Estrogen receptor-β signaling modulates epithelial barrier function

Mirjam Looijer-van Langen,1 Naomi Hotte,1 Levinus A. Dieleman,1 Eric Albert,1 Chris Mulder,2 and Karen L. Madsen1

1Division of Gastroenterology, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada; and 2Department of Gastroenterology and Hepatology, VU University Medical Center, Amsterdam, The Netherlands

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Looijer-van Langen M, Hotte N, Dieleman LA, Albert E, Mulder C, Madsen KL. Estrogen receptor-β signaling modulates epithelial barrier function. Am J Physiol Gastrointest Liver Physiol 300: G621–G626, 2011. First published January 20, 2011; doi:10.1152/ajpgi.00274.2010.—Impaired epithelial barrier function and estrogens are recognized as factors influencing inflammatory bowel disease (IBD) pathology and disease course. Estrogen receptor-β (ERβ) is the most abundant estrogen receptor in the colon and a complete absence of ERβ expression is associated with disrupted tight-junction formation and abnormal colonic architecture. The aim of this study was to determine whether ERβ signaling has a role in the maintenance of epithelial permeability in the colon. ERβ mRNA levels and colonic permeability were assessed in IL-10-deficient mice and HLA-B27 rats by RT-PCR and Ussing chambers. ERβ expression and monolayer resistance were measured in HT-29 and T84 colonic epithelial monolayers by RT-PCR and electric cell-substrate impedance sensing. The effect of 17β-estradiol and an estrogen agonist [diarylpropionitrile (DPN)] and antagonist (ICI 182780) on epithelial resistance in T84 cells was measured. Expression of ERβ and proinflammatory cytokines was investigated in colonic biopsies from IBD patients. Levels of ERβ mRNA were decreased, whereas colonic permeability was increased, in IL-10-deficient mice and HLA-B27 transgenic rats prior to the onset of colitis. T84 cells demonstrated higher resistance and increased levels of ERβ mRNA compared with HT-29 cells. 17β-estradiol and DPN induced increased epithelial resistance in T84 cells, whereas an ERβ blocker prevented the increased resistance. Decreased ERβ mRNA levels were observed in colonic biopsies from IBD patients. This study suggests a potential role for ERβ signaling in the modulation of epithelial permeability and demonstrates reduced ERβ mRNA in animal models of colitis and colon of patients with inflammatory bowel disease.

estrogen receptor-β, intestinal permeability; inflammation; inflammatory bowel disease

Inflammatory bowel disease (IBD) is a group of chronic, inflammatory diseases affecting the gastrointestinal tract. Two main disease phenotypes are Crohn’s disease and ulcerative colitis. IBD is regarded as a complex disease with a multifactorial pathophysiological basis, but the exact pathogenesis is still unclear. Genetic susceptibility, immune dysregulation driven by a response to commensal flora, triggering environmental factors, and impaired epithelial barrier function are recognized as influencing IBD pathology (19). Additionally, intestinal permeability has been shown to be increased in Crohn’s disease patients (27). This phenomenon is also observed in healthy first-degree relatives of patients, suggesting that this defect can occur independently of inflammation (16, 20, 28, 33). Furthermore, an increase in intestinal permeability has been shown to predict relapse in patients with Crohn’s disease (3). However, whether increased gut permeability is a causative event or merely an effect of the inflammatory milieu is still unknown.

Contraceptive use, pregnancy, and hormone replacement therapy have been reported to influence the risk of developing IBD or influencing disease course (5, 8, 18). However, the role of estrogen or gender in IBD pathology remains inconclusive. Estrogen mediates its action via estrogen receptors and is involved in modulating many biological processes, including immune responses and gastrointestinal physiology (9). Two estrogen receptors have been identified: estrogen receptor-α (ERα) and estrogen receptor-β (ERβ), both of which have their own distinct physiological function and distribution (9, 22). The results of ERα action are well known and involve classical estrogen effects, such as development of secondary sex characteristics and regulation of the menstrual cycle in females and sperm maturation in males (9). ERβ was discovered in 1996 and its biological effects have not been completely unraveled (22). Whereas ERβ plays a minor role in mediating action in classical estrogen target tissues, a dominant role appears to exist for ERβ in the brain, cardiovascular system, and colon where it is expressed primarily on epithelial cells (6, 15, 21, 36).

Research has demonstrated a role for ERβ in animal models, in that anti-inflammatory effects of estrogen or ERβ agonists have been described in models of chronic intestinal inflammation (14). In ERβ knockout mice an increase in epithelial cell proliferation, decrease of apoptosis, and decrease in cellular adhesion molecules was found. These mice also had disrupted tight-junction formation and abnormal colonic architecture (35).

