basal and acid-stimulated DMBSs in vivo are higher in female than male mice

As stated previously the gender differences in the prevalence in duodenal ulcer have long been observed in humans (9–13); however, the causes of these gender differences are currently destitute. Because DMBS is a major defense mechanism for acid-induced duodenal injury (4), we hypothesized that DMBS might contribute to the observed gender differences in duodenal ulcer. To this end, we assessed basal DMBS in female and age-matched male mature Harlan C-57 black mice and their DMBS in response to a physiological stimulant acid. The basal HCO_3^- output was 3.2 ± 0.3 μmol/cm·h in female mice (n = 8) *vs.* 2.1 ± 0.4 μmol/cm·h in age-matched male mice (n = 5; $P < 0.05$). As shown in Fig. 1A, in a control experiment, duodenal luminal perfusion of saline (no HCl) in female mice did not alter their basal DMBS, which kept consistent for at least 1 h. However, duodenal luminal perfusion of 10 mM HCl resulted in a robust DMBS increase in the mice of both genders, which rapidly increased and maximized at approximately 30 min after HCl stimulation. Afterwards, the acid-stimulated DMBS gradually declined. The time course of acid-stimulated DMBS was similar in female and age-matched male mice; however, the magnitude of acid-stimulated DMBS in female and male mice was significantly different (Fig. 1A). The net peak HCO_3^- secretion, calculated from the difference between the baseline and the peak value at 30 min, was used to represent acid-stimulated HCO_3^- secretion (Fig. 1B). Acid-stimulated DMBS was significantly higher in female than age-matched male mice (net peak DMBS: 20.3 ± 0.5 *vs.* 8.4 ± 1.5 μmol/cm·h; $P < 0.001$; n = 5 for both genders). The male to female ratio of net peak murine DMBS was 1:2.4, which is inversely correlated with the male to female ratio of incidence of human duodenal ulcer (2.2:1) (10, 12). Therefore, these data implicate that the gender difference in acid-stimulated DMBS may contribute to the clinically observed gender difference in the incidence of duodenal ulcer.

Acid-stimulated DMBS in vivo can be inhibited by tamoxifen and ICI 182, 780

To study whether ERs are involved in the gender associated acid-stimulated DMBS, a selective ER modulator (SERM) tamoxifen was tested in the first set of experiments. As shown in Fig. 2A, administration of tamoxifen (10 mg/kg, ip) significantly attenuated acid-stimulated DMBS in mice of both genders. Tamoxifen inhibited the acid-stimulated net peak HCO_3^- secretion by 80% in female and male mice (Fig. 2B). Because tamoxifen is not a complete ER antagonist that has agonistic action in some tissues (such as the uterus), a near complete ER antagonist ICI 182,780 was tested in another set of experiments. As shown in Fig. 2B, ICI 182,780 (3 mg/kg, ip) also significantly attenuated acid-stimulated DMBS in mice of both genders. ICI 182,780 inhibited the net peak HCO_3^- secretion by 98% in female mice and by 50% in male mice (Fig. 2B). The blockade of acid-stimulated DMBS by ER antagonists suggests that estrogen and ERs may play a direct role in murine DMBS.

E_2 stimulates DMBS in vivo, which is inhibited by tamoxifen and possibly mediated by both ERα and ERβ

We then tested the effects of estrogenic compounds on DMBS *in vivo*. In one set of experiments, the effect of the natural ER agonist, E_2, on DMBS was tested. As shown in Fig. 3, A and B, E_2 (1–10 mg/kg, ip) dose dependently stimulated DMBS, which was also significantly attenuated by tamoxifen (10 mg/kg, ip). In control experiments the same amount of vehicle (DMSO, ip) did not significantly alter DMBS (Fig. 3A). The biological actions of estrogen are largely mediated by two subtypes of ER, ERα and ERβ. To differentiate which subtype(s) is involved in estrogen stimulation of DMBS, the effects of selective ERα and ERβ agonists on DMBS were tested. PPT and DPN have been developed as highly selective ERα and ERβ agonists, respectively. A bolus injection of either PPT or DPN (1 mg/kg, ip) significantly stimulated DMBS with similar efficacies (Fig. 3B). Therefore, highly selective ERα and ERβ agonists are also stimulators of DMBS *in vivo*.

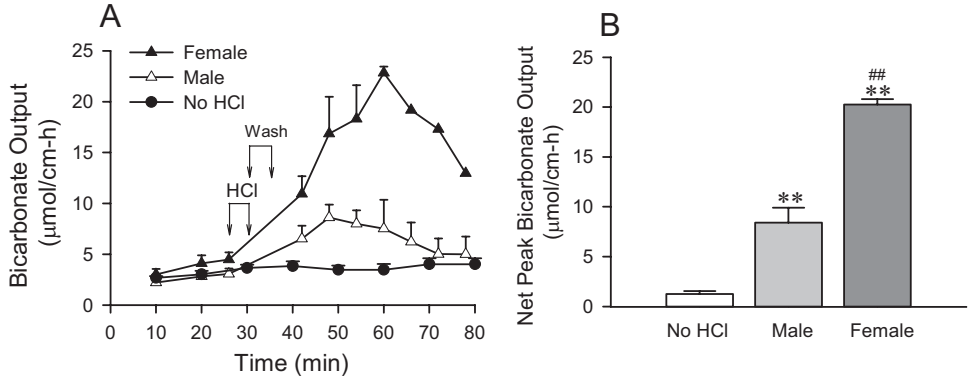

FIG. 1. Gender differences in acid-stimulated murine DMBS *in vivo*. A, Time course of acid-stimulated DMBS in female and male C57 mice. After luminally perfused with saline for 30 min as baseline, the murine duodenum was perfused with 10 mM HCl for 5 min in female and male mice, or perfused with saline in female mice as control (no HCl). B, Acid-stimulated net peak murine DMBS calculated from the difference between baseline and the peak value at 30 min after stimulation. Values are expressed as means ± SE for five experiments. **, $P < 0.01$ *vs.* the negative control (no HCl). ##, $P < 0.01$ *vs.* male mice. Two-way ANOVA and one-way ANOVA were used for A and B, respectively.

