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Changes in the subcellular and tissue location of estrogen and progesterone receptors in rat uterus after long-term treatment with analogs of gonadoliberin

Zmiany w subkomórkowej i tkankowej lokalizacji receptorów estrogenowych i receptora progesteronowego w macicy szczurów poddanych długoterminowemu działaniu analogów gonadoliberyny

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Abstract

Objectives: *Certain therapies with the use of analogs of gonadotropin-releasing hormone (GnRH, gonadoliberin) aim at achieving the effect of desensitization of the pituitary gland that causes inhibition of the hypothalamic-pituitary-gonadal axis. The resulting hormonal changes may influence the location and expression of estrogen and progesterone receptors, as well as their endogenous functions.*

The aim: *The aim of the study was to investigate whether long-term administration of low doses of dalarelin (GnRH agonist) and cetrorelix (GnRH antagonist) affected subcellular and tissue-specific location of ERα and ERβ estrogen receptors and progesterone receptor (PR) in rat uterus, as well as explore the extent to which the changes were reversible.*

Material and methods: *Analogs were administered to SPD adult females in the course of 3 months, at a dose of 6 μg/kg b.w. Afterwards, the ovaries and the uterus were resected – in the course of 4 weeks after treatment completion. Tissue paraffin-embedded samples were stained with hematoxyline-eosin for morphological studies or incubated with specific antibodies for the immunohistochemical studies (ABC method).*

Results: *GnRH analogs induced desensitization, resulting in specific and relatively persistent histological changes in the ovaries and the uterus. Strong nuclear reaction for ERα in the lining and the glandular epithelial cells in dalarelin-treated rats, and lack of expression changes in cetrorelix-treated rats, were observed in the uterus. Epithelial ERα expressions were accompanied by diminished ERβ and elevated PR expression, as well as diminished ERα and ERβ expression, and unchanged PR expression in the stromal and muscle cells, in both dalarelin- and cetrorelix-treated rats. The majority of the changes were reversible after treatment discontinuation.*

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Conclusions: *Long-term exposure to low doses of GnRH analogs causes morphological changes in the uterine tissues, accompanied by reversible changes of the ERα, ERβ and PR expression, possibly influencing tissue sensitivity. These changes indicate that agonist and antagonist regulate ERα expression by means of different mechanisms. A functional interaction between the receptors, depending on ERβ expression, direct influence of analogs on the local hormonal axes, and dose-dependent effects, cannot be excluded. After discontinuation of the analog treatment, the time needed for stabilization of ER and PR expression is shorter than the period of time required to restore histological structure of the uterus.*

Key words: **estrogen receptor α** / **estrogen receptor β** / **progesterone receptor** / / **GnRH agonist** / **GnRH antagonist** / **dalarelin** / **cetrorelix** / **long-term treatment** / **uterus** /

Streszczenie

Wstęp: *W terapiach hormonalnych z użyciem analogów GnRH, dąży się do uzyskania efektu desensytyzacji przysadki mózgowej powodującego zahamowanie osi podwzgórze-przysadka-jajnik. Wywołane zmiany hormonalne mogą wpływać na lokalizację i ekspresję receptorów estrogenowych i receptora progesteronowego w macicy, istotnie modyfikując ich endogenne funkcje.*

Cel: *Celem pracy było zbadanie, czy długoterminowe podawanie szczurom niskiej dawki agonisty receptora GnRH - dalareliny i antagonisty receptora GnRH – cetroreliksu, wpływa na subkomórkową i tkankową lokalizację receptorów estrogenowych ERα i ERβ oraz receptora progesteronowego (PR) w macicy, a także, w jakim stopniu te zmiany są odwracalne.*

Materiał i Metody: *Badania wykonano na dojrzałych samicach szczurów Spraque-Dawley. Oba analogi były podawane w ciągu trzech miesięcy, w dawce 6 μg/kg m.c. Po tym okresie czasu oraz w ciągu miesiąca od zakończenia iniekcji, pobierano jajniki oraz macicę. Do badań morfologicznych, skrawki parafinowe barwiono hematoksyliną i eozyną. Do badań immunohistochemicznych, skrawki inkubowano z odpowiednimi przeciwciałami poliklonalnymi. Wizualizacji kompleksów antygen-przeciwciało dokonano za pomocą metody ABC.*

Wyniki: *Analogi GnRH wykazały działanie desensytyzacyjne skutkujące charakterystycznymi i stosunkowo trwałymi zmianami histologicznymi w jajnikach i w macicy. W macicy zaobserwowano pojawienie się silnego jądrowego odczynu ERα w komórkach nabłonka wyścielającego i gruczołowego u szczurów traktowanych dalareliną oraz brak zmian tej ekspresji w komórkach szczurów, którym podawano cetroreliks. Zmianom nabłonkowym towarzyszyły: obniżona ekspresja ERβ oraz podwyższona ekspresja PR, a także obniżona ekspresja ERα i ERβ oraz niezmieniona ekspresja PR w komórkach zrębowych endometrium i mięśniowych myometrium zarówno szczurów traktowanych dalareliną, jak i cetroreliksem. Większość tych zmian była odwracalna zaraz po zaprzestaniu iniekcji.*

