HHS Public Access

Author manuscript

Clin Immunol. Author manuscript; available in PMC 2017 November 07.

Published in final edited form as:

Clin Immunol. 2017 October ; 183: 174–180. doi:10.1016/j.clim.2017.08.019.

Estrogen decreases tight junction protein ZO-1 expression in human primary gut tissues

Zejun Zhou[a,*], **Lumin Zhang**[b,*], **Miao Ding**[a], **Zhenwu Luo**[a], **Shao Yuan**[c], **Meena B. Bansal**[b], **Gary Gilkeson**[d], **Ren Lang**[e,#], and **Wei Jiang**[a,f,#]

[a]Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC, USA, 29425

[b]Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, NY, USA, 10029

[c]The Biorepository & Tissue Analysis Shared Resource, Hollings Cancer Center, Medical University of South Carolina, SC, USA, 29425

[d]Division of Rheumatology, Department of Medicine, Medical University of South Carolina, Charleston, SC, USA, 29425

[e]Department of Hepatobiliary Surgery, Beijing Chaoyang Hospital, Capital Medical University, Beijing, China, 10020

[f]Divison of Infectious Diseases, Department of Medicine, Medical University of South Carolina, Charleston, SC, USA, 29425

Abstract

Females have a higher prevalence of most autoimmune diseases; however, the mechanism is unknown. In this study, we examined the expression of tight junction protein zonula occludens 1 (ZO-1) and estrogen receptor (ER)-α/β in human primary gut tissues by immunohistochemistry, immunofluorescence and qPCR. The expression of ZO-1 and ER-β but not ER-α was present in both male and female gut tissues. There was no sex difference in ER-β expression, but ZO-1 expression was decreased in females compared to males. *In vitro*, estrogen treatment decreased ZO-1 mRNA and protein expression, ZO-1 promoter activity, IL-6 production, and NF-κB activation in human primary gut tissues or the Caco-2 cells, but increased the ER-β expression in Caco-2 cells. Consistently, plasma IL-6 levels in females were reduced relative to males *in vivo*. Our finding indicates that estrogen may play a role in gut tight junction expression and permeability.

Keywords

Sex differences; estrogen; ZO-1; gut tissues

[#]Corresponding author, Wei Jiang, 173 Ashley Ave. BSB208D, Charleston, SC, 29425; jianw@musc.edu; and Ren Lang, Department of Hepatobiliary Surgery, Beijing Chaoyang Hospital, Capital Medical University, Beijing, Chaoyang Qu, China, 10020; dr-langren@hotmail.com.

[*]The two first-authors contribute equal amount of work

Introduction

There is accumulating evidence that sex has profound effects on the immune system [1–4]. The fundamental differences between males and females, including the number of specific immune cell types and their activation and function in response to immunological challenge following vaccination or exposure to a pathogen, are likely to result in the different susceptibility to a variety of pathogens and incidence of autoimmune diseases [5–7]. In general, males are more susceptible and females are more resistant to pathogenic infections, which are especially prominent following from puberty until menopause [8–10]. In contrast, females have a higher prevalence of most autoimmune diseases after adolescence [11, 12]. Several mechanisms may take part in these sex-biased immune responses, including sex hormones, genetic and environmental factors [13–15]. However, the exact mechanisms underlying this phenomenon are largely unknown.

The prevalence of various autoimmune diseases in females after puberty suggests that female sex hormones, like estrogen, may play a major role in autoimmune responses [14, 16]. 17β-estradiol may protect females from viral-mediated pathogenesis by suppressing inflammatory responses [17]. Estrogen has also been reported to mediate immune activation and trigger different immune responses through estrogen receptor (ER) dependent or independent pathways [1, 18]. There are two functional ER subtypes ER-α and ER-β in the ER dependent pathway. However, ER-α and ER-β exhibit differential expression among immune cell subsets; ER-α was expressed at higher levels in $CD4^+$ T cells rather than B cells. Instead, B cells expressed high levels of ER-β [19, 20]. Furthermore, IL-6 expression is downregulated by estrogen receptor through a NF-κB dependent pathway [21]. The ER independent pathway also is implicated in immune cell responses, enabling protein-protein interactions between ERs and ER independent transcription factors, including NF-κB, specific protein 1 (SP1) and activator protein 1 (AP-1) [22].

In addition to sex-biased immune responses, the loss of the protective function of mucosal barriers that interact with the environment (mainly the gastrointestinal mucosa) is necessary for development of autoimmunity [23]. As we know, tight junctions are composed of occludin, claudins, and zonula occludens (ZO)-1, -2, -3, which govern the paracellular permeability of endothelial and epithelial cells, and provide a barrier function such as inhibit bacterial invasion [24]. Several autoimmune diseases, including celiac disease, type 1 diabetes, multiple sclerosis, and rheumatoid arthritis, are characterized by increased intestinal permeability that allow the translocation of antigens (e.g., microbial products) from the intestinal flora, challenging the immune system to produce an aberrant immune responses and inflammation [25–28]. For example, both ZO-1 protein levels and mRNA were clearly reduced in patients with active celiac disease, which may be responsible for the increased paracellular permeability [29, 30]. A study has showed that altered expression of claudin lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease [31]. Specifically, in some female-preponderant autoimmune diseases such as Sjögren's syndrome and autoimmune thyroid diseases, disrupted tight junction integrity is an important contributor to the dysfunction of immune system [32, 33].

Although the importance of tight junction that influences the autoimmune systems of female and male differently, it is presently unclear about the role of sex hormones (e.g., estrogen) on tight junction protein expression in human primary gut tissues. Therefore, this study sought to determine the expression of ER-α/β and tight junction protein ZO-1 in primary gut tissues of both male and female. Moreover, we further investigated the inhibitory effect of estrogen on ZO-1 expression, IL-6 production and NF-κB activation *in vitro*.