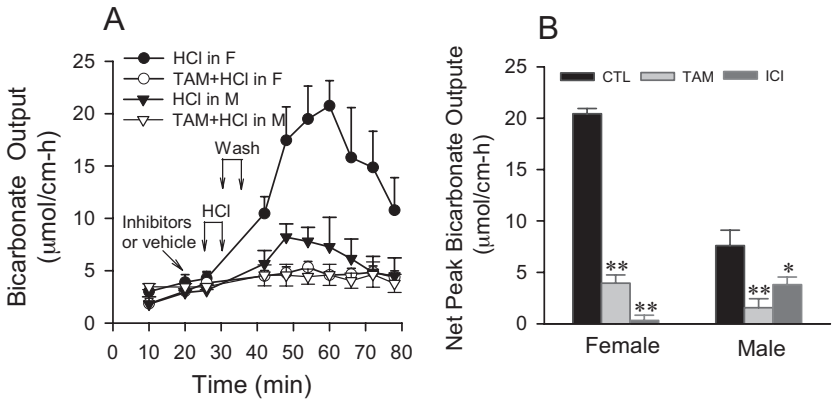

FIG. 2. Inhibition of acid-stimulated murine DMBS by ER antagonists *in vivo*. A, Time course of acid-stimulated DMBS in the presence or the absence of ER antagonists in female (F) and male (M) C57 mice. After pretreatment with tamoxifen (TAM) (10 mg/kg, ip), ICI182,780 (ICI) (3 mg/kg, ip), or vehicle (CTL) (DMSO) for 5 min in female and male mice, the murine duodenum was luminally perfused with 10 mM HCl for another 5 min. B, Acid-stimulated net peak murine DMBS calculated from the difference between baseline and the peak value at 30 min after stimulation. Values are expressed as means ± SE for five to six experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* the control in the absence of ER antagonists. Two-way ANOVA and one-way ANOVA were used for A and B, respectively.

E_2, but not progesterone, stimulates DMBS in vitro

In the aforementioned studies, we injected higher doses of estrogenic compounds (E_2, PPT, and DPN) into mice, which might be physiologically irrelevant. Therefore, we further tested whether these findings with pharmacological doses of E_2 can be reproduced with a relative low dose of E_2 via duodenal luminal perfusion. As shown in Fig. 4, A and B, in the control experiment, duodenal perfusion of saline in female mice slightly increased the basal DMBS; however, luminal perfusion of a lower dose of E_2 (100 nM) significantly increased DMBS in female mice, and thereby the net peak DMBS induced by E_2 was higher than that induced by saline (2.6 ± 0.5 μmol/cm·h for E_2 *vs.* 1.5 ± 0.4 μmol/cm·h for saline, n = 5; $P < 0.01$). Thus, these findings support the notion that E_2 may be a physiological stimulator or modulator of DMBS.

Besides E_2, progesterone is another important sex hormone in the female body. Thus, we also tested whether progesterone is involved in the regulation of DMBS. As Fig. 4B illustrates, duodenal perfusion of progesterone (1 μM) in female mice did not significantly stimulate DMBS (0.9 ± 0.6 μmol/cm·h for progesterone *vs.* 1.4 ± 0.1 μmol/cm·h for saline, n = 5; $P > 0.05$). In addition, administration of progesterone (1 μM) to both apical and basolateral sides of the female duodenal tissues in Ussing chambers did not significantly stimulate DMBS (n = 5, data not shown). Therefore, progesterone appears not to be a causative factor for the gender-associated DMBS, which also suggests the specific action of E_2 in this physiological process.

Cl^-/HCO_3^- anion exchanger in E_2-stimulated HCO_3^- secretion

After demonstrating E_2 specific stimulation of DMDS, we further investigated the mechanisms underlying this physiological process. Because Cl^-/HCO_3^- anion exchanger expressed on the apical membrane of duodenal epithelium likely plays an important role in secretagogue-stimulated DMBS (4, 41), we tested whether it is involved in E_2-stimulated HCO_3^- secretion. To this end, in the first set of experiments, the duodenal lumen was perfused with DIDS (100 μM), an inhibitor of Cl^-/HCO_3^- anion exchanger, which itself did not significantly affect basal DMBS (Fig. 4A). After

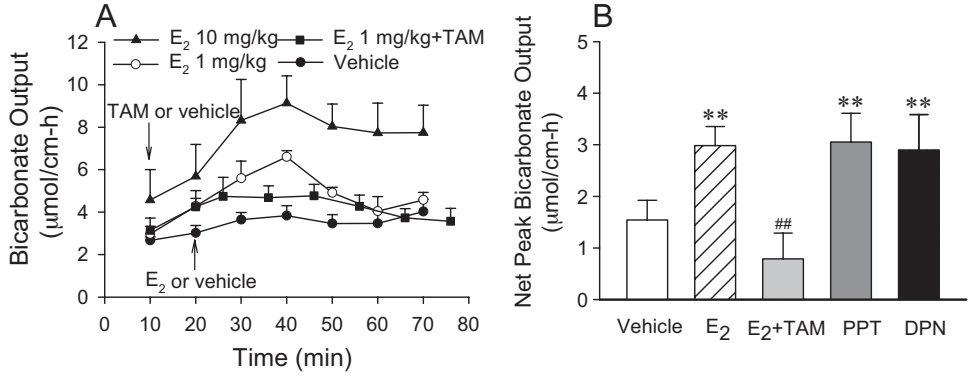

FIG. 3. Effects of ER agonists and antagonists on murine DMBS in female C57 mice *in vivo*. A, Time course of DMBS stimulated by vehicle (DMSO, ip), different doses of E_2 (1–10 mg/kg, ip), or inhibition of E_2-stimulated DMBS by tamoxifen (TAM) (10 mg/kg, ip). B, Stimulation of net peak murine DMBS calculated from the difference between baseline and the peak value by vehicle, E_2, PPT, or DPN (1 mg/kg, ip for all drugs), or inhibition of net peak DMBS by tamoxifen before E_2. Values are expressed as means ± SE for five to six experiments. **, $P < 0.01$ *vs.* vehicle. ##, $P < 0.01$ *vs.* E_2. Two-way ANOVA and one-way ANOVA were used for A and B, respectively.