Wnioski: *W tkankach macicy, długoterminowe podawanie niskiej dawki analogów GnRH wywołuje zmiany morfologiczne, którym towarzyszą odwracalne zmiany ekspresji receptorów ERα, ERβ i PR mogące wpływać na wrażliwość tkanek. Zmiany te wskazują, że agonista i antagonista regulują ekspresję ERα poprzez różne mechanizmy. Nie można wykluczyć interakcji funkcjonalnej pomiędzy receptorami, zależnej od ekspresji ERβ, bezpośredniego oddziaływania analogów na lokalne osie hormonalne w macicy oraz efektów zależnych od wielkości dawki. Po odstawieniu analogów, czas powrotu ekspresji ER i PR do wartości kontrolnych jest krótszy niż czas potrzebny do przywrócenia struktury histologicznej macicy.*

Słowa kluczowe: **receptor estrogenowy α** / **receptor estrogenowy β** / / **receptor progesteronowy** / **agonista receptora GnRH** / **antagonista receptora GnRH** / / **dalarelina** / **cetroreliks** / **działanie długoterminowe** / **macica** /

Abbreviations: PR, progesterone receptor; ER, estrogen receptor; GnRH, gonadoliberin
Skróty: PR, receptor progesteronowy; ER, receptor estrogenowy; GnRH, gonadoliberyna

Introduction

Biological activity of gonadotropin-releasing hormone (GnRH, gonadoliberin) during the menstrual cycle consists mostly in direct regulation of pituitary gonadotropin secretion and indirect regulation of ovarian hormone synthesis, particularly estradiol and progesterone. Both steroids affect the target organs *via* receptors from the nuclear hormone receptor superfamily [1].

Estradiol may bind to two nuclear receptors – ERα and ERβ, and cell membrane receptors [2]. ERα and ERβ are encoded separately, by two different genes, but they share homology – particularly distinct in the DNA binding domain. Most authors claim that ERα acts as a dominant isoform [3, 4], and has a different role than ERβ. In cases when both isoforms are present in a cell, the latter is responsible for silencing the action of the former, whereas in the absence of ERα, ERβ can partially replace it [5, 6]. ER expression is most common in the reproductive tract, especially the uterus [4, 7].

It is also present in the bones, cardiovascular system, central nervous system and thymus. The level of expression of both ER receptors in particular tissues varies, and the effects of their activation – caused by estradiol binding – are the result of various transcriptional profiles [8]. In the uterus, ERα binds to 5184 sites even in the absence of ligand, and to over 17 thousand sites in chromatin if the ligand is present [9].

ER receptor expression depends on the phase of the menstrual cycle [7, 10, 11], age [12, 13], ovariectomy [3, 11, 14], estradiol or progesterone supply [3, 12, 14], or exposure to xenobiotics [15]. Expression of ER mRNA and protein was demonstrated in human uterus [16], as well as numerous species of mammals, among others, rats [3, 10, 17, 18], mice [19, 20, 21, 22], sheep [13], and monkeys [11]. Reproductive cycle in rats is infinitely shorter (4-5 days) than in humans, and has been divided into 4 phases: proestrus, estrus, metestrus and diestrus. Ovulation appears between proestrus and estrus, whereas corpora lutea develop during metestrus and function during diestrus. Despite these differences, ER expression in the epithelium, endometrial stroma and myometrial smooth muscle cells has been confirmed in both species. Trans-species tendency to higher ERα expression in those stages of the menstrual cycle that correspond to the proliferative phase, as compared to the secretory one, has been demonstrated [7, 10, 11]. ERβ expression in animals, including rats, remains relatively stable during the entire estrous cycle [10, 11]. Both, higher [7], and lower [23] ERβ expressions during the proliferative phase have been reported in humans. The exact mechanism of hormonal regulation of ER expression remains to be fully elucidated. In ovariectomized rats, estradiol has been demonstrated to decrease ERα expression in the lining and glandular epithelium [12], as well as cause the opposing effect, i.e. lower the expression in the glandular cells and increase it in the cells of the lining epithelium [19].