Materials and Methods

Study subjects

This study was approved by IRB from the Medical University of South Carolina (MUSC, Pro00037761), and all participants provided written informed consents. Human fresh colon biopsies of non-cancer tissues from males and postmenopausal females with an annual intestinal microscopy test were obtained at Hollings Cancer Center, biorepository at MUSC.

Immunohistochemical analysis of ZO-1, ER-α and ER-β under normal physiological conditions

Immunohistochemical assay was performed according to the previous description with little modification [34]. Gut samples from 8 males and 7 females (table 1) were fixed in 10% formalin at room temperature for 24 h, faded in 70% ethanol 2 h for four times, and dehydrated in 80%, 95% and 100% ethanol sequentially. Then the samples were embedded in paraffin wax, sectioned (5 μm) and mounted on slides. The slides were deparaffinated in xylene, rehydrated in diluted ethanol series from 95% up to distilled water. After antigens were refolded in sodium citrate-hydrochloric acid buffer, the slides were blocked with 3% BSA in PBST at 37°C for 30 min. To examine whether ER-α and ER-β were expressed in human primary gut tissues, mouse anti-human ER-α antibody (diluted 1:500 in 3% BSA, Dako, Glostrup, Denmark) and mouse anti-human ER-β antibody (diluted 1:500 in 3% BSA, Invitrogen, Carlsbad, CA, USA) were added to the slide. The slide was incubated at 37°C for 1 h. After washing with PBST, the sheep anti-mouse second antibody conjugated with horseradish peroxidase (1/1000 dilution, Jackson ImmunoResearch, West Grove, PA, USA) was incubated with the sections at 37°C for 1 h. Proteins were visualized using DAB staining with a fluorescence microscopy (Zeiss Axio Vet. A1, Germany). To examine ZO-1 expression in human primary gut tissues, rabbit anti-human ZO-1 antibody (diluted 1:500 in 3% BSA, Invitrogen), rabbit anti-human cytokeratin antibody (diluted 1:500 in 3% BSA, Invitrogen) or mouse anti-human ER-β antibody (diluted 1:500 in 3% BSA, Invitrogen) was added to the slide. The slide was incubated at 37°C for 1 h and washed in PBST. DyLight 594 labeled goat anti-rabbit second antibody (KPL, Gaithersburg, MD, USA) and Alexa Flour 488 labeled sheep anti-mouse second antibody (1/1000 dilution, Jackson ImmunoResearch) were added to the slide. The slide was incubated at 37°C. After 1 h, the slide was washed with PBST three times and observed under a fluorescence microscope (Zeiss Axio Vet. A1).

qPCR analysis of ZO-1 mRNA expression under estrogen treatment

Gelfoam (Gelfoam absorbable gelatin sponge, Pfizer Inc., NY, USA) were distributed into 12-well tissue culture plates. Gut samples from postmenopausal females including 3 whites

and 3 Africa Americans (3 mm × 3 mm) were placed on the Gelfoam and then cultured at 37°C with RPMI-1640 medium (Gibco) containing penicillin (100 unit/ml), streptomycin (100 μg/ml) and 10% charcoal stripped FBS (Gibco). The tissues were treated with different concentrations (0, 0.2, 2 and 20 ng/ml) of 17β-estradiol (Alfa Aesar, Tewksbury, USA) for 24 h. Samples were extracted total RNA by EZNA Total RNA Kit (Omega Bio-tek, Doraville, GA, USA). cDNA was synthesized from 5 μg of each RNA sample using the SuperScript III First-Strand Synthesis System (Invitrogen) followed by PCR. Primer sequences, reaction conditions, and optimal cycle numbers of PCR were described previously [35]. The expression level of ZO-1 was analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with GAPDH as an internal reference [36]. The expression level of medium without estrogen treatment was set as 1. The experiment was performed three times.

Cell culture

Caco-2 cell line, a human epithelial colorectal cancer cell line, was purchased from ATCC (Manassas, VA, USA). Caco-2 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM, phenol red-free, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20 % charcoal stripped fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 2 mM L-glutamine, 10mM HEPES, 100 unit/ml penicillin and 100 μg/ml streptomycin (Gibco) in a humidified 37°C, 5% CO_2 incubator.

Effect of estrogen on ZO-1 expression determined by Western blotting

Caco-2 cells were cultured as above and then incubated with different concentrations (0, 0.2, 2, 20 and 200 ng/ml) of 17β-estradiol for 24 h. The cells were harvested for preparation of whole-cell extracts. The whole-cell extracts were loaded in each lane for 10% SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was blocked with (10 mM Tris-HCl, 100 mM NaCl, 0.1% (w/v) Tween 20, pH 8.0) containing 5% skim milk for 2 h, and incubated with rabbit anti-human ZO-1 antibody (1:1000, v/v, Invitrogen) at room temperature for 1 h. The rabbit anti-β-actin antibody (1:1000, v/v, Santa Cruz, CA, USA) was employed as a negative control. After washing with TBST for three times, the membranes were incubated with 1: 2000 (v/v) horseradish peroxidase-conjugated goat anti-rabbit IgG (Invitrogen) for 1 h, followed by thoroughly washing. The target proteins were developed with TMB 1-component membrane solution (KPL). The experiment was performed three times.