Based on these findings, we hypothesized a role for ERβ in the maintenance of epithelial permeability in the colon. In the present study, we demonstrate reduced ERβ mRNA expression and increased gut permeability to precede the onset of colitis in two animal models. Furthermore, we observed decreased ERβ mRNA levels in colonic biopsies from IBD patients. Finally, in vitro experiments demonstrated a clear association between ERβ signaling and epithelial barrier function. These data support our hypothesis that ERβ signaling has a potential role in maintaining epithelial barrier function.

Materials and Methods

Animal models of inflammation. For this study, two chronic models of intestinal inflammation were examined. HLA-B27 transgenic (TG) rats spontaneously develop colitis and systemic inflammation in a specific pathogen-free (SPF) environment (14). TG rats and their healthy nontransgenic littermates (NTG) were maintained under SPF conditions with free access to food (LabDiet rodent diet 5001) and water. Rats were maintained at 23°C with a 12:12-h light-dark cycle. A second model of inflammation studied was the interleukin-10 (IL-10−/−) gene-deficient mouse bred on the 129 Sv/Ev background.
These mice develop a patchy inflammation primarily in the colon, beginning at 6 – 8 wk of age (26). IL-10 and wild-type 129 Sv/Ev controls (wild type) were housed under SPF conditions and fed a standard diet consisting of 9% fat rodent blocks. All experiments were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee.

Measurements in animal models. At death, small intestinal and colonic tissue were removed, and total RNA was isolated in Trizol (Invitrogen, Burlington, ON, Canada) using the RNA easy kit (Qia-gen, Mississauga, ON, Canada) in accordance with the manufacturer’s instructions. RNA concentrations and purity were determined using the nd-1000 spectrophotometer (NanoDrop Technologies Wilmington, DE). For reverse transcription, mastermix was produced containing 1 μl random primers, 2 μl DNTP mix, 9.5 μl RNase free H2O, 4 μl 5X first-strand buffer, 0.5 μl RNase out, 2 μl 0.1M DTT, 1 μl Superscript II Reverse Transcriptase (Invitrogen), and 1 μg RNA. Real-time PCR analysis was performed using the HT-7900 sequence detector (Applied Biosystems, Foster City, CA) with GAPDH used as the endogenous control. The mouse, rat, and human ERβ target primers were purchased as ready-to-use inventoried gene expression assays from TaqMan (Applied Biosystems, Streetville, ON, Canada). Analysis was performed using the Sequence Detection Software version 2.3, which is supplied with the Applied Biosystems HT-7900 instrument.

Intestinal permeability measurement. At death, segments of colon and small intestine were removed and mounted in lucite Ussing chambers for assessment of intestinal permeability. Serosal and mucosal surfaces were exposed to 10 ml Ringers buffer (in mmol/l: 115 NaCl, 8 KCl, 1.25 CaCl2, 1.2 MgCl2, 2 KH2PO4, 225 NaHCO3; pH 7.35). The buffers were maintained at 37°C by a heated water jacket and circulated by CO2. Fructose (10 mmol/l) was added to both sides. For measurement of basal mannitol fluxes, 1 mM of mannitol with 10 μCi [3H-mannitol] was added to the mucosal side. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short-circuit current (Isc) with an automatic voltage clamp (DVC 1000 World Precision Instruments, New Haven, CT), except for 5–10 s every 10 min when PD was measured by removing the voltage clamp. Tissue ion conductance (G) was calculated from PD and Isc according to Ohm’s Law (7). Isc is expressed as μA/cm², and G as mS/cm².

Measurements in colonic epithelial cell lines. HT-29 and T84 cells were purchased from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 media and DMEM/F12 media, respectively, with 5% and 10% heat-inactivated FBS. All experiments were performed using the HT-7900 sequence detector (Applied Biosystems, Foster City, CA) with GAPDH used as the endogenous control. The mouse, rat, and human ERβ target primers were purchased as ready-to-use inventoried gene expression assays from TaqMan (Applied Biosystems, Streetville, ON, Canada). Analysis was performed using the Sequence Detection Software version 2.3, which is supplied with the Applied Biosystems HT-7900 instrument.

Fig. 1. Colonic permeability and estrogen receptor-β (ERβ) expression levels in IL-10−/− mice. A: mannitol fluxes were measured in Ussing chambers and were significantly increased in IL-10−/− mice compared with wild-type controls. B: levels of mRNA for ERβ are expressed as fold change compared with wild-type small intestine. Decreased ERβ expression was observed in the colon of IL-10−/− mice compared with wild-type littermates. Values are means ± SE for 6 – 8 mice in each group. *P < 0.01 compared with age-matched wild-type males. **P < 0.01 compared with age-matched wild-type females.