The progesterone receptor (PR) exists as two basic isoforms: PRA and PRB. They are encoded by the same gene but independently regulated by ovarian steroids in a tissue-specific manner. The level of their expression, both in the ovary and the endometrium, varies during different phases of the estrous cycle [24, 25]. They are susceptible to pharmacological modulation, among others, the analogs of gonadoliberin [26, 27]. The analysis of PR transcripts in breast cancer revealed their heterogeneity at the 5' region. The third discovered PR isoform - PRC – is characterized by molecular mass of approximately 60 kDa [28, 29]. Probably, the formation of different PR isoforms is connected with alternative splicing and insertion of extra exons [30, 31]. PRC has the shortest amino acid chain of all three PR isoforms and lacks transcriptional activity [30].

Regulation of expression of PRA and PRB receptor isoforms varies, depending on different uterine tissues and physiological conditions [25, 32]. In immature rats, a positive reaction to PRA and PRB is strongly manifested in the uterine epithelium and glandular cells, while remaining significantly weaker in stromal and myometrial cells. During a physiological cycle in mature rats, PR demonstrates immunoreactivity in the nuclei of uterine epithelial and stromal cells, as well as myometrial smooth muscle cells, although the reaction in the lining epithelium and the glands is weaker, as compared to younger individuals, and stronger in the endometrial stroma and the myometrium [33]. During the proliferative phase of the menstrual cycle in humans, estradiol is the positive regulator of receptor mRNA and protein synthesis, both PRA and PRB. Gradual increase of estrogen concentration is followed by a subsequent increase of PR expression in the glandular epithelium and stromal cells, reaching its maximum intensity between the proliferative phase and periovulatory period. Progesterone-dependent down-regulation of PR expression takes place during the secretory phase, when serum estrogen levels decrease and progesterone, released by the corpus luteum, starts to play the dominant role [34, 35]. PRB isoform expression in the glandular epithelium is extremely weak at the beginning of the proliferative phase, reaching maximum intensity mid-phase, and gradually decreasing toward the end. PRB expression in stromal cells remains to be rather low. During the secretory phase, both isoforms are detectable in perivascular stromal cells, what suggests progestin influence on the morphology and function of the spiral arteries [24, 36]. In myometrial cells of the uterine fundus, PRA and PRC isoforms have been found to be located predominantly in the cytoplasm, with the PRB isoform mostly traced in cell nuclei. PRC overexpression inhibits the activation of PRB isoform, what might be the ground for initiation of labor in humans [28, 30, 37]. mRNA expression level for PR is not as unstable as of the corresponding proteins, what indicates that receptor expression might be regulated *via* post-translational modification [33].

In some hormonal therapies, the effect of decreasing sex hormone synthesis and secretion, as well as inhibition of their action on target organs, can be achieved [38, 39]. It results from so called 'desensitization' or 'down regulation' of the pituitary gland and subsequent inhibition of the hypothalamic-pituitary-gonadal axis. Chemically modified GnRH analogs with long half-lives, ranging from several minutes to tens of hours, have been used to achieve such an effect during pharmacological treatment [40, 41, 42]. They are resistant to enzymatic degradation and their inhibitory action on the hypothalamic-pituitary-gonadal axis lasts long [38]. GnRH agonists trigger the release of gonadotropins (the flare up effect), which is accompanied by a temporary increase in the concentration of ovarian steroids, and followed by inhibition of gonadotropin secretion. The binding of antagonists to GnRH receptor (GnRHR) immediately inhibits FSH and LH, as well as steroid hormone secretion [43]. That mechanism has been applied in the treatment of hormone-dependent cancers, endometriosis, uterine leiomyomas, and precocious puberty [44, 45]. Hormonal changes observed during long-term therapies with GnRH analogs may influence ER and PR location and expression. As a result, analogs can significantly modify endogenous functions of these receptors and tissue sensitivity to hormones during therapy, as well as after treatment discontinuation.

The aim of the study was to investigate whether a long-term administration of a low dose of dalarelin and cetrorelix to rats:

1) affected ERα, ERβ and PR subcellular and tissue-specific location in the uterus,
2) the extent to which these changes were reversible.

Material and Methods

Animals

The study was performed on sexually mature female Spraque-Dawley rats, weighing 200-220 g. The animals were purchased from the Center for Experimental Medicine, Medical

University of Silesia. During the experiment, they were housed in cages, in standard conditions, a 12hr/12hr light cycle, and free access to feed and water. Each week, the animals were weighed to adjust the dose of analogs. The study protocol was accepted by the Local Ethics Committee.

GnRH analogs

Dalarelin acetate (BAPEX, Ryga) – courtesy of prof. F. Ryszka (Biochefa; Sosnowiec, Poland), and cetrorelix acetate (ChemPep, Inc**.** USA), were used in the experiment. Dalarelin {(D-Ala^6, NHET10)-GnRH} is a new GnRH agonist that has not yet been introduced into clinical practice [46]. Cetrorelix {(Ac-D-Nal(2)^1, D-Phe(4Cl)^2, D-Pal(3)^3, D-Cit^6, D-Ala^{10})-GnRH} is a known GnRH antagonist [47]. Both analogs were dissolved in 0.9% sodium chloride for the injection.

