Effect of 17β-oestradiol on the leptin receptor expression in the colon of ovariectomized rats

M. YILDIZ1* and K. ALTUNBAS2

1Department of Histology and Embryology, Institute of Health Sciences, University of Adnan Menderes, 09016 Aydin, TURKEY.
2Department of Histology and Embryology, Faculty of Veterinary Medicine, University of Afyon Kocatepe, 03200 Afyonkarahisar, TURKEY.

*Corresponding author: mustayildiz17@yahoo.com

SUMMARY

The aim of this study is to determine the effects of ovariectomy and 17β-oestradiol on the leptin receptor expression in colon in rats. A total of 36 six-month-old Sprague-Dawley ovariectomized rats were divided into oestrogener (25 mg 17β-oestradiol, intramuscularly) and control (0.2 mL sesame oil, intramuscularly) groups and were slaughtered at 18th, 90th and 162nd hours (6 control and 6 treated animals for each time point). The leptin receptor expression in colon was analysed by immunohistochemistry. Immunoreactivity was seen in the colonic crypt epithelium in both control and oestrogener groups and was significantly higher in the treated animals at the 90th and 162nd hours. Immunolabelling of plexus myentericus was only observed in the oestrogener groups and gradually declined according to time. These results demonstrate that 17β-oestradiol up-regulates the leptin-receptor expression in the colon in rats suggesting that some colon disorders related to decreased leptin receptor expression may be induced by oestrogen depletion in females.

Keywords: Rat, ovariectomy, leptin receptor, 17β-oestradiol, colon, immunohistochemistry

RÉSUMÉ

Effet du 17β-oestradiol sur l’expression du récepteur de la leptine dans le côlon de rats ovariectomisés

Le but de cette étude est de déterminer les effets d’une ovariectomie et du 17β-oestradiol sur l’expression du récepteur de la leptine dans le côlon de la ratte. Au total, 36 rattes Sprague Dawley de 6 mois ont subi une ovariectomie et ont été traitées par du 17β-oestradiol (25 mg par voie intramusculaire) ou ont servi de contrôles (0.2 mL d’huile de sésame par voie intramusculaire) avant d’être euthanasiées à 18, 90 ou 162 heures (6 animaux traités et 6 contrôles pour chaque échéance). L’expression du récepteur de la leptine dans le côlon a été analysée par immunohistochimie. Une immuno-réactivité des épithéliums des cryptes dans le côlon a été mise en évidence et chez les contrôles et chez les rattes traitées. L’expression du récepteur de la leptine dans les cryptes a été significativement plus élevée chez les rattes traitées à 90 et 162 heures. Le marquage des plexus myoentériques n’a été observé que chez les rattes traitées et a progressivement diminué au cours du temps. Ces résultats montrent que le 17β-oestradiol induit l’expression du récepteur de la leptine dans le côlon chez le rat, ce qui suggère que certaines pathologies du côlon liées à une expression diminuée du récepteur de la leptine pourraient être induites par un déficit en oestrogènes chez les femelles.

Mots-clés : Ratte, ovariectomie, récepteur de la leptine, 17β-oestradiol, côlon, immunohistochimie

Introduction

The action of leptin is mediated by leptin receptor [14]. The leptin receptor [obesity receptor (Ob−R)] is a member of the class I cytokine receptor family [40]. Six isoforms of Ob−R have been identified [38]. The long form, Ob-Rb has a long cytoplasmic region containing several motifs required for signal transduction [12]. The four short isoforms, Ob-Ra, Ob-Rc, Ob-Rd and Ob-Rf, have shorter intracellular tails [38]. Ob-Re has no transmembrane domain and is a soluble form of the receptor [34]. Leptin receptor is found in stomach [33], small intestine [2] and colon in the gastrointestinal tract [14]. There are some effects of leptin such as up-regulation of the butyrate uptake [6], proliferation [14] and angiogenesis [4]. Additionally it accelerates the healing of colonic anastomoses [35], controls the functions of mucus-secreting goblet cells [26] and shows an anti-inflammatory effect in colitis [5, 8].