Immunofluorescence analysis of ZO-1 and ER-β expression under estrogen treatment

Caco-2 cells were cultured as above and treated with or without 20 ng/ml of 17β-estradiol for 24 h. The cells were washed with PBST and incubated with 4 % paraformaldehyde for 30 min. After incubation, rabbit anti-human ZO-1 antibody (1/1000, v/v, as described above) was added and incubated at 37°C for 2 h, and washed 3 × with PBST. FITC labeled goat anti-rabbit antibody (1/1000, v/v, KPL) was added to the cells and incubated at 37°C for 1 h. To examine ER-β expression, Caco-2 cells were cultured as above and treated with different concentrations (0, 2 and 20 ng/ml) of 17β-estradiol for 24 h. After incubation, mouse anti-human ER-β antibody and Alexa Flour 488 labeled sheep anti-mouse second antibody were added to the slide as described above. The cells were washed twice with PBST and stained for 5 min at room temperature in DAPI (Invitrogen). After washing, the cells were observed

with fluorescent microscope (Zeiss Axio Vet. A1). The experiment was performed three times.

Fluorescence intensity analysis

Images were taken as above with the ×20 objective using a red fluorescence filter for each staining, whereas their corresponding ER-β images were taken using a green fluorescence filter. All images were taken using identical camera, microscope lens, and light settings. Fluorescence intensity analysis was performed using ImageJ software (ImageJ, Bethesda, USA). The mean intensity values were corrected for background differences by dividing the measured intensities with the average intensity of a cell-free region in each section. Briefly, images were inverted to gray-scales and were drawn a region of interest at first. Next, images were calibrated the optical density and then measured the areas and integrated densities. The same method was used for analyzing the intensities of background regions. The percentages of changes in immunofluorescent intensity were shown after subtracting values in background (%fluorescence intensity = integrated densities/areas).

ZO-1 reporter activity in response to estrogen treatment

For ZO-1 promoter assay, Caco-2 cells were cultured in 6-wells plate (5×10^5 cells/well) with Eagle's minimal essential medium (MEM) and transfected with TJP1 reporter plasmid (s709631, Switchgear Genomics, CA, USA) or control reporter plasmid (s790001, Switchgear Genomics) using Lipofectamine™ 2000 (Invitrogen) according to the instructions of the manufacturer. After transfection, the cells were divided into 96-wells plate and then treated with or without 20 ng/ml 17β-estradiol for 24 h. The chemiluminescence assay was performed using LightSwitch Assay Reagent (Switchgear Genomics). The experiment was performed three times.

ELISA analysis of IL-6 expression in Caco-2 cell culture supernatants and human plasma

Caco-2 cells were cultured as above and incubated with 17β-estradiol for 24 h. IL-6 protein was measured in supernatants by ELISA according to the manufacturers' instruction (eBioscience, San Diego, CA, USA). To measure plasma IL-6 level in healthy people, plasmas from 10 healthy males and 10 healthy females (table 1) were isolated and stored in a 1.5-ml microtube at 80°C until thawing once for study. IL-6 protein was measured by ELISA.

NF-κB reporter activity in response to estrogen treatment

Caco-2 cells were resuspended in MEM medium and distributed into 96-well culture plates (1×10^4 cells/well). Cells were transfected with 0.5 μg pNFκB-SEAP-1 (Clontech, Mountain View, CA, USA) and 10 ng pMet-Luc plasmid (an internal control, Clontech). After transfection, the cells were treated with or without 20 ng/ml 17β-estradiol for 24 h. The activities of SEAP and Met-Luc in the supernatant were analyzed by chemiluminescence as previously described [37]. The experiment was performed three times.

Statistical analysis

Data from repeated experiments were averaged and expressed as means ± standard deviations. Statistical analysis was performed by GraphPad Prism 6.0 (GraphPad, San Diego, USA) using the Mann Whitney U test (non-paired). P values of < 0.05 were considered statistically significant.

Results

Immunohistochemical analysis of ZO-1, cytokeratin, ER-α and ER-β in human gut tissues

The human intestinal epithelium is formed by epithelial cells and connected by tight junctions, which play an important role in the integrity of epithelium formed mucosal barrier. The ER-α expression was essentially negative while ER-β is the predominant ER subtype in the human colon by immunohistochemistry assays (Fig. 1), which is in agreement with others [38, 39]. Thus, to explore sex difference in the expression of tight junction related protein (ZO-1), we examined the expression of ZO-1 as well as ER-β in male and female primary gut tissues by immunofluorescence assays. The cytoskeleton protein cytokeratin was used as an epithelial cell marker. The results showed that ZO-1, ER-β and cytokeratin were all detected in male and female primary gut tissues (Fig. 1 and Fig. 2).

Fluorescence intensity analysis of ZO-1, cytokeratin and ER-β

Next, we analyzed the expression levels by fluorescence intensities. Of interest, the fluorescence intensity of ZO-1 in male gut tissues was significantly higher than those in female gut tissues ($P = 0.02$, Fig. 2C). No sex differences in the fluorescence intensities of ER-β ($P = 0.79$, Fig. 2D) and cytokeratin ($P = 0.85$, Fig. 2E) were found.

Effect of estrogen on ZO-1 expression

To determine the effect of estrogen on ZO-1 expression, primary female gut tissues were incubated with different concentrations of 17β-estradiol, and the ZO-1 mRNA expression relative to GAPDH was subsequently determined by qPCR. Dose dependent decreases of ZO-1 expression were observed in gut tissues by 17β-estradiol treatment *in vitro* (Fig. 3A). To further verify the effect of estrogen on ZO-1 expression, Caco-2 cells were treated with 17β-estradiol and examined for ZO-1 expression by western blotting. Consistently, dose dependent decreases of ZO-1 expression were observed in Caco-2 cells by 17β-estradiol *in vitro* (Fig. 3B). Moreover, ZO-1 protein expression was decreased by 17β-estradiol treatment in Caco-2 cells by fluorescence microscopy *in vitro* (Fig. 3C and 3D). ZO-1 promoter activity was significantly decreased by 17β-estradiol in Caco-2 cells, confirming the inhibitory effect of 17β-estradiol on the transcription of ZO-1 expression (Fig. 3E). Interestingly, we also found that ER-β expression was increased after estrogen treatment in Caco-2 cells (Fig. 3F and 3G).