Procedure

The animals were divided into 6 control and 12 study groups (6 rats in each). Every morning, animals from the study groups received subcutaneous injections of dalarelin or cetrorelix at a dose of 6 µg/kg b.w. The control rats received a placebo solution of 0.9% sodium chloride (Table 1). Both, analogs and placebo were administered for 1, 2 and 3 months. After a specified period of time, as well as after 1, 2 and 4 weeks following the 3-month treatment, the animals were sectioned to excision of the ovaries and uterus. Liquid was removed from the uteri by making small incisions on both uterine horns and drying the organs with blotting-paper. The control rats, at the moment of decapitation, were at proestrus phase of the reproductive cycle.

Histological staining

Tissue samples taken from the ovary and the uterus were fixed in 10% (v/v) solution of buffered formalin for 24 h at 4°C, and then dehydrated, cleared in xylenes and embedded in paraffin. Tissue sections (5 µm) were mounted on silane coated slides, de-waxed, rehydrated and stained with hematoxyline-eosin.

Immunohistochemical studies

Deparaffinized sections were treated with 10 mM citrate buffer, pH 6, or Tris-EDTA pH 9 in water bath (45 min. at 95°C) for antigen retrieval, and cooled for at least 30 min. Afterwards, the slides were washed in distillated water and phosphate buffered saline (PBS) containing 0.05% Tween 20 (v/v). Nonspecific binding was reduced by incubation in 1% BSA for 60 min. Next, the slides were incubated with rabbit anti-progesterone receptor (sc-538), anti-estrogen receptor α (sc-543; Santa Cruz Biotech. Inc., USA), and anti-estrogen receptor β (ab3577; Abcam, Cambridge, USA) antibodies in a humidified chamber for 22h at 4°C. Endogenous peroxidase activity was blocked for 30 min. with 0.3% H_2O_2. After washing in PBS-Tween 20, the sections were incubated with biotinylated goat anti-rabbit immunoglobulins (Vector Laboratories Inc., Burlingame, USA) for 30 min., and then with avidin-biotinylated peroxidase complex (Vector) for 30 min. Bound antibodies were visualized with diaminobenzidine (DAB) and H_2O_2 in PBS, pH 7.5, according to the manufacturer's instructions (Vector). Finally, the tissues were stained with Gill's hematoxylin, dehydrated, and cover-slipped. Negative controls were performed by substituting the primary antibodies with rabbit IgG.

Results

Ovarian and uterine tissue morphology

The ovaries and uteri of control rats exhibited features typical of the follicular/proliferative phase. The applied dose of GnRH agonist and antagonist induced antiovulatory effects, resulting in various histological changes in rats. The histological changes caused by dalarelin and cetrorelix treatment had been described in detail earlier [48] and are discussed below. These changes confirmed the desensitization efficiency of the applied dose of analogs.

ER and PR immunodetection in the rat uterus

ERα (Figure 1)

Controls: A relatively strong ERα receptor expression in the cytoplasm of the lining epithelium, glandular, and myometrial cells, and weak expression in the connective tissue stromal cells of the endometrium, were observed. Extremely weak nuclear reaction was detectable in single glandular cells and a greater number of myometrial smooth muscle cells. Vascular endothelial cells did not exhibit ERα expression.

Dalarelin: In the course of dalarelin treatment, nuclear reaction was noted in most cells of the uterine lining and glandular epithelium. Loss of ERα expression was observed in the endometrial stromal and myometrial cells: significant in the stroma – distinctly visible since the first month, and gradual in the myocytes – until the third month of the experiment.

Cetrorelix: The antagonist did not cause visible changes of the ERα nuclear expression in the uterine lining and glandular epithelium, but diminished the cytoplasmic reaction of the receptor protein. In the endometrial stromal cells, the positive reaction started disappearing already in the first month, and disappeared altogether in the uterine myometrium in the third month after treatment commencement.

Table I. Control and experimental groups of rats used in the study. Rats from I, II, III and III+4 groups were 4-, 5-, 6- and 7-month-old, respectively, n=6.