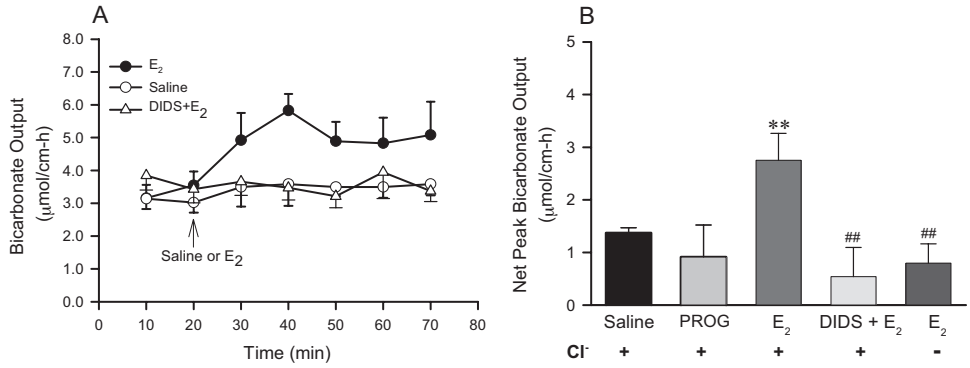

FIG. 4. Effects of E_2 or progesterone luminal perfusion on murine DMBS in female C57 mice *in vivo*. A,. Time course of DMBS after duodenal luminal perfusion of E_2 (100 nM), DIDS (100 μM) plus E_2, or saline as a control. After anesthetization the female mouse abdomen was opened, and the proximal duodenum was isolated. E_2, DIDS plus E_2, or saline was administered by duodenal luminal perfusion as indicated. B, Net peak murine DMBS calculated from the difference between baseline and the peak value by saline, progesterone (PROG) (1 μM), E_2 (100 nM), DIDS (100 μM) plus E_2 in the presence or the absence of Cl^-. Values are expressed as means ± SE for five to eight experiments. **, $P < 0.01$ *vs.* saline. ##, $P < 0.01$ *vs.* E_2 alone in the presence of Cl^-. Two-way ANOVA and one-way ANOVA were used for A and B, respectively.

10 min the duodenal lumen was perfused with DIDS plus E_2 (100 nM) in saline. As shown in Fig. 4, A and B, perfusion of DIDS significantly inhibited E_2-stimulated HCO_3^- secretion (2.8 ± 0.5 μmol/cm·h for E_2 alone *vs.* 0.5 ± 0.5 μmol/cm·h for E_2 plus DIDS, n = 5 for both groups; $P < 0.01$). In the second set of experiments, E_2-stimulated HCO_3^- secretion was tested in Cl^--free solutions in which NaCl was replaced by Na-gluconate. As Fig. 4B illustrates, after Cl^- was removed from the apical side of epithelial cells to inactivate the Cl^-/HCO_3^- anion exchanger, luminal perfusion of E_2 could not induce significant HCO_3^- secretion (2.8 ± 0.5 μmol/cm·h for Cl^--containing solutions *vs.* 0.8 ± 0.4 μmol/cm·h for Cl^--free solutions, n = 5 for both groups; $P < 0.01$). Together, these data suggest that apical Cl^-/HCO_3^- anion exchanger plays a role in E_2-stimulated HCO_3^- secretion.

CFTR in E_2-stimulated HCO_3^- secretion in vivo

Because CFTR channels are essential for DMBS stimulated acid and most secretagogues (6–8), we further tested whether CFTR channels are required for the E_2-stimulated DMBS. $CFTR^{+/+}$ and $CFTR^{-/-}$ female mice were used to compare systematically the E_2-stimulated DMBS. As shown in Fig. 5, A and B, E_2 (1 mg/kg, ip) significantly stimulated DMBS in $CFTR^{+/+}$ female mice but failed to do so in $CFTR^{-/-}$ female mice (3.8 ± 0.4 μmol/cm·h for $CFTR^{+/+}$ mice *vs.* 0.7 ± 0.2 μmol/cm·h for $CFTR^{-/-}$ mice, n = 5; $P < 0.001$). Administration of the same amount of vehicle (0.2 ml saline, ip) did not significantly alter basal DMBS in $CFTR^{+/+}$ female mice (Fig. 5, A and B). Thus, these data show that CFTR channels in the duodenal epithelium are essential for E_2 stimulation of DMBS.

E_2 specifically stimulates DMBS without altering duodenal I_{sc} in vitro

In Ussing chamber assay, net ion transport across the duodenal mucosa (a combination of Cl^- and HCO_3^- secretion) was assessed further as I_{sc} and HCO_3^- secretion was determined by pH-stat. As shown in Fig. 6A, E_2 dose dependently (10–100 nM) and rapidly (within 5 min) evoked HCO_3^- secretion from the duodenal mucosa in female mice. Tamoxifen (10 μM) alone did not affect basal DMBS; however, it significantly inhibited E_2-induced net peak DMBS (Fig. 6B). These data suggest that tamoxifen acts as an ER antagonist in the duodenum. In the duodenal mucosa, most secretagogues stimulate DMBS and I_{sc} simultaneously (42–44); however, interestingly, as shown in Fig. 7A, even at the

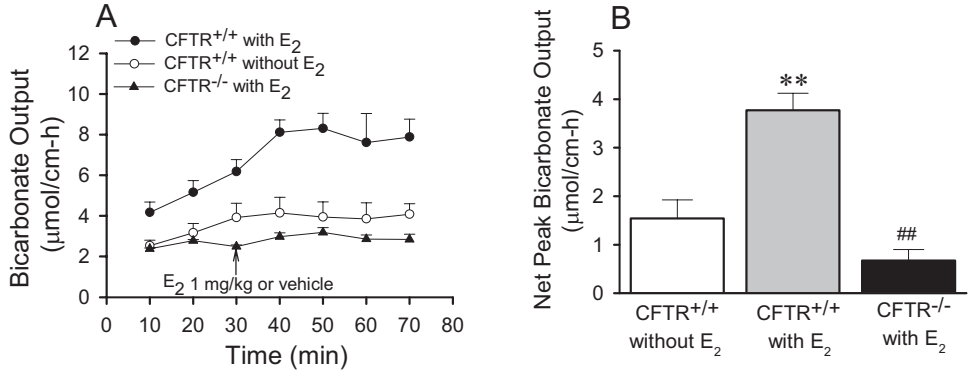

FIG. 5. E_2 stimulation of murine DMBS in CFTR wild-type ($CFTR^{+/+}$) but failure in CFTR knockout ($CFTR^{-/-}$) female mice *in vivo*. A, Time course of DMBS stimulated by vehicle or E_2 (1 mg/kg, ip) in $CFTR^{+/+}$ or $CFTR^{-/-}$ female mice. B, Vehicle- or E_2-stimulated net peak murine DMBS calculated from the difference between baseline and the peak value in $CFTR^{+/+}$ or $CFTR^{-/-}$ female mice. Values are expressed as means ± SE for five experiments. **, $P < 0.01$ *vs.* vehicle. ##, $P < 0.01$ *vs.* $CFTR^{+/+}$ mice. Two-way ANOVA and one-way ANOVA were used for A and B, respectively.