Effect of estrogen on IL-6 expression

IL-6 promoter has been reported to be inhibited by estrogen through NF-κB [40]. To determine the effect of estrogen on IL-6 production, Caco-2 cells were treated with estrogen *in vitro*. We found that IL-6 production in Caco-2 cell culture supernatants was reduced by

17β-estradiol compared to medium alone (Fig. 4A). To verify our finding *in vivo*, we measured plasma IL-6 levels in healthy males and females. Consistent with previous studies [41], plasma levels of IL-6 were decreased in females compared to males (P = 0.03, Fig. 4B).

Effect of estrogen on NF-κB activation

Since NF-κB activation is essential for the regulation of IL-6 production by estrogen, a NF-κB reporter was employed to assess the impact of estrogen on NF-κB activation. Luciferase activity was analyzed in Caco-2 cells after the NF-κB reporter pNFκB-SEAP-1 transfection. We found significantly decreased luciferase activity in Caco-2 cells incubated with 17β-estradiol compared to medium alone (P = 0.002, Fig. 5). These results suggest that estrogen reduces NF-κB activity.

Discussion

Tight junctions are essential for establishing a mucosal barrier, and they act as a selective gate to control paracellular diffusion of ions and solutes [24]. Mutations in genes encoding tight junction proteins have been linked to a range of human diseases [23, 31–33, 42]. In addition, studies examined the impact of estrogen on vascular permeability in mammals suggest that 17β-estradiol has an effect on vascular endothelial growth factor (VEGF) through ER-α and ER-β, resulting in the increased permeability of blood vessels and tumor metastasis [43–45]. To investigate the role of estrogen in human gut permeability, we analyzed the expression of ZO-1 in male and female primary gut tissues. We found that ZO-1 expression in females was significantly lower compared to males, indicating that sex hormones may be involved in the regulation of gut tight junction. Moreover, estrogen inhibited ZO-1 mRNA and protein expression and ZO-1 promoter activity in human primary gut tissues and the Caco-2 cell line *in vitro*. Although the effect of estrogen on tight junctions of epithelial cells has been studied previously [46–48], our study is the first one to show the inhibitory effect of estrogen on ZO-1 expression in primary human gut tissues.

Previous studies reported that estrogen reduces IL-6 production and therefore inhibits carcinogenesis and osteoporosis [41, 49–51]. More importantly, estrogen may have an anti-inflammatory effect due to inhibition of NF-κB activation [52]. These studies are consistent with our results that estrogen-mediated inhibition of IL-6 production and NF-κB activation in gut epithelial cells, which may contribute to anti-inflammation immune responses at local sites.

The fact that the expression of ZO-1 is inhibited by estrogen indicates estrogen may increase gut permeability and as a consequence of systemic microbial translocation and its associated inflammation. If so, multiple microbial products such as RNA, DNA, peptidoglycan and flagellin derived from bacteria, viruses, fungi and other gastrointestinal residents may translocate into systems and cause systemic immune activation [53–55]. Indeed, our recent work shows that monocytes were activated *in vivo* in women compared to men, and plasma level of soluble CD14, a marker of monocyte activation by LPS, was higher in women relative to men [56], suggesting a role of systemic bacterial products in immune activation and inflammation. In addition, our recent studies have revealed that the heightened plasma

level of LPS play an important role on T cell and B cell activation in HIV pathogenesis [57, 58]. Therefore, women should have overall higher levels of certain inflammation (e.g., sCD14) and increased proportions of activated monocyte subset (CD14−CD16+) compared to men [56]. Paradoxically, in the current study, we found that the plasma IL-6 level in female was reduced compared to male *in vivo*. This result is in line with previous studies, which showed that decreased plasma IL-6 levels are due to the estrogen treatment [59, 60]. Given the results we observed that IL-6 production and NF-κB activation were inhibited by estrogen, we propose that plasma IL-6 may be impacted through a direct estrogen-mediated mechanism *in vivo*, which appears to be different from monocyte activation and its associated inflammation in women through an indirect mechanism of estrogen-mediated gut permeability and microbial translocation.

The main limitation of our study is that all female gut tissues were from postmenopausal women, given the difficulty to get the normal gut tissues from premenopausal women, we are unable at this time to identify the expression of ZO-1 and ER-β in primary gut tissues from premenopausal women. After menopause in females, follicles are depleted and estrogen concentration reduces to very low levels [1, 42, 61]. Therefore, the expression of ZO-1 in primary gut tissues from premenopausal women may be different from postmenopausal women.

In conclusion, the strengths of this study are the demonstration of ZO-1 expression on human primary gut tissues, and the inhibitory effect of estrogen on ZO-1 expression in gut tissues and human gut epithelial cell line. The *in vitro* inhibitory effect of estrogen on IL-6 and NF-κB activation supports a role for estrogen in the regulation of host immune response in the gut. These observations provide the first insights to the sex-based differences of gut ZO-1 expression and inflammation.