Fig. 2. Colonic permeability and ERβ expression levels in HLA-B27 rats. A: mannitol fluxes were measured in Ussing chambers and were significantly increased in transgenic HLA-B27 rats compared with nontransgenic littermates. B: levels of mRNA for ERβ are expressed as fold change compared with nontransgenic small intestine. Decreased ERβ expression was observed in the colon of HLA-B27 rats compared with nontransgenic littermates. Values are means ± SE for 6 – 8 mice in each group. *P < 0.05 compared with age-matched nontransgenic males. **P < 0.05 compared with age-matched nontransgenic females.
were carried out in optiMEM (Invitrogen, Burlington, ON, Canada) without FBS supplementation to remove endogenous estrogen. For measurement of ERα and ERβ mRNA levels, confluent monolayers were trypsinized and collected in Trizol. RNA isolation and RT-PCR were performed as described above. For measurements of epithelial barrier function, impedance and resistance were measured using the electric cell substrate impedance sensing (ECIS) 1600R (Applied Biophysics, Troy, NY). ECIS plates with 10 electrodes (8W10E) were incubated with 400 µl RPMI medium per well for at least 3 h at 37°C, after which the electrodes were stabilized following the manufacturers’ instructions. T84 cells were grown until confluence. Cells were then trypsinized and resuspended with 1 × 10^6 cells in 400 µl media seeded per well. After cells were confluent, monolayers were treated with either 17β-estradiol (1–1,000 nM), diarylpropionitrile (DPN; 1–1,000 nM) or the estrogen receptor blocker ICI 182,780 (Sigma, Oakville, ON, Canada). Changes in resistance were measured for up to 15 h. The acquired data were analyzed for changes in resistance using the ECIS software.

Assessment of human biopsies. Biopsies were obtained from healthy individuals (n = 13) undergoing screening colonoscopy and from patients with documented Crohn’s disease (n = 18) or ulcerative colitis (n = 15) either currently in remission or suffering from a relapse in their disease. All protocols and procedures were approved by the University of Alberta Health Research Ethics board (no. 7113). All subjects provided written consent for tissue analysis. In all cases, biopsies were taken from macroscopically noninvolved tissue in the transverse colon, snap-frozen in liquid nitrogen, and stored at −80°C until further processing. RNA isolation and RT-PCR were performed for measurement of ERβ, TNF-α, and IFNγ mRNA levels. Relative expression data for each cytokine are shown as normalized to the average ΔCT of the control group of patients for the respective cytokine (31). The control group was normalized against itself but does not appear as “1” due to patient variability (24).

Statistics. Data are presented as means ± SE. Data were tested for normality of distribution, and analyses were performed using the statistical software SigmaStat (Jandel, San Rafael, CA). Comparisons between multiple groups were carried out using one-way ANOVA with a post hoc test of significance between individual groups. Differences were considered significant when P was < 0.05.

RESULTS

Intestinal ERβ mRNA levels and permeability in IL-10−/− mice. We demonstrated IL-10−/− mice have increased gut permeability compared with wild-type mice both prior to and following the onset of colitis (25). In this study, we confirmed the increased colonic permeability in IL-10−/− mice prior to the onset of colitis. As seen in Fig. 1A, both male and female IL-10−/− mice had increased colonic permeability compared with wild-type mice as shown by increased mannitol flux in Ussing chambers. There was no histological inflammation seen in the colon of these mice (data not shown). We then measured the mRNA levels of ERβ to determine whether alterations in permeability were associated with ERβ expression. As seen in Fig. 1B, reduced ERβ mRNA was seen in the colon of IL-10−/− mice compared with wild-type littermates. Wild-type females showed a higher level of ERβ expression than wild-type males; however, no such differences were seen in IL-10−/− mice. ERβ was not detectable in the small intestine in either wild-type or IL-10−/− mice (data not shown).