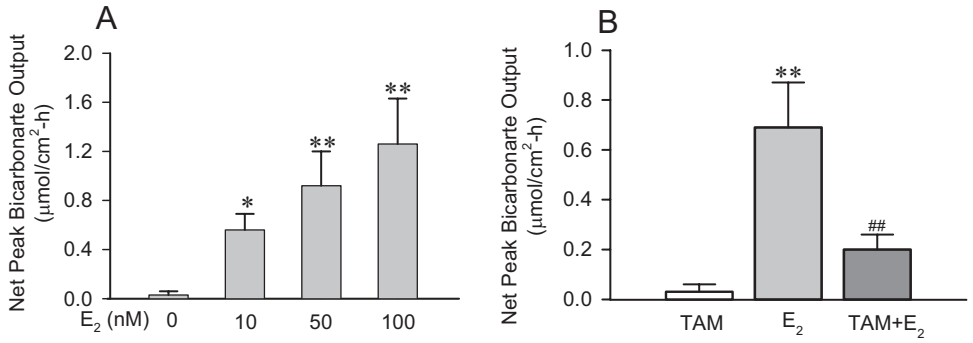

FIG. 6. E_2 concentration-dependent stimulation and inhibition by tamoxifen of murine DMBS from duodenal tissues in female C57 mice *in vitro*. A, Net peak murine DMBS calculated from the difference between baseline and the peak value was stimulated by different concentration of E_2 (10–100 nM), which was measured by pH-stat method in Ussing chambers. B, Inhibition of E_2 (10 nM)-stimulated net peak DMBS by tamoxifen (TAM) (10 μM). Values are expressed as means ± SE for six to seven experiments. In A, *, $P < 0.05$; **, $P < 0.01$ *vs.* control without E_2. In B, **, $P < 0.01$ *vs.* tamoxifen alone. ##, $P < 0.01$ *vs.* E_2 alone. One-way ANOVA was used for A and B.

highest concentration of 100 nM, E_2 did not alter murine duodenal I_{sc}, a well-recognized index of the intestinal Cl^- secretion (45, 46). Thus, E_2 may specifically stimulate DMBS via an ER-dependent pathway without altering duodenal Cl^- secretion. To investigate further whether the DMBS response to E_2 differs in genders, we systematically compared E_2-induced HCO_3^- secretion from male to that of female murine duodenal tissues in Ussing chambers. As shown in Fig. 7B, E_2 (100 nM) stimulated HCO_3^- secretion from duodenal tissues of both female and male mice but with different magnitudes according to gender. E_2-induced net peak HCO_3^- secretion was significantly higher in female than in male duodenal tissues (1.3 ± 0.4 *vs.* 0.3 ± 0.1 μmol/cm²·h, n = 6; $P < 0.01$). The female to male ratio in E_2-stimulated DMBS was 4.3. Therefore, E_2-stimulated DMBS was more potent in female than male mice and the aforementioned observed gender differences of DMBS *in vivo* were confirmed using the *in vitro* system.

E_2 stimulates DMBS without changing duodenal transepithelial resistance (TER)

Bicarbonate secretion may be resulted from transepithelial and/or paracellular pathways (3, 4). To study if E_2 induces transepithelial and/or paracellular HCO_3^- secretion, we measured the basal TER of mouse duodenal mucosa and tested the effect of E_2 on TER. As shown in Fig. 8A, the basal TER of mouse duodenal mucosa was stable in the period of 30-min measurement, at which time point the average basal TER was 26.9 ± 1.8 Ω.cm² (n = 20). This basal TER is close to that reported by Tuo *et al.* (47) in the mouse duodenal mucosa. Addition of E_2 (100 nM) to Ussing chambers did not alter basal TER (Fig. 8A). As shown in Fig. 8B, the average TER after addition of E_2 for 30 min was 26.2 ± 2.1 Ω.cm² (n = 20; $P > 0.05$ *vs.* the absence of E_2), suggesting that E_2 specifically induces transepithelial HCO_3^- secretion rather than paracellular HCO_3^- secretion by changing TER.

Ca^{2+} signaling in E_2-stimulated DMBS

$[Ca^{2+}]_{cyt}$ plays a pivotal role in regulating intestinal epithelial ion transports (42, 48, 49). We then asked whether $[Ca^{2+}]_{cyt}$ is involved in E_2 stimulation of DMBS. First, we used digital Ca^{2+} imaging to assess the ability of E_2 to mobilize Ca^{2+} in SCBN cells, a well-characterized nontransformed duodenal epithelial crypt cell line (34). As illustrated in Fig. 9, E_2 at 10 nM significantly increased $[Ca^{2+}]_{cyt}$ within 30 sec. E_2 induced a transient increase in $[Ca^{2+}]_{cyt}$ in Ca^{2+}-free solutions (Fig. 9A), but a sustained increase in $[Ca^{2+}]_{cyt}$ in Ca^{2+}-containing solutions (Fig. 9B). In comparison, progesterone at 100 nM did not alter $[Ca^{2+}]_{cyt}$ in either Ca^{2+}-free or Ca^{2+}-containing solutions (data not shown). These data suggest that E_2, but not progesterone, may induce both Ca^{2+} release from the intracellular Ca^{2+} store and extracellular Ca^{2+} influx in duodenal epithelial cells.