Acknowledgments

The funding support was from the National Institute of Allergy and Infectious Diseases grant AI091526 (Jiang) and AI128864 (Jiang), National Institute of Arthritis and Musculoskeletal and Skin Diseases grant P60 AR062755 (Gilkeson), UL1 RR029882, the Medical Research Service at the Ralph H. Johnson VA Medical Center Merit grant VA CSRD MERIT (CX001211, Gilkeson), BLRD MERIT (BX000470, Gilkeson), the Biorepository & Tissue Analysis Shared Resource, Hollings Cancer Center, Medical University of South Carolina (P30 CA138313), and Beijing Natural Science Foundation (Grant No. 7152063) and Scientific Research Common Program of Beijing Municipal Commission of Education (KM2016100250017).

We thank that Dr. Alter Harvey kindly provide NF-κB reporter plasmid and reagents.

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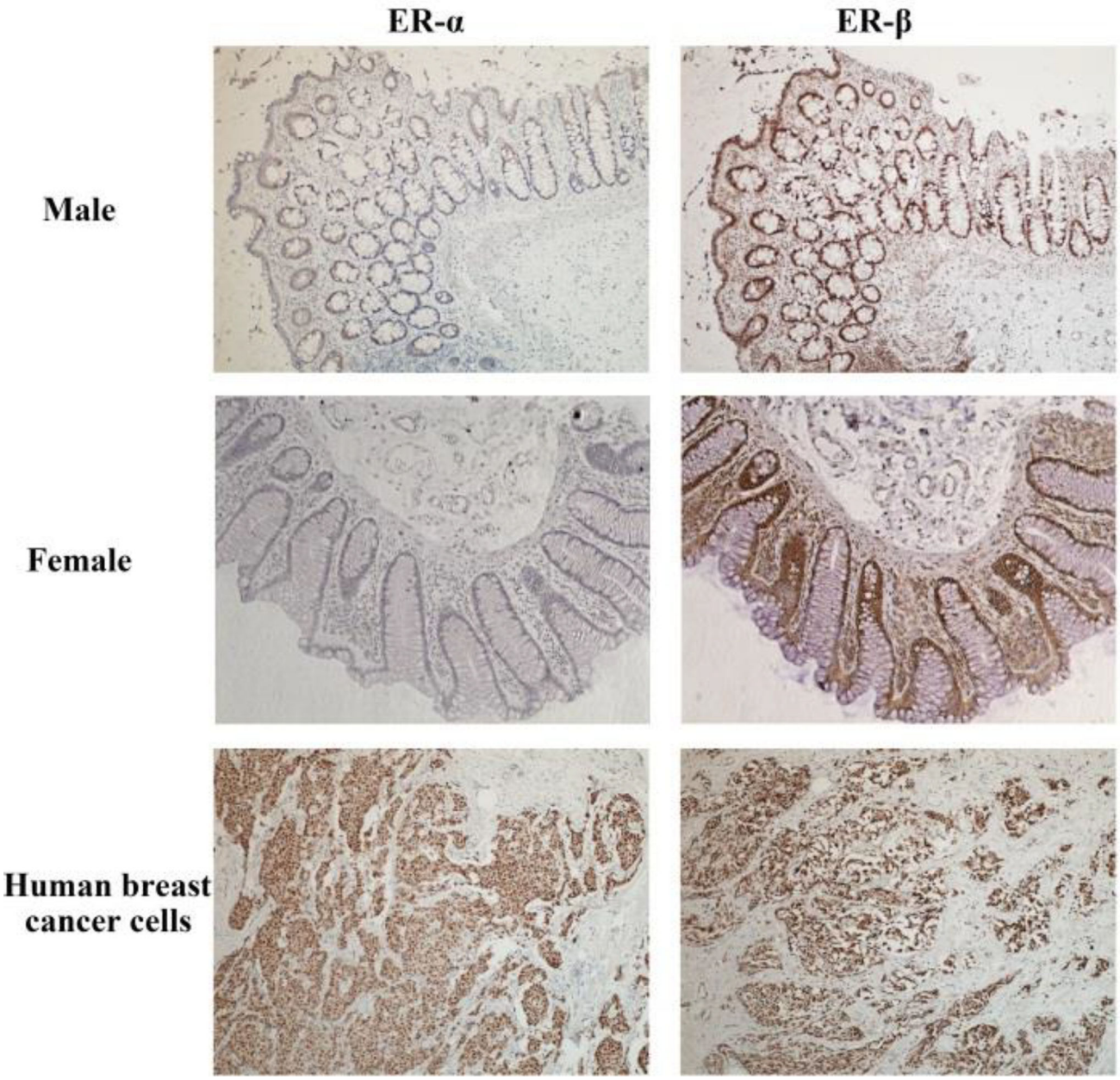

Figure 1.

Estrogen receptor (ER) α and β expression in human primary gut tissues. ER-α and ER-β in male and female primary gut tissues were detected by immunohistochemistry (100 ×). ER-α and ER-β staining in primary human breast cancer cells as positive controls were detected by immunohistochemistry (100 ×).