Intestinal ERβ mRNA levels and permeability in HLA-B27 rats. To determine whether the reduction in ERβ was specific to the IL-10−/− mouse, a second model of chronic intestinal inflammation was examined. HLA-B27 TG mice spontaneously develop colitis beginning at 8–12 wk of age (14). Similar to what was seen in the IL-10−/− mouse, ERβ expression was significantly reduced in the colon at 6 wk of age, again in the

<table>
<thead>
<tr>
<th>Lsc/cm²</th>
<th>Conductance. mS/cm²</th>
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</thead>
<tbody>
<tr>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>NTG, n = 5</td>
<td>TG, n = 5</td>
</tr>
<tr>
<td>8.0 ± 2.6</td>
<td>13.4 ± 2.9</td>
</tr>
<tr>
<td>28.4 ± 4.6</td>
<td>20.7 ± 2.6</td>
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</table>

Values are means ± SE. Lsc, short-circuit current.
absence of any histological inflammation, compared with NTG littermates (Fig. 2B). There was no difference in ERβ expression between males and females. Levels of ERβ in the small intestine were barely detectable (data not shown). To determine whether HLA-B27 TG rats also demonstrated alterations in intestinal permeability, colonic tissue was studied in Ussing chambers. At 6 wk of age, mannitol flux was significantly increased in the colon of both male and female HLA-B27 TG rats compared with NTG littermates (Fig. 2A). There were no significant differences in $I_{sc}$ or conductance (Table 1).

Stimulation of ERβ increases epithelial resistance. To further examine the relationship between ERβ signaling and epithelial permeability, we utilized two human colonic cell lines that have been shown to express ERβ but not ERα (2, 6). In agreement with previously reported findings (2), we found that neither cell line had any detectable expression of ERα (data not shown). The levels of ERβ mRNA were significantly higher in T84 (5.4 ± 0.9-fold change gene expression) compared with HT-29 (1.1 ± 0.3-fold change) cells, and this was associated with increased epithelial resistance in T84 (2,491 ± 56 ohms/cm²) compared with HT-29 (824 ± 13 ohms/cm²). Treatment of T84 cells with the ERβ agonists, 17β-estradiol (1–1,000 nM) (Fig. 3A) or DPN (1–1,000 nM) (Fig. 3B) resulted in enhanced monolayer resistance in a dose-dependent fashion. Furthermore, treatment of T84 epithelial cells with the estrogen receptor blocker ICI 182,780 blocked the effects of 17β-estradiol (Fig. 3A). These data support a role for ERβ signaling in modulating epithelial resistance.

ERβ mRNA levels in human biopsies. Several studies have demonstrated IBD patients to have increased intestinal permeability (28, 33). To determine whether ERβ expression was altered in IBD patients, colonic biopsies were obtained from healthy individuals undergoing screening colonoscopy ($n = 13$) and ERβ mRNA levels were measured and compared with levels in Crohn’s disease patients in remission ($n = 13$) or relapse ($n = 5$) and ulcerative colitis patients in remission ($n = 9$) or relapse ($n = 6$). Characteristics of each group are listed in Table 2. All biopsies were taken from grossly noninflamed tissue of the transverse colon. Similar to our observations in the animal models of colitis, a reduction in ERβ mRNA levels was observed in colonic biopsies from the Crohn’s disease patients in relapse, but interestingly, not the patients in remission. In contrast, in the ulcerative colitis patients, both the patients in remission and the patients suffering from relapse demonstrated reduced levels of ERβ mRNA (Fig. 4). There were no apparent differences in ERβ mRNA levels between males and females in this small sample size. To determine whether the reduction in ERβ levels was related to enhanced levels of proinflammatory cytokines, TNF-α and IFNγ expression were measured. As seen in Table 3, there was no apparent relationship between levels of these cytokines and expression of ERβ. In fact, patients with Crohn’s disease actually had reduced levels of IFNγ compared with controls.

**DISCUSSION**

In this study, increased colonic permeability and decreased levels of ERβ mRNA were found in colonic tissue prior to the onset of inflammation in two rodent models of chronic intestinal inflammation. This reduction in ERβ mRNA expression was also seen in grossly noninflamed colonic biopsies from ulcerative colitis patients and Crohn’s disease patients who were suffering from a relapse. Furthermore, this study showed that treatment of T84 cells with either 17β-estradiol or the ERβ agonist, DPN, resulted in a dose-dependent increase in monolayer resistance.