Subsequently, we tested if Ca^{2+} signaling is involved in

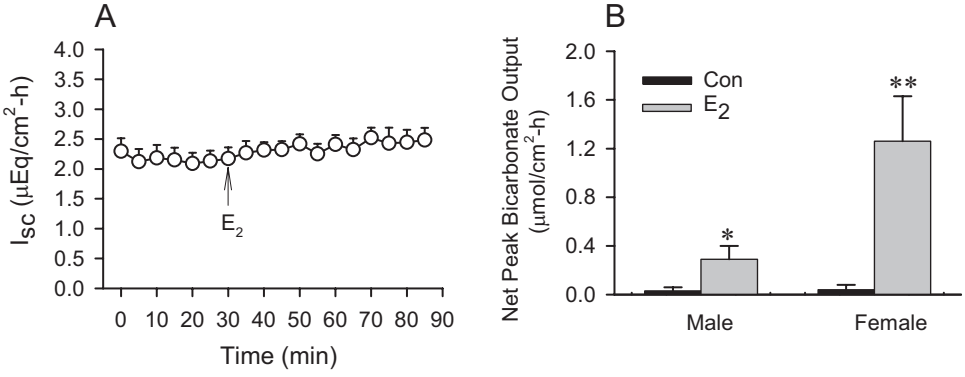

FIG. 7. Effects of E_2 on duodenal (I_{sc} and gender difference in E_2 stimulation of murine DMBS *in vitro*. A, Time course of duodenal I_{sc} in female C57 mice after addition of E_2 (100 nM) to Ussing chambers as indicated. B. Gender-dependent DMBS stimulated by E_2 (100 nM) from duodenal tissues in male *vs.* female C57 mice. Values are expressed as means ± SE for nine experiments in A and five to six experiments in B. *, $P < 0.05$; **, $P < 0.01$ *vs.* control vehicle. One-way ANOVA and the Student's *t* test were used for A and B, respectively. Con, Control.

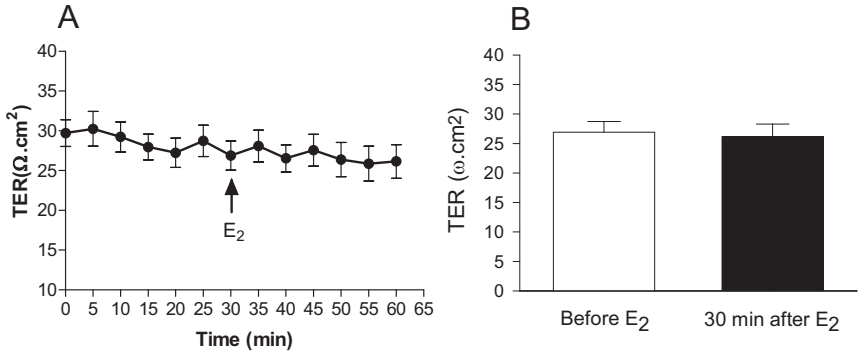

FIG. 8. Effects of E_2 on TER in murine duodenal mucosae from female C57 black mice. A, Time course of TER in murine duodenal mucosae before or after addition of E_2 to Ussing chambers as indicated. After TER was measured for 30 min as a baseline, E_2 (100 nM) was added, and then TER was measured for an additional 30 min. B, TER of murine duodenal mucosae before addition of E_2 or 30 min after addition of E_2. Values are expressed as means ± SE for 20 experiments. There is no statistically significant difference between them. One-way ANOVA and the Student's *t* test were used for A and B, respectively.

E_2-stimulated murine DMBS *in vivo*. As Fig. 9C illustrates, in the control experiment, luminal perfusion of E_2 (100 nM) significantly increased DMBS in female C-57 black mice. However, after duodenal lumen was pretreated with BAPTA-AM (100 μM) for 10 min, a cell-permeable Ca^{2+} chelator, E_2 failed to induce DMBS (net peak DMBS: 3.0 ± 0.8 μmol/cm·h for E_2 alone *vs.* 0.4 ± 0.4 μmol/cm·h for BAPTA-AM plus E_2, n = 5; $P < 0.01$). BAPTA-AM did not significantly alter basal DMBS. Thus, these findings indicate that $[Ca^{2+}]_{cyt}$ plays an important role in E_2-stimulated DMBS (Fig. 9C).

mRNA and protein expressions of ERs in duodenal epithelial cells

Two specific ERs, ERα and ERβ, have been identified to date. Our foregoing functional experiments suggest that E_2 may cause biological effects via specific ERs in duodenal epithelial cells. To determine whether duodenum is a direct target for E_2 action, we then assessed expression of ERα and ERβ mRNAs and proteins in freshly isolated mouse duodenal epithelial cells and in SCBN cells. This was done using RT-PCR analysis to determine the expression of mRNAs and Western blotting for measuring proteins specific for the two subtypes of ERs in freshly isolated mouse duodenal epithelium. Figure 10A shows that transcripts for two subtypes of ERs, ERα and ERβ, were readily detected in C57 black mouse duodenal epithelium of both sexes. Apparently, no gender difference was found in the expression levels of ERα or ERβ mRNAs. By using Western blot analysis with antibodies as described in one of our recent studies (39), ERα protein was detected in duodenal epithelium isolated from both male and female mice, and in the SCBN duodenal epithelial cell line (Fig. 10B). The apparent molecular massof mouse duodenal ERα protein migrates on a SDS-PAGE similarly to that in the sheep uterine artery endothelial cells (38) and the recombinant human ERα (~67 kDa). ERβ protein was also detected in all the samples and migrated on SDS-PAGE similarly to that in the sheep uterine artery endothelial cells with an apparent molecular mass of approximately 56 kDa (39), whereas the approximate 59-kDa recombinant human ERβ migrated slower on the SDS-PAGE than the native cellular ERβ. Moreover, the levels of ERβ protein were lower than that of ERα in both genders. No gender difference existed in the expression levels of ERα or ERβ protein in the mouse duodenal epithelium. In addition, both ERα and ERβ proteins were detectable in the SCBN cell line. Interestingly, the immortalized SCBN cells also expressed high levels of ERβ that only expressed at a very low level in freshly isolated mouse duodenal epithelial cells, presumably due to up-regulation of ERβ during the development of the cell line.

Discussion

Although the gender differences in duodenal ulcer have been clinically observed for many years, little is known about