Injections	Duration of the treatment			Time after treatment discontinuation		
	1 month (I)	**2 months** (II)	**3 months** (III)	**1 week** (1)	**2 weeks** (2)	**4 weeks** (4)
Placebo (K)	IK	IIK	IIIK	IIIK+1	IIIK+2	IIIK+4
Dalarelin (D)	ID	IID	IIID	IIID+1	IIID+2	IIID+4
Cetrorelix (C)	IC	IIC	IIIC	IIIC+1	IIIC+2	IIIC+4

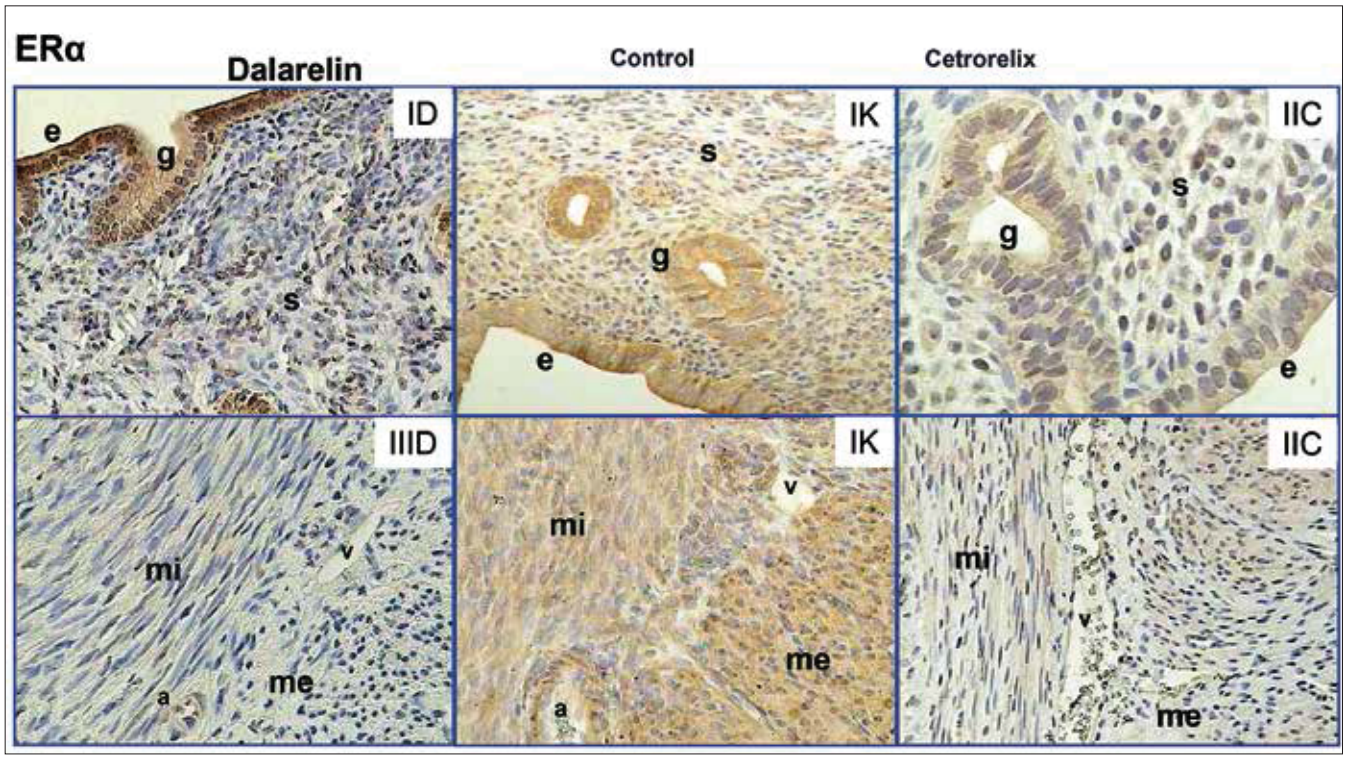

Figure 1. ERα immunolocalization in the uterus of rats administered placebo for one month (IK), dalarelin for one (ID) and three months (IIID) and cetrorelix for two months (IIC). Abbreviations: e – lining epithelium; g – glandular epithelium, s – endometrial stroma, mi – muscularis interna, me – muscularis externa, a – arteries, v – veins. Microscope magnification: 200x.

ERβ (Figure 2)

Controls: ERβ expression was observed in epithelial, glandular, stromal and smooth muscle cells in the uteri of control rats. In each of the locations, nuclear and cytoplasmic expression was visible in most cells. Also, ERβ expression was found in vascular endothelial cells located within the endometrial stroma and uterine muscularis.

Dalarelin: Dalarelin lowered ERβ expression in the uterine epithelium and glandular cells already after 1 month of treatment. A similar tendency was noted in stromal and myometrial cells since the second month of the analog therapy, both in the nuclei and the cytoplasm.

Cetrorelix: The antagonist evoked a stronger inhibiting effect on receptor expression as compared to the agonist in all uterine tissues. However, loss of expression in smooth muscle and stromal cells started since the first month of treatment.

PR (Figure 3)

Controls: Positive reaction to PR, of varying intensity, was observed in some nuclei of the lining and glandular epithelium, stromal endometrial cells and cells of myometrium. Diminished immunoreactivity was found in the cytoplasm of epithelial and smooth muscle cells.