Oestrogens have several effects on leptin production and leptin receptor expression. For example, serum leptin concentration decreases after ovariectomy [20, 32] but oestradiol supplement reverses the effect of ovariectomy on circulating leptin concentrations. Moreover, the serum leptin concentrations in post-menopausal women are lower than in premenopausal women [32]. The previous studies reported that Ob-Rb expression was lower in adipose tissue of ovariectomized (ovx) rats and 17β-oestradiol treatment restored the physiological receptor protein expression in the long-term study [19]. Besides, it was observed that 17β-oestradiol can increase the expressions of Ob−R protein and mRNA in ovariectomized rat dorsal root ganglion [9].

There are some evidences about changing bowel function in the postmenopausal period. It was stated that 38% of postmenopausal women reported altered bowel function, as opposed to 14% of premenopausal women. It was determined that laxative usage, gaseousness/excessive flatulence, and heartburn/acid regurgitation were more common among postmenopausal women than premenopausal women. Besides, it was found that the prevalence of irritable bowel syndrome (IBS)-type complaints peaked to 36% during the climacteric period (40–49 years) [36] and postmenopausal women with IBS had numerous symptoms relating to gas and bloating (abdominal distension, bloating, intestinal gas) than
menstruating women with IBS [7]. Also, it was emphasized that oestrogen usage did not affect gastrointestinal symptoms both premenopausal women and postmenopausal women [36]. On the other hand, it was determined that of faecal incontinence [24] and of constipation in postmenopausal women were high [23, 25] and it was stated that history of perianal surgery and presence of haemorrhoids were associated with constipation [23].

It is not clear if there is a crosstalk between oestrogen hormone and leptin receptor in the regulation of colonic function. Therefore, depending on the reduction in the concentrations of steroid hormones, especially oestrogen hormone in ovariectomized animals and postmenopausal women, change in the leptin receptor expression may occur in colon. As a result, because receptor signal transduction will be affected, the functions of the colon may be impaired and colon disorders may occur as irritable bowel syndrome. The aim of the present study is to directly investigate the effects of oestrogen on leptin receptor in colon of ovariectomized rats.

Material and Methods

ANIMALS AND EXPERIMENTAL DESIGN

In the present study, a total of 36 six-month-old Sprague-Dawley female rats were used. Rats were obtained from the Department of Laboratory Animals, Gülhane Military Medical Academy Research Centre, Ankara, Turkey. The rats were kept in a room with a temperature between 20 and 24°C. The animals were housed under a 12:12 hours light: dark (light from 08.00 to 20.00) cycle with free access to standard diet and water. All procedures were approved by the Ethics Committee at Afyon Kocatepe University, Turkey.

Rats were anaesthetized by ketamine (Alfamine, Alfasan Int. BV3440 AB, Voerden Hollanda) 21.2 mg/kg i.p. and xylazine (Rompun* enj, Bayer Turk Kimya San. Ltd. Sti. Istanbul) 4.2 mg/kg i.p. and underwent bilateral ovariectomy. Small incisions were made laterally on the sides of the back to expose the ovaries retroperitoneally. The ovaries were clamped and removed and the uterine tubes were ligated. The muscle and skin were then sutured.

A week after the operation, the ovariectomized rats were randomly divided into oestrogen-treated [sacrificed later at the 18th hour (O1), at the 90th hour (O2) and at the 162nd hour (O3)] and control [sacrificed later at the 18th hour (C1), at the 90th hour (C2) and at the 162nd hour (C3)] groups. Oestrogen treated ovariectomized (ovx) rats were intramuscularly injected with 25 µg 17β-oestradiol (Sigma E8875) diluted in sesame oil once a day, one, three and six times in O1, O2 and O3 groups (6 animals per group) respectively, whereas 0.2 mL sesame oil was administrated in control ovx rats one time (C1 group), 3 times (C2 group) or 6 times (C3 group) (6 animals per group) one a day.