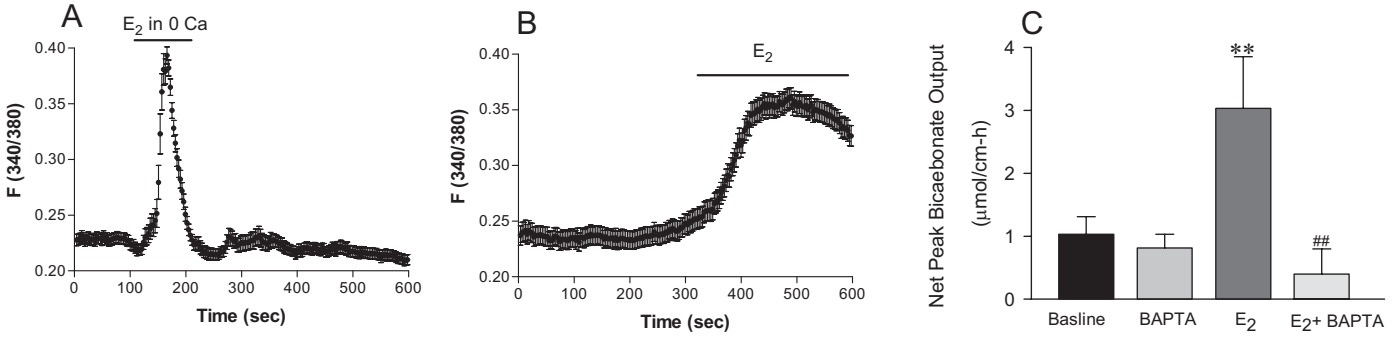

FIG. 9. Effects of E_2 on $[Ca^{2+}]_{cyt}$ in SCBN cells and role of $[Ca^{2+}]_{cyt}$ in E_2-stimulated DMBS. After loaded with 5 μM Fura 2-AM, coverslips with SCBN cells were mounted on a Nikon microscope stage. $[Ca^{2+}]_{cyt}$ was measured using cell digital Ca^{2+} imaging during the perfusion of solutions containing different components. E_2 (10 nM) induced transient $[Ca^{2+}]_{cyt}$ signaling in Ca^{2+}-free solutions (A), but increased sustained $[Ca^{2+}]_{cyt}$ signaling in Ca^{2+}-containing solutions (B). Values are expressed as means ± SEM for 40–50 cells in each condition. C, Effect of BAPTA-AM (BAPTA) (100 μM) on DMBS induced by luminal perfusion of E_2 (100 nM) in female C57 mice. Values are expressed as means ± SE for five experiments. **, $P < 0.01$ *vs.* baseline. ##, $P < 0.01$ *vs.* E_2. One-way ANOVA was used for C.

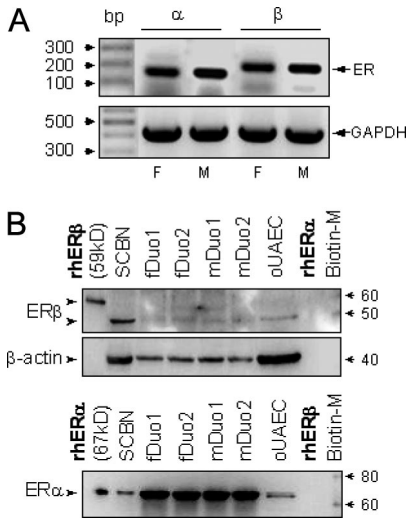

FIG. 10. Expression of ERs (ERα and ERβ) in duodenal epithelial cells. A, ERα and ERβ mRNA expression in freshly isolated duodenal mucosae derived from male (M) and female (F) mice. RT-PCR was performed to determine the expression of ER mRNA. Five micrograms of total RNA isolated from C57 black mouse duodenal epithelium of both sexes were used in each reaction. DNA ladder (bp) was indicated on the *left*. The predicated product sizes of ERα and β are 155 and 187 bp, respectively. These data are representative of three experiments conducted on different male and female C57 black mice with identical results. B, Western blot analysis was performed to determine the expression of ER proteins in extracts of SCBN cells (20 μg), freshly isolated duodenal epithelial cells (100 μg) of female (fDuo) and male (mDuo) mice, and uterine artery endothelial cells (UAEC) (40 μg) as a positive control (38). Recombinant human (rh) ERα and ERβ proteins were also used as controls for cross-reactivity of the ERα and ERβ antibodies. Anti-ERα and -ERβ antibodies recognized two proteins with molecular masses of approximately 67 and 56 kDa, respectively, corresponding to the native ERα and ERβ proteins. β-Actin was measured to monitor the protein loading in the mouse duodenal cells. Protein markers (kDa) are indicated on the *right*. Images shown represent one of three similar experiments. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

the causes and underlying mechanisms (9–13). According to our current knowledge on the pathogenesis of duodenal ulcer, three possibilities might exist. First, gastric acid secretion may differ in males and females. If males secreted more gastric acid than females, then the incidence of duodenal ulcer would be higher in males than females. However, this is very unlikely because previous studies have shown that gastric acid secretion is approximately the same in male and female humans and rodents (50, 51). Second, the prevalence of *H. pylori* infection may differ in males and females. However, previous studies again have excluded *H. pylori* infection to be a deciding factor for the observed gender differences in duodenal ulcer because: 1) the male to female ratio of the prevalence of duodenal ulcer is similar in *H. pylori* positive as well as *H. pylori* negative patients (1.7:1 for both categories) in Europe (52); and 2) no gender differences in prevalence of *H. pylori* infection were found in Asian patients with peptic ulcers (13, 53). Third, DMBS may differ in males and females. In this study we showed that basal and acid-stimulated DMBS was 1.5- to 2.4-fold higher in female than male mice. In a parallel study, we also observed that substantial greater basal and acid-stimulated DMBS in premenopausal women than age-matched men (Tuo, B., *et al.*, unpublished observations). In keeping with the fact that proximal DMBS is impaired in patients with duodenal ulcer and, thus, has been generally recognized as the predominant defending mechanism against the gastric acid-induced duodenal injury (1, 3, 54), our current findings clearly suggest that gender difference in DMBS is the most likely cause attributing to the gender differences in the prevalence in duodenal ulcer.

Our data showing significant greater basal and acid-stimulated DMBS in female than male mice suggest that DMBS is physiologically gender-related because basal DMBS is a physiological phenomenon, and acid is a physiological stimulus of DMBS (1, 3). In this study we found that E_2 at 1 and 10 mg/kg body weight stimulated DMBS in intact female and male mice *in vivo*. In a recent study, similar high concentration of E_2 (1 mg/kg) was reported to effectively attenuate stress-induced gastric mucosal injury by inhibiting decreases in gastric tissue levels of calcitonin gene-related peptide in ovariectomized rats (55). Although the doses of estrogen tested in gastrointestinal tract are pharmacological, our current Ussing chamber data may suggest that the effect of estrogen on DMBS is of physiological importance because in these studies as low as 10 nM E_2 stimulated DMBS *in vitro*.