Dalarelin: PR expression in the nuclei of glandular and epithelial cells was significantly stronger as compared to controls, whereas in the smooth muscle cells it was similar to controls. An additional cytoplasmic reaction, especially in the lining epithelial and glandular cells, was noted after 2 and 3 months of dalarelin treatment. A positive reaction in endometrial stromal cells, comparable to controls, was present mostly within the cell nuclei. In the myometrial cells, the positive reaction, both cytoplasmic and nuclear, also remained at the level comparable to controls.

Cetrorelix: After 1 month of cetrorelix administration, immunoreactivity in the surface epithelial and glandular cells remained at the level of controls. During the second and third month of the analog injections, PR nuclear expression in some of these cells was notably stronger as compared to the first month, though no-reaction sites were also detectable. The reaction within endometrial stromal cells, although stronger in some sites, in fact remained at the level of controls and was located predominantly in cell nuclei. No distinct changes in PR expression in smooth muscle cells, located in the cytoplasm and cell nuclei, were noted after 1, 2 and 3 months of injections.

ERα, ERβ and PR location changes after discontinuation of analog treatment (Figure 4)

Discontinuation of both, dalarelin and cetrorelix injections resulted in restoration of primary subcellular and tissue location of ERβ and ERα receptors already in the first and second week of the observation, respectively.

In the course of a 30-day period following dalarelin discontinuation, nuclear reaction to PR in the majority of epithelial and glandular cells was less distinct than during the 2- and 3-month analog therapy, whereas the cytoplasmic reaction remained at a level similar to the corresponding control group. Regardless, a small part of epithelial cells continued to demonstrate strong immunoreactivity. Smooth muscle and stromal cell immunoreactivity did not significantly alter after analog treatment discontinuation.