Eighteen hours after the last injection, rodents were again anaesthetized and sacrificed by cervical dislocation; consequently, rats from the O1 and C1 groups were slaughtered at the 18th hour, those from the O2 and C2 at the 90th hour and those from the group O3 and C3 at the 162nd hour after the first injection. Tissue samples were taken from the distal colon and fixed immediately in 10% buffered formalin. After routine histological processing, tissues are embedded in paraffin and 4-5 µm sections were used for immunohistochemical staining.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry for Ob-R was performed using indirect Streptavidin–Biotin Peroxidase method. After deparaffinization and dehydration, to unmask antigens, an antigen-retrieval procedure was performed by treating the samples five times in a microwave oven at 700 W for 5 minutes each time in 10 mM citrate buffer (pH:6). After cooling at room temperature, the sections were rinsed in phosphate buffered saline (PBS) for 3 x 5 minutes. Then, slides were treated with 3% hydrogen peroxide (H₂O₂) in methanol for 10 minutes and washed with PBS for 3 x 5 minutes. After that, the sections were incubated overnight at 4°C with goat polyclonal Ob-R antibody diluted to 1:25 (Santa Cruz, m-18: sc-1834). Negative control slides were processed using PBS instead of the primary antibody. Following rinsing in PBS for 3 x 5 minutes, the sections were incubated with the biotinylated secondary antibody (Universal Dakocytomation LSAB + Kit, Peroksidase K0690) for 25 minutes at room temperature. After washing in PBS for 3 x 5 minutes, they were incubated with streptavidin–horseradish peroxidase (HRP) (Universal Dakocytomation LSAB + Kit, Peroksidase K0690) for 25 minutes at room temperature. Following rinsing in PBS for 3 x 5 minutes, the sections were incubated in DAB (3,3'-diaminobenzidine) (Zymed, 00-2020) for 3 minutes to reveal peroxidase. Then, the sections were rinsed in distilled water and all sections were counterstained with Harris’s haematoxylin.

Relative leptin receptor immunoreactivity was evaluated by two independent observers in a blinded fashion and given a score as follows: 0- no immunoreactivity (-); 1- weak immunoreactivity (+); 2- moderate immunoreactivity (++); 3- strong immunoreactivity (+++) [14].

STATISTICAL ANALYSIS

The degree of immunoreactivity between control and experimental groups were compared using Mann-Whitney U test of SPSS 10.0 package program. Values are presented as means ± standard errors. The values of p < 0.05 and p < 0.01 were considered as statistically significant.

Results

The Ob-R expression was observed in the cytoplasm in the crypt epithelium, plexus myentericus and also on a few connective tissue cells of lamina propria in colon. No Ob-R

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immunostaining was detected in negative controls in the colonic crypt epithelium or in the plexus myentericus.

The Ob-R expression was observed in the colonic crypt epithelium in both control and oestrogen groups (Figure 1). While the Ob-R expression was observed on the upper parts of crypts in the control groups (Figures 1A, 1B and 1C), the expression was determined to extend towards to the lower parts from the upper parts of the crypts in the oestrogen groups (Figures 1D, 1E and 1F). In addition, the staining intensity weakly decreased in the lower parts of the crypts. However, the immunolabelling was moderate both in control and oestradiol treated ovariectomized rats 18 hours after the first injection (Figures 1A and 1D) and the staining intensity was not significantly different between the C1 and O1 groups. By contrast, the Ob-R expression in the colonic crypt epithelium appeared as strengthened in oestrogen supplemented rats at the 90th (Figures 1B and 1E).