When considering any gender-related responses, reproductive hormones mainly from the female ovary (*i.e.* estrogen and progesterone) and the male testis (*i.e.* testosterone) are often first taken into consideration. In the present study, we focused on the effects of female sex hormones on DMBS because in a recent cohort study (n = 55,336), we found no significant difference in the prevalence of duodenal ulcer among native Chinese men ranging 10–80 yr of age (13). These data implicate that hormones from the testis might not play a critical role in the gender difference of this disease. Our current study suggests that E_2 specifically stimulates DMBS based on series of experiments showing that:

1) E_2, in physiological concentration range (10 nM), increased $[Ca^{2+}]_{cyt}$ in duodenocytes (Fig. 9), and E_2 at 10–100 nM stimulated mouse DMBS *in vitro* using Ussing chamber assay (Figs. 5 and 6).
2) Our whole animal studies using pharmacological doses of E_2 also showed that E_2 stimulated DMBS, which was inhibited by an ER antagonist ICI 182,780 and a widely used SERM tamoxifen (Figs. 3 and 4).
3) E_2-stimulated DMBS was confirmed using two different techniques to measure HCO_3^-, *i.e.* pH-stat method *in vitro* (Figs. 5 and 6) and CO_2-sensitive electrodes *in vivo* (Figs. 1–4). Because the pH-stat method detects either H^+ loss or HCO_3^- increase, the former measurement may be confounded by epithelial H^+ secretion. However, the latter method with CO_2-sensitive electrodes measures a true HCO_3^- concentration (56).
4) E_2 specifically induced transepithelial HCO_3^- secretion without changing TER (Fig. 7) and duodenal Cl^- secretion (Fig. 6).
5) E_2 stimulated DMBS via Cl^-/HCO_3^- exchanger and CFTR channels (Figs. 4 and 8), which accords with our previous findings that a phytoestrogen, genistein, stimulated DMBS in CFTR wild-type mice only, but not in CFTR-null mice (57).
6) Both ERα and ERβ mRNAs and proteins were ex-

pressed in mouse duodenal epithelium and SCBN cell model (Fig. 10).

7) Progesterone could neither raise $[Ca^{2+}]_{cyt}$ in duodenocytes nor induce murine DMBS (Fig. 4), excluding a role of progesterone in DMBS that further supports the specificity of E_2 in the gender-associated DMBS.

Both ERα and ERβ have been identified in intestinal epithelium (23, 58–61). It has been suspected that ERβ might mediate the inhibitory effects of E_2 on active colonic Cl^- secretion (62, 63). However, very little is known about the expression and function of ERs in the duodenum. In the present study, we have detected expression of both ERα and ERβ mRNAs and proteins in murine duodenal epithelium and SCBN cells. The expression of both subtypes of ERs implicates that duodenal epithelium is a direct target of estrogen action. Obviously, an important question that needs to be addressed is which receptor subtype (ERα *vs.* ERβ) plays a dominant role in mediating estrogen stimulation of DMBS. In a previous study, Frasor *et al.* (64) generated the dose-response curves for uterine hypertrophic responses to PPT and DPN in immature mice. They showed that PPT at 100 μg/mice was uterotrophic, whereas DPN was not uterotrophic up to 500 μg/mice tested. We showed in this study that both ERα and ERβ agonists PPT and DPN were capable of stimulating similar DMBS responses in 2- to 3-month-old mature mice (body weight ~20–30 g). Although immature mice would have provided a potentially better model for addressing estrogen regulation of DMBS *in vivo*, mature mice were used by us and others because surgical procedures required for measuring mouse DMBS *in vivo* are not feasible in practice with immature mice. The doses of PPT and DPN tested in our current intact mouse studies were 1 mg/kg body weight. This dose is equivalent to approximately 20 μg/mice, which is at the low end of the uterotrophic response curve in immature mice (64). Thus, our data showing expression of ERα and ERβ mRNAs and proteins and stimulation of DMBS by E_2 and both ERα and ERβ selective agonists *in vivo* may suffice to draw a conclusion that ERα and ERβ play a comparably similar role in estrogen stimulation of DMBS. However, this conclusion may need additional experiments to solidify with ERα and ERβ knockout mouse models.

Of note is that in our *in vivo* studies, the dose of E_2 (1 mg/kg body weight) used is not comparable to its concentrations in any physiological conditions. Thus, we have demonstrated pharmacologically a stimulatory effect of estrogen on DMBS *in vivo*. Our *in vitro* Ussing chamber studies tested much lower doses of E_2 (10–100 nM) that may suggest a physiological role of estrogen in regulating DMBS. However, whether estrogen plays a physiological role in DMBS *in vivo* awaits further investigations to be established. It is speculating that DMBS may display cyclical changes *in vivo* during estrous/menstrual cycle as estrogen levels fluctuate. Thus, further experiments are warranted to investigate DMBS in different stages of the estrous/menstrual cycle and/or pregnancy, which will correlate endogenous estrogen levels with DMBS *in vivo*. In addition, administration of estrogen to ovariectomized animals is an important estrogen therapy model to verify the stimulatory effects of estrogen on DMBS *in vivo*.

Both genomic and nongenomic pathways have been ascribed for mediating estrogen action (65, 66). Genomic action is characterized by a latency of onset of more than 2 h, starting with the binding of estrogen to its intracellular receptors and then initiating transcription and *de novo* protein synthesis in the nucleus. In contrast, nongenomic action is rapid, with a latency of seconds to minutes in a variety of cells, by interacting with plasma membrane receptors to activate various intracellular second messengers (63, 67, 68). We have demonstrated that E_2 rapidly increased $[Ca^{2+}]_{cyt}$ in SCBN cells likely by evoking both Ca^{2+} release from the intracellular Ca^{2+} store and extracellular Ca^{2+} entry in duodenal epithelial cells. These experiments were performed with the SCBN cell model because: 1) the cell line is a well-characterized nontransformed duodenal epithelial crypt cell line (35); 2) it expresses functional CFTR channels and has been widely used in the study of Ca^{2+}-dependent Cl^- secretion (36, 69); 3) it secretes HCO_3^-, as demonstrated by us (unpublished observations) and others (34); and 4) it expresses both ERα and ERβ (Fig. 10). Our data obtained from SCBN cells are in agreement with a previous report (24) showing that E_2 can rapidly stimulate Ca^{2+} entry into rat duodenal enterocytes through phospholipase C-dependent mechanism involving store-operated Ca^{2+} channels. These effects are E_2 specific because they are not activated by other steroids such as progesterone or testosterone (24). All these findings, including our current data, suggest that $[Ca^{2+}]_{cyt}$ plays an important role in mediating membrane ER signaling in the duodenocytes. Elevated $[Ca^{2+}]_{cyt}$ mediates both acute and chronic cell activities, including gene expression (70). We showed that E_2-induced DMBS was relatively slow and lasted for at least 1 h. We speculate that both genomic and nongenomic mechanisms are involved in E_2-induced DMBS and potential duodenal protection. However, an in-depth understanding of the molecular mechanisms underlying estrogen regulation of DMBS and other responses in the duodenum awaits further investigation.