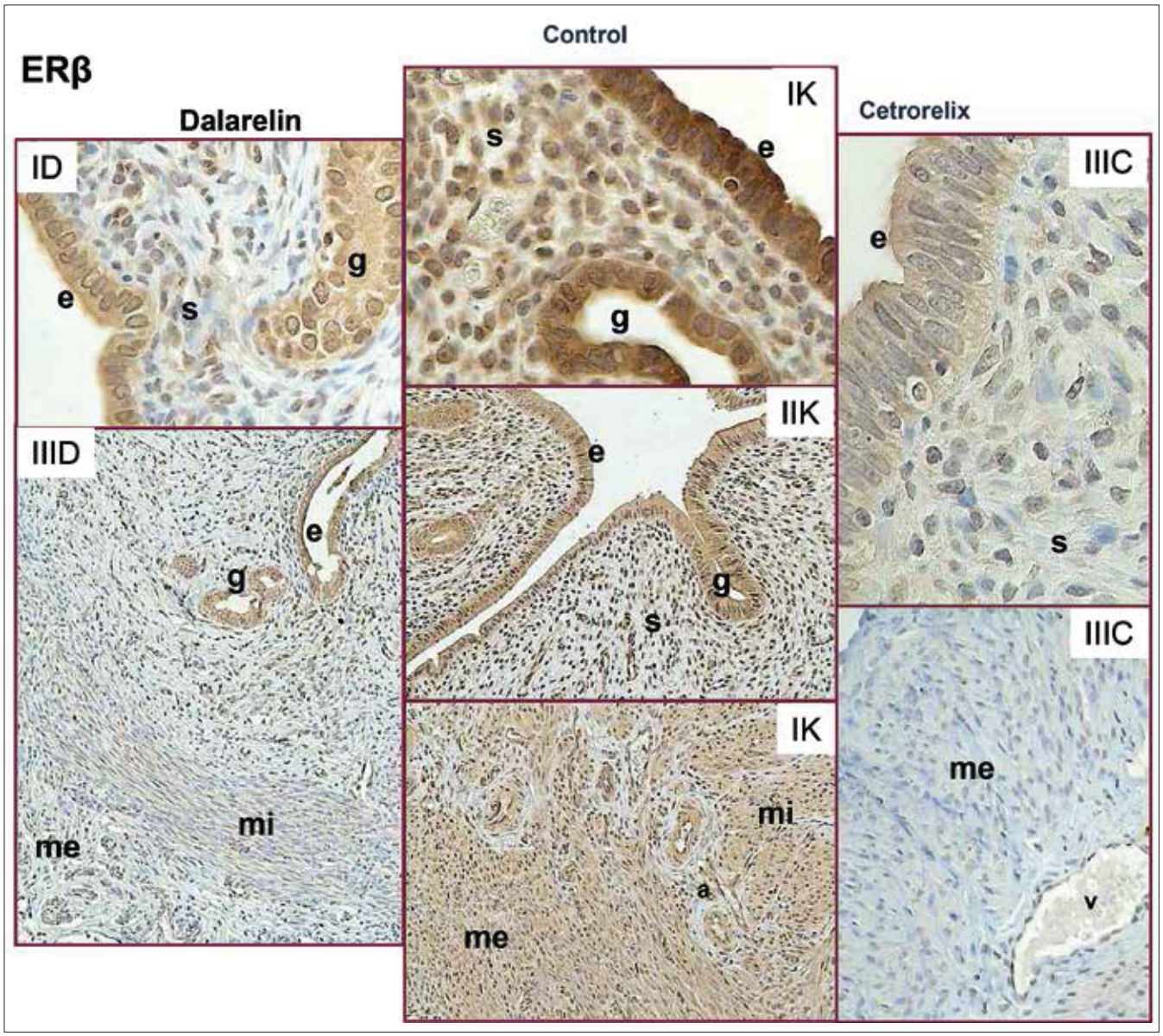

Figure 2. ERβ immunolocalization in the uterus of rats administered placebo for one (IK) and two months (IIK), dalarelin for one (ID) and three months (IIID) and cetrorelix for three months (IIIC). Abbreviations: e – lining epithelium; g – glandular epithelium, s – endometrial stroma, mi – muscularis interna, me – muscularis externa, a – arteries, v – veins.
Microscope magnification: upper pictures: 400x; IIK and lower IIIC: 200x; IIID and lower IK: 100x.

Simultaneously, cetrorelix discontinuation did not result in changed PR expression in smooth muscle and stromal cells, either as compared to controls or the study groups. Receptor expression within the epithelium and the glands was weaker as compared to group III, locally it was lost completely, but some cells maintained strong immunoreactivity.

Discussion

Certain hormonal therapies with chemically modified GnRH agonists and antagonists aim to achieve the effect of desensitization of the pituitary gland. In rats, doses of GnRH antagonists as low as 2 μg/rat, or lower, have been known to cause desensitization, resulting in the suppression of GnRH-dependent hormonal axes and inhibition of ovulation [47]. Such doses were sufficient to cause GnRHR-dependent decrease in FSH and LH concentration in the blood [49]. Chronic treatment of adult rat females with GnRH agonists interrupted the cycles, caused ovarian atrophy, and a decrease in the weight of the uterus. These changes were reversible after treatment discontinuation [50].

Previously, we described various histological changes in the ovaries and in uterus of rats after long-term treatment with dalarelin and cetrorelix, at a dose of 6 μg/kg b.w. [48]. During the entire period of exposure, an antiovulatory effect of both GnRH analogs was reached, although the agonist and antagonist seemed to have acted through different mechanisms. They resulted in structural changes of the uterine wall tissues. After long-term treatment with dalarelin the weight of the ovaries approximately doubled the ovarian weight studied in controls. There were features of luteinization in the ovaries and features specific for early secretory phase in the uteri. It seems that the