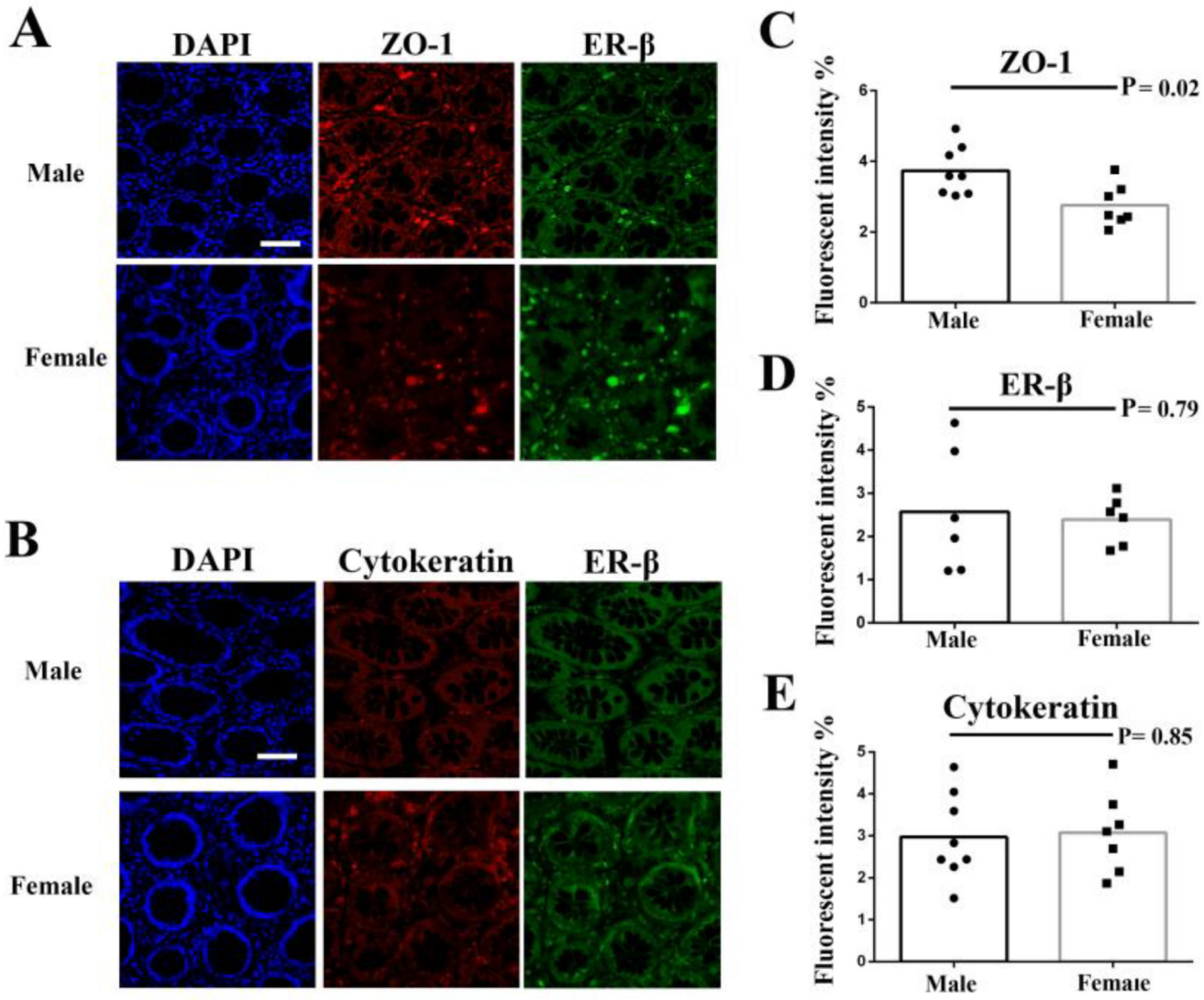

Figure 2.

Immunofluorescence analysis of the distribution of ZO-1, cytokeratin and ER-β in male and female gut tissues. The nuclei of cells were stained blue by DAPI in single section. ZO-1 and cytokeratin were detected by DyLight 594-labeled antibody. ER-β was detected by Alexa Flour 488-labeled antibodies (A and B). Bar: 100 μm. Images were then taken with a fluorescence microscopy; Fluorescence intensity of ZO-1 (C), cytokeratin (D) and ER-β (E) was analyzed using ImageJ software. *P* values were analyzed by Mann Whitney test (non-paired).