A role for ERβ in maintenance of colonic homeostasis and barrier function has been suggested by the findings of Wada-Hiraike et al. (36) in their study utilizing ERβ−/− mice. Our findings support a possible role for ERβ signaling in modulating colonic barrier function, in that reduced levels of ERβ were found in conjunction with increased colonic permeability in both the IL-10−/− mouse and the HLA-B27 rat. Unfortunately, protein expression of ERβ was not able to be measured in these studies due to problems with specificity with existing antibodies (32), which is a limitation of these studies. Proinflammatory cytokines have been shown to downregulate estrogen receptors in epithelial cells (23, 34); thus the reduction in ERβ mRNA levels prior to the onset of colitis in these rodent models could have been related to increased levels of proinflammatory cytokines that are present prior to the onset of inflammation in these models (30). However, decreased levels of ERβ expres-

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**Table 2. Patients demographic profile**

<table>
<thead>
<tr>
<th>Group</th>
<th>Gender</th>
<th>Age (mean and range)</th>
<th>Disease Status</th>
<th>Biopsy Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, $n = 13$</td>
<td>Female</td>
<td>7</td>
<td>53 (18–88)</td>
<td>N/A Transverse colon</td>
</tr>
<tr>
<td>Crohn’s disease, $n = 18$</td>
<td>Male</td>
<td>6</td>
<td>42 (24–62)</td>
<td>Remission, $n = 5$; remission, $n = 13$ Transverse colon</td>
</tr>
<tr>
<td>Ulcerative colitis, $n = 15$</td>
<td></td>
<td>10</td>
<td>39 (18–64)</td>
<td>Remission, $n = 9$; relapse, $n = 6$ Transverse colon</td>
</tr>
</tbody>
</table>

NA, not applicable.

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**Fig. 4.** ERβ mRNA levels in colonic biopsies from healthy individuals and patients with Crohn’s disease (CD) or ulcerative colitis (UC). CD patients in relapse and UC patients had significantly reduced levels of ERβ expression compared with healthy controls. All biopsies were taken from noninflamed regions of the transverse colon. Rem, remission. Values are means ± SD. *$P < 0.05$ compared with controls.
sion were also observed in colonic biopsies from IBD patients in grossly uninflamed regions of the intestine, and this decrease did not correlate with increased levels of either TNF-α or IFNγ in individual patients. Thus it is unlikely that the reduction in ERβ mRNA expression in IBD patients was due to increased TNF-α or IFNγ levels.

The finding that patients with Crohn’s disease in remission had normal levels of ERβ mRNA expression, while patients in relapse had reduced levels is particularly interesting in view of the findings that increased intestinal permeability is predictive of subsequent relapse (3). However, as with the animal studies, protein levels of ERβ could not be determined; thus future studies are required to determine the exact role of ERβ signaling in patients with IBD. The underlying cause for this decrease in ERβ expression in IBD patients cannot be determined from this study, but may be either genetically determined, or alternatively, related to the nutritional status of these patients. IBD patients commonly exhibit reduced plasma zinc levels, which can directly influence expression and sensitivity of estrogen receptors (10). Zinc deficiency also has numerous effects on the immune system that ultimately result in increased levels of oxidative stress and proinflammatory cytokine release, both of which can have a role in the breakdown of gut barrier function (29). Future studies are necessary to determine the underlying mechanism responsible for these observed decreased levels of ERβ, and also whether alterations in ERβ expression are linked with changes in intestinal permeability in IBD patients.

Beneficial effects of estrogen and ERβ agonists in different animal models of chronic colitis have been reported (13–15, 17). Harnish et al. (14) and Harris (15) reported improved histological scores, decreased inflammatory cell infiltration, and improvement of chronic diarrhea by estradiol treatment or administration of ERβ agonist ERB-041. Although intestinal permeability was not measured in these studies, we previously showed that treatment aimed at correcting the barrier defect in the IL-10−/− mouse is beneficial in both preventing the onset or attenuating existing colitis, (4, 11). These findings support a role for gut permeability in modulating colonic inflammation in this animal model.

Previous studies demonstrated variable levels of ERβ expression and no ERα expression in the T84 and HT-29 cell lines (2, 6). We confirmed that the T84 cell line used in these studies expressed significantly higher levels of ERβ mRNA compared with HT-29 cells, and that neither cell line had ERα expression. This increased level of ERβ mRNA was associated with a significantly higher resistance in T84 cells compared with HT-29 cells. Exposure of cell monolayers to either 17β-estradiol or the ERβ agonist DPN increased resistance, strongly supporting a role for ERβ signaling in modulating epithelial permeability. Supporting this hypothesis are the data showing that blocking of estrogen receptors with ICI 182,780 prevented the increase in the presence of 17β-estradiol. Interestingly, this increased resistance in response to 17β-estradiol is in contrast to effects in endothelial cells or vaginal-cervical epithelial cells, which both exhibit a decreased resistance when exposed to estrogen (1, 12). However, these cells primarily express ERα, suggesting that ERβ signaling may have opposing effects on epithelial permeability.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

ERβ AND EPITHELIAL PERMEABILITY


12. G626 ER