It is well known that an increase in $[Ca^{2+}]_{cyt}$ of epithelial cells could activate apical CFTR channels and Cl^-/HCO_3^- exchanger in pancreatic duct cells (71) and murine duodenal epithelium (37, 51), and inhibit ilea brush-border Na^+/H^+ exchanger (72–74). $[Ca^{2+}]_{cyt}$ has also increased basolateral Na^+-HCO_3^- cotransport (NBC) activity in murine colonic crypts (75) and activated basolateral intermediate Ca^{2+} activated K^+ channels (IK_{Ca}) in murine duodenal epithelium to provide a driving force for HCO_3^- secretion (34). All of these actions of $[Ca^{2+}]_{cyt}$ in epithelial cells may contribute to the molecular mechanisms underlying ER-Ca^{2+}-mediated DMBS observed in the present study. In this study our data demonstrate that ER-Ca^{2+}-CFTR and ER-Ca^{2+}-Cl^-/HCO_3^- exchanger are the major pathways mediating the E_2-stimulated DMBS. E_2 specific stimulation of DMBS without simultaneously altering duodenal I_{sc} is in agreement with our previous notion that different processes and/or reg-'ulatory mechanisms may exist in intestinal HCO_3^- and Cl^- secretion (43).

One of the most intriguing and unexpected findings in the present study is that SERMs largely blocked acid-stimulated

DMBS. These results strongly suggest that ERs are involved in acid-stimulated DMBS, but also raise an interesting question in ER biology as to how acid activates them. Although acid might directly activate ERs, we do not think this is likely at the moment because virtually nothing is available regarding this possibility. How estrogen and ERs are involved in the acid-stimulated DMBS need further study. However, our results suggest that ligand levels (*i.e.* estrogen concentration in the male or female body) may play a more critical role in the gender difference of basal and acid-stimulated DMBS than expression levels of duodenal ERs. This is because no gender difference was observed in the expression of duodenal epithelial ERs, and acid-stimulated DMBS were blocked by SERMs in both male and female mice. It is well known that luminal acid stimulates release of multiple biological factors from the duodenum, such as secretin, 5-HT, ACh, PGE_2, *etc.* (37, 43). Thus, acid may induce the release of endogenous estrogens from the bloodstream in the duodenum and then activate ERs on enterocytes indirectly.

It is noteworthy that E_2 stimulated more than 4-fold greater DMBS in female than male mice *in vitro*. This result suggests that the duodenum is more responsive to estrogen stimulation in females than males. However, the mechanism(s) underlying this gender difference is obscure. Similar expression levels of ER mRNAs and proteins in female and male duodenum suggest that mediators other than ERs are involved. We showed that $[Ca^{2+}]_{cyt}$, CFTR and Cl^-/HCO_3^- exchanger, are linked to estrogen stimulation of DMBS. As mentioned previously, estrogen may potentiate acid-stimulated DMBS via CFTR channels, which has been revealed in different systems previously (76, 77). Thus, one likely scenario for explaining gender difference in estrogen stimulation of DMBS is that duodenal CFTR and Cl^-/HCO_3^- exchanger, as well as channels responsive for mobilizing $[Ca^{2+}]_{cyt}$, including Na^+/Ca^{2+} exchanger (37, 42), may express at higher levels and/or possess greater responsiveness to estrogen stimulation in female than male mice. However, this idea awaits further investigation.

What is the physiological and clinical significance of the present study? Given the fact that estrogen is a hormone mainly present in the female body and, in comparison to men, women of reproductive age also experience a significant lower incidence in duodenal ulcer (9–12), our findings showing E_2 stimulation of DMBS may have offered a reasonable explanation for the clinically observed gender difference in duodenal ulcer. One may argue about the effective role of estrogen in protecting the duodenum against gastric acid-induced injury in the female because estrogen seems to elevate in women of reproductive age only during the menstrual cycle when ovarian follicles develop to preovulatory stage, whereas the development of duodenal ulcer seems to occur chronically. However, estrogen may also act chronically to protect the female duodenum due to the following reasons. First, higher basal DMBS in females than males is associated with constantly higher estrogen level in female than male, which is a chronically physiological phenomenon. A brief elevation of serum estrogen in females before ovulation during the menstrual cycle may function as a single exposure to high E_2 on a menstrual cycle basis to prime deuterium with higher basal and greater responsiveness to acid stimulation in females than males chronically. Second, we showed in Figs. 3A, 4A, and 8A, E_2-induced DMBS *in vivo* kept relatively stable for at least 50 min after a single exposure to E_2. Third, E_2 may potentiate other chronic stimulant-induced DMBS via CFTR channels, as exemplified by the fact that both E_2 and a phytoestrogen genistein potentiate membrane currents through CFTR channels (76, 77). Fourth, although the development of duodenal ulcer seems to occur chronically, the gastric acid-induced duodenal insults are relatively acute, which usually happens shortly after meals and simultaneously with gastric acid-induced DMBS (1). Fifth, it was reported recently that: 1) female mice had lower gastric ulcer index than male mice; and 2) gastric mucosal injury was exacerbated by ovariectomy, which was reversed by 1 mg/kg E_2 replacement (55, 78). These data strongly suggest protection of ovarian estrogen against the development of gastric mucosal injury in mice. Finally, clinical observations showing that pregnant women with high estrogen levels of placental origin barely develop ulcers and women taking oral contraceptives (estrogen/progesterone compounds) are with a significantly reduced incidence of ulcers (25, 26) strongly support a protective role of estrogen in the duodenum.

Altogether, we have revealed the gender-specific duodenal protection by estrogen in terms of HCO_3^- secretion, and the underlying molecular mechanisms of estrogen stimulation of DMBS that is linked to ER-Ca^{2+}-CFTR and Cl^-/HCO_3^- exchanger pathways. These observations may have provided evidence for a reasonable explanation on the cause of gender differences in duodenal ulcer. One may be concerned that the extrapolation of data obtained from animal to human is speculative; however, more recently we have revealed that gender differences of DMBS exist in humans as well (Tuo, B., *et al.*, unpublished observations). Thus, our current study may have provided an initial rationale for a potential hormone (*e.g.* estrogen) therapy to protect the gastrointestinal tract in postmenopausal women.

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