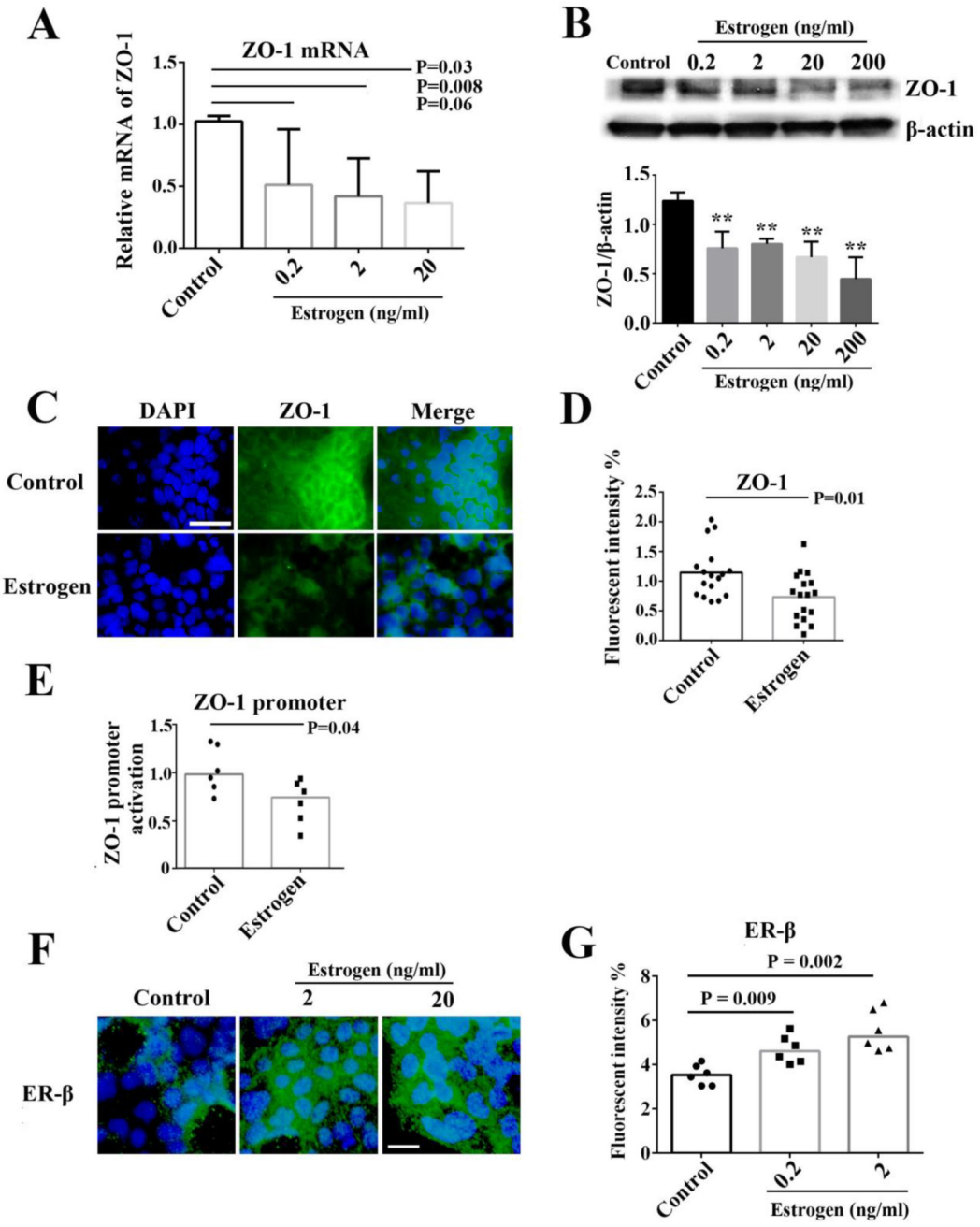

Figure 3.

Effect of estrogen on ZO-1 expression. Female primary gut tissues or Caco-2 cells were treated with medium alone (control) or different concentrations of estrogen and examined for ZO-1 expression. ZO-1 mRNA expression was analyzed by qPCR in human postmenopausal female gut tissues (n = 6) (A). Western blot analysis and densitometry quantification of ZO-1 expression normalized to total β-actin in Caco-2 cells, repeated three times (B). ZO-1 expression and nuclei were stained by FITC-labeled antibody and DAPI respectively in Caco-2 cells (C). Bar: 50 μm. After immunofluorescence assay in Caco-2 cells, images were taken with a fluorescence microscopy, and fluorescence intensity of ZO-1

was then analyzed using ImageJ software (D). Caco-2 cells were transiently transfected with TJP1 reporter plasmid, and then treated with or without estrogen; ZO-1 promoter activation was examined by luminescent assay (E). ER-β expression and nuclei were stained by Alexa Flour 488-labeled antibody and DAPI respectively in Caco-2 cells (F). Bar: 25 μm. The fluorescence intensity of ER-β was then analyzed using ImageJ software as above (G). *P* values were analyzed by Mann Whitney test (non-paired).

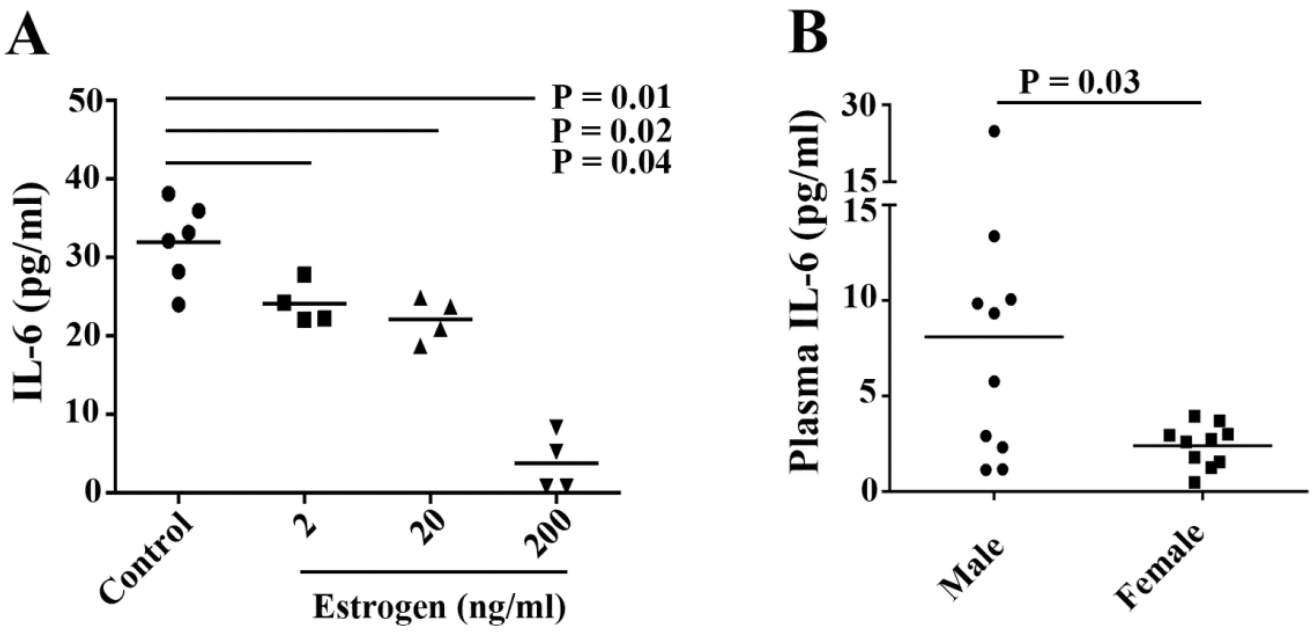

Figure 4.