Figure 1: Ob-R immunoreactivity in the colon crypt epithelium from Sprague-Dawley ovariectomized rats intramuscularly injected with 25 mg 17β-oestradiol one, three and 6 times and sacrificed at the 18th, 90th and the 162nd hours after the beginning of the treatment, respectively (groups O1, O2 and O3) or injected only with the diluent (0.2 mL sesame oil) (groups C1, C2 and C3). The immunolabelling of the crypt epithelium and connective tissue (arrow) was moderate in controls at the 18th (1A) and 90th hours (1B) and weak at the 162nd hour (1C) whereas it was moderate in oestradiol-treated rats at the 18th (1D) and 162nd hours (1F) and strong at the 90th hour (1E). Immunohistochemistry (Streptavidin-Biotin Peroxidase method, counterstaining with Harris’s haematoxylin), Bar 100 μm.
and the 162nd (Figures 1C and 1F) hours compared to the corresponding controls (being moderate to strong instead of moderate in the group O2 and moderate instead of weak in the group O3); as reported in the Table I, differences between controls and treated animals were statistically significant (p < 0.05 at the 90th hour and p < 0.01 at the 162nd hour).

The expression of the Ob-R in the *plexus myentericus* was not detected in ovariectomized control rats (Figure 2A) whatever the time after injection and was only observed in the oestrogen treated groups (Figures 2B, 2C and 2D). Moreover, the staining intensity in treated rats gradually depressed between the 18th and the 162nd hours and while

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<tr>
<td>Control groups</td>
<td>(C1) 1.67 ± 0.21</td>
<td>(C2) 1.66 ± 0.21</td>
<td>(C3) 0.16 ± 0.16</td>
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<td>Treated groups</td>
<td>(O1) 2.17 ± 0.17</td>
<td>(O2) 2.50 ± 0.22</td>
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<td>p</td>
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Table I: Ob-R immunoreactivity in the colonic crypt epithelium from Sprague-Dawley ovariectomized rats intramuscularly injected with 25 mg 17β-oestradiol one, three and 6 times and sacrificed at the 18th, 90th and the 162nd hours after the beginning of the treatment, respectively (groups O1, O2 and O3) or injected only with the diluent (0.2 mL sesame oil) (groups C1, C2 and C3). Results are expressed as means ± standard errors (6 animals per group).

The Ob-R expression in the *plexus myentericus* was highly increased compared to the controls at the 18th and 90th hours (p < 0.01), no difference with the corresponding control group was obtained at the last time point (Table II).

**Discussion**

The leptin receptor expression has been determined in intestinal tract of various species such as humans [1] and chickens [22]. APARICIO et al. [1] reported that it was a faint Ob-Rb immunolabelling in the cytoplasm of large intestine epithelial cells and observed a moderate to strong expression on the membranes, especially apical membranes

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**Figure 2**: Ob-R immunoreactivity in the *plexus myentericus* from Sprague-Dawley ovariectomized rats intramuscularly injected with 25 mg 17β-oestradiol one, three and 6 times and sacrificed at the 18th, 90th and the 162nd hours after the beginning of the treatment, respectively (groups O1, O2 and O3) or injected only with the diluent (0.2 mL sesame oil) (groups C1, C2 and C3). No immunolabelling of *plexus myentericus* was seen in controls at the 18th (2A), 90th and 162nd hours whereas it was observed in oestradiol-treated rats and gradually declined between the 18th (2B), the 90th (2C) and the 162nd hours (2D). Immunohistochemistry (Streptavidin-Biotin Peroxidase method, counterstaining with Harris’s haematoxylin), Bar 100 μm.
The enteric nervous system regulates intestinal motility [3]. *Myentericus plexus* is characteristically found between the circular and longitudinal layers of smooth muscle that form the gut *muscularis externa* [13] and mainly regulates muscle activity [31]. In the present study, the leptin receptor expression was absent in the *plexus myentericus* from the ovariectomized rats but was restored in oestradiol treated ones suggesting that leptin may have important role on smooth muscle contraction under the control of oestrogen; therefore deficiency in oestrogen and the consecutive disappearance of the Ob-R expression in plexus could lead to colon motility irregularities and constipation in the postmenopausal period in women.