Measurement of IL-6 levels. Caco-2 cells were treated with or without different concentrations of estrogen and then examined for IL-6 in cell culture supernatants by ELISA (A). Plasma IL-6 levels in healthy males and females were examined by ELISA (B). *P* values were analyzed by Mann Whitney test (non-paired).

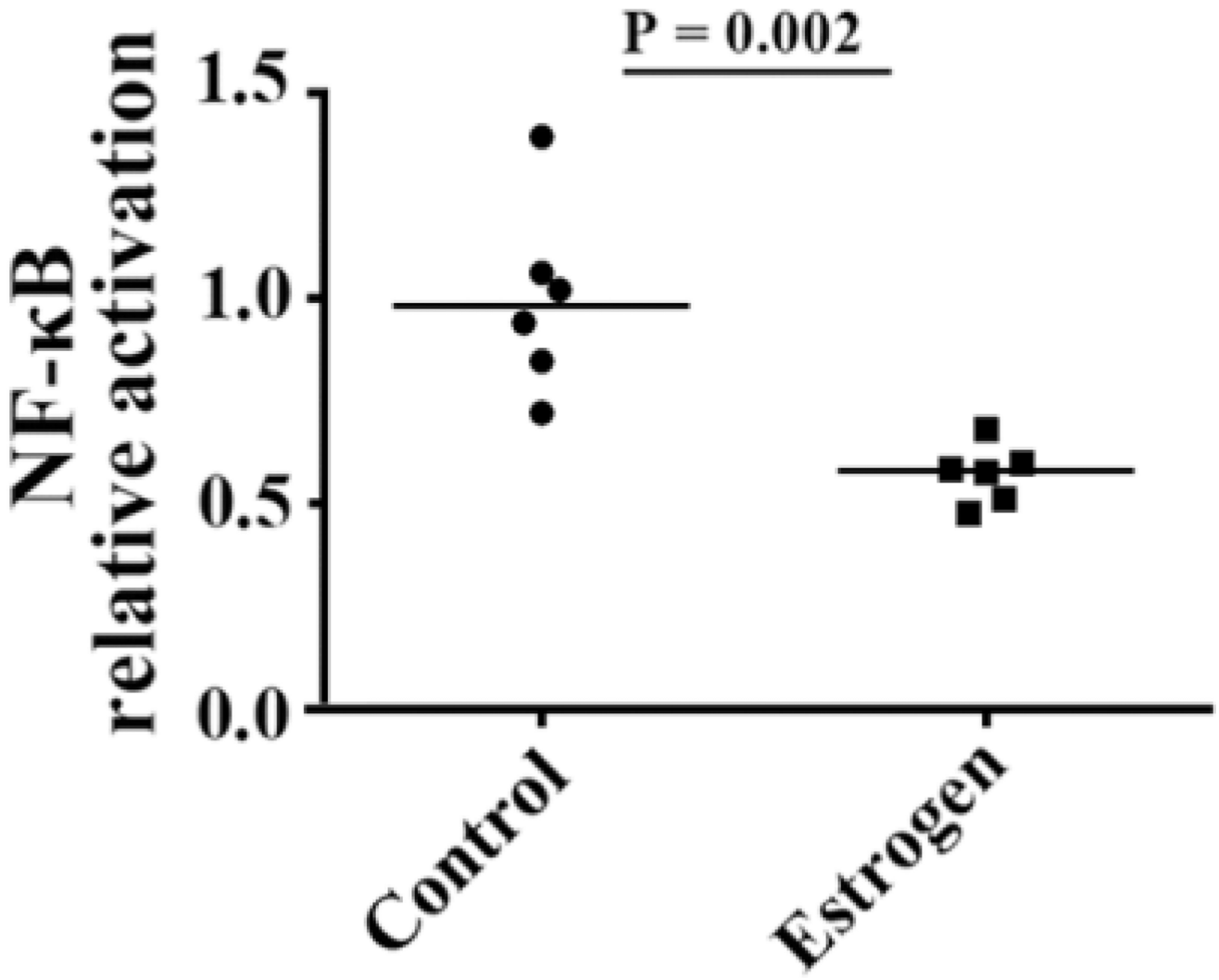

Figure 5.

Effect of estrogen on NF-κB activation. Caco-2 cells were transiently transfected with pNFκB-SEAP-1 and then treated with or without estrogen. NF-κB activation in the estrogen-treated group relative to the control group was examined by luminescent assay. *P* values were analyzed by Mann Whitney test (non-paired).

Table 1

Clinical characteristics

	Male	Female	*P* value [a]
Immunohistochemistry assay			
Numbers of subjects	8	7	
Age (years) median [b]	67.5 (45–71)	61 (57–74)	0.71
Ethnicity (No.) [b]			> 0.99
White	4	3	
Africa American	2	2	
East Asian	2	2	
Tissue	Normal colon	Normal colon	
Phase of menstrual cycle		Post-menopausal	
ELISA for Plasma IL-6 levels test			
Numbers of subjects	10	10	
Age (years) median [b]	47 (43–59)	49 (45–58)	0.75
Ethnicity (No.) [b]			> 0.99
White	5	5	
Africa American	5	5	
Phase of menstrual cycle		Post-menopausal	
Hormone replacement therapies	No	No	
Systemic healthy and no smokers	Yes	Yes	
Systemic antibiotic treatment (past 6 months)	No	No	

[a] *P* values compared between the two study groups were analyzed by Mann Whitney U test (non-paired).

[b] Data are median (interquartile range) values.