Butyrate is a well known colonic luminal short chain fatty acid (SCFA) [21] and a product of colonic fermentation of dietary fibres [10] that appears as important for the energy metabolism and normal development of colonic epithelial cells and has a mainly protective role in relation to the colonic disease [28]. Butyrate regulates colonic motility, increases colonic blood flow and may enhance colonic anastomosis healing. It may reduce the symptoms from ulcerative colitis and it may prevent the progression of colitis [37]. Butyrate inhibits cell proliferation by blocking cells in the G1/G0 phase of the cell cycle [10]. It induces Caco-2 cell apoptosis via the mitochondrial pathway [30]. On the contrary, KIEN et al. [15] stated that intracolonic butyrate may enhance intestinal growth during infancy. BUYSE et al. [6] reported that the luminal leptin up-regulated butyrate uptake in Caco2-BBE cell monolayer and ROUET-BENZINEB et al. [29] reported that leptin promoted HT-29 colon cancer cell survival from butyrate apoptosis. In the present study, reduction of the leptin receptor expression in the crypt epithelium of control groups may impair effects of leptin on butyrate function in colon. Also, it may induce irregularities in proliferation and apoptosis mechanisms and some colonic diseases such as colitis may occur in postmenopausal women and in ovariectomized animals.

Mucins protect the epithelium against constant attacks such as digestive fluids, microorganisms, polluting agents and toxins. They can distinguish two main families: secreted mucins or gel-forming mucins (MUC2, MUC5AC, MUC5B and MUC6) and membrane-bound mucins (MUC1, MUC3, MUC4, MUC12 and MUC17) [27]. EL HOMSI et al. [11] reported that leptin may contribute significantly to membrane-associated and secreted mucin production via a direct stimulation of colonic epithelial cells and the activation

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<td>(C1) 0.00 ± 0.00</td>
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<td>Treated groups</td>
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<td>(O1) 1.33 ± 0.21</td>
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<td>(O2) 0.66 ± 0.21</td>
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<td>(O3) 0.33 ± 0.21</td>
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Table II: Ob-R immunoreactivity in the plexus myentericus from Sprague-Dawley ovariectomized rats intramuscularly injected with 25 mg 17β-oestradiol one, three and 6 times and sacrificed at the 18th, 90th and the 162nd hours after the beginning of the treatment, respectively (groups O1, O2 and O3) or injected only with the diluent (0.2 mL sesame oil) (groups C1, C2 and C3). Results are expressed as means ± standard errors (6 animals per group).
of leptin receptors. They stated that leptin increased mucin expression by activating PKC-, phosphatidyl inositol 3-kinase-, and MAPK-dependent pathways. PLAISANCIE et al. [26] demonstrated that intracolonic leptin increased numbers of stained mucus cells and leptin acting from the luminal side was a powerful colonic secretagogue. They also obtained evidence that leptin increased rMuc2 mRNA contents in the colonic mucosa. Besides, YAN et al. [39] stated that the number of intestinal goblet cells decreased in ovariectomized rats, whereas it was significantly increased when oestriadiol benzoate was given to the ovariectomized rats. In the present study, the reduction in the leptin receptor expression in the crypt epithelium after ovariecctomy may alleviate the leptin effects on mucus secretion, leading to depletion in the protective and barrier function of mucus in colon mucosa. Therefore, some disorders or symptoms such as irritable bowel syndrome and constipation may occur.

As a conclusion, ovariecctomy reduces the leptin receptor expression in colon from rats whereas 17β-oestradiol treatment restores it, suggesting that the oestrogen hormone regulates the Ob-R expression in colon. Because of the recently demonstrated various roles of leptin and leptin receptor on the regulation of the intestinal motility, the crypt epithelium turn-over associating proliferation and apoptosis and on the protective mucin secretion, oestrogen depletion may induce decreases in the leptin function leading to pathogenesis of some colon disorders. However, further experiments are required for studying the direct involvement of sexual steroid on the regulation of the Ob-R expression in the colon and for elucidating the molecular roles of leptin receptor in colon function.

References