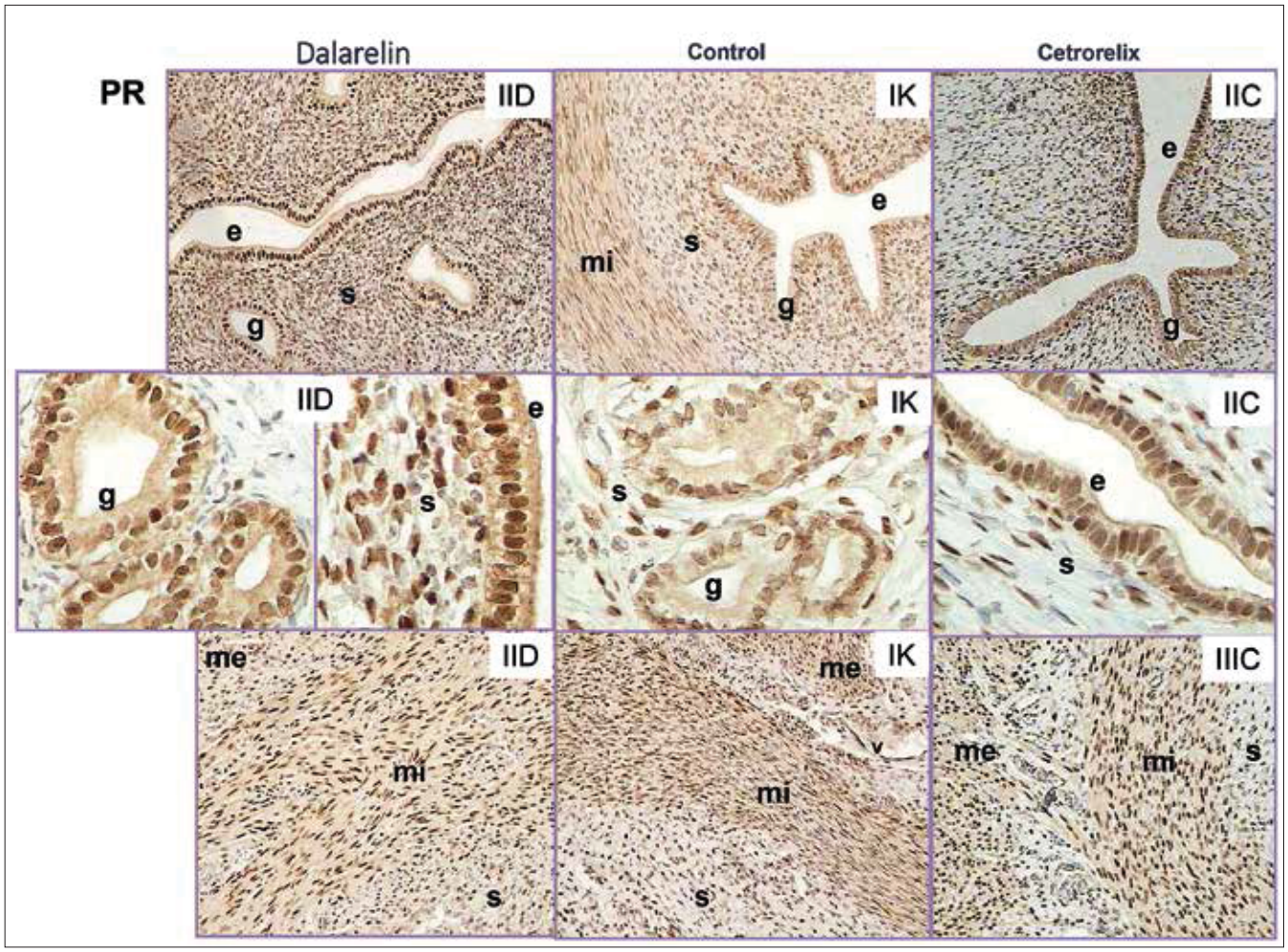

Figure 3. PR immunolocalization in the uterus of rats administered placebo for one month (IK), dalarelin for two months (IID) and cetrorelix for two (IIC) and three months (IIIC). Abbreviations: e – lining epithelium; g – glandular epithelium, s – endometrial stroma, mi – muscularis interna, me – muscularis externa, v – veins. Microscope magnification: upper and lower pictures: 100x; other pictures: 400x.

secretory activity (i.e. progesterone secretion) of these corpora lutea might have been very poor because the cells in uteri of dalarelin-treated rats were characterized by features specific for early, but not for advanced, secretory phase, and the weight of the uteri of dalarelin-rats was about 50% lower as compared to controls. On the other hand, it is known that short-term treatment of rats with GnRH agonist triptorelin or antagonist cetrorelix, at a dose of 10 µg/kg b.w., slightly decreased or did not cause any changes in the weight of the uterus, respectively [43].

In turn, we found that in cetrorelix-treated rats, the development of ovarian follicles was inhibited, mostly at the stage of maturing follicles, and corpora lutea were absent [48]. In the uterus, the expansion of the uterine endometrium, resembling tissues of late proliferative phase in which glands do not display secretory activity, was observed. These changes were accompanied by a 50% decrease in the weight of the ovaries and no significant changes in the weight of the uteri, when compared to the control group. In other studies, a 3-month treatment of rats and monkeys with detirelix, a GnRH antagonist, resulted in a significantly decreased weight of the uterus [51].

In general, the histological changes observed in the ovaries and in the uterus, were stable and persisted for 2-4 weeks after the last injection of both analogs [48]. A lengthy period of time required for uterine recovery after discontinuation of the analog treatment indicated that long-lasting hormonal dysfunction could occur after therapy with both synthetics.

Studies with ERα, ERβ and PR knockout mice revealed that the uterine structure and weight were closely connected with the function of estrogen and progesterone receptors. Receptor proteins, which are products of these genes, have different functions in uterine tissues. ERα knockout mice exhibit a hypoplastic uterus with significantly thinner walls, and the mice are infertile [22, 52]. In turn, the uteri of ERβ knockout mice are more expanded, and contain numerous glands. Their cells exhibit higher proliferative index, PR expression and growth factor secretion. At the same time, the mice are fertile but the litter sizes are smaller [20]. In general, ERα is believed to have an uterotrophic effect, while ERβ retards growth. PR knockout female mice are also infertile. Their uteri exhibit abnormal response to estradiol and progesterone. Morphologically, they resemble organs exposed to estrogens alone, i.e. show signs of hypertrophy, swelling of the walls with inflammatory infiltration and hyperplastic epithelium. Decidual transformation is not possible in such uteri [53, 54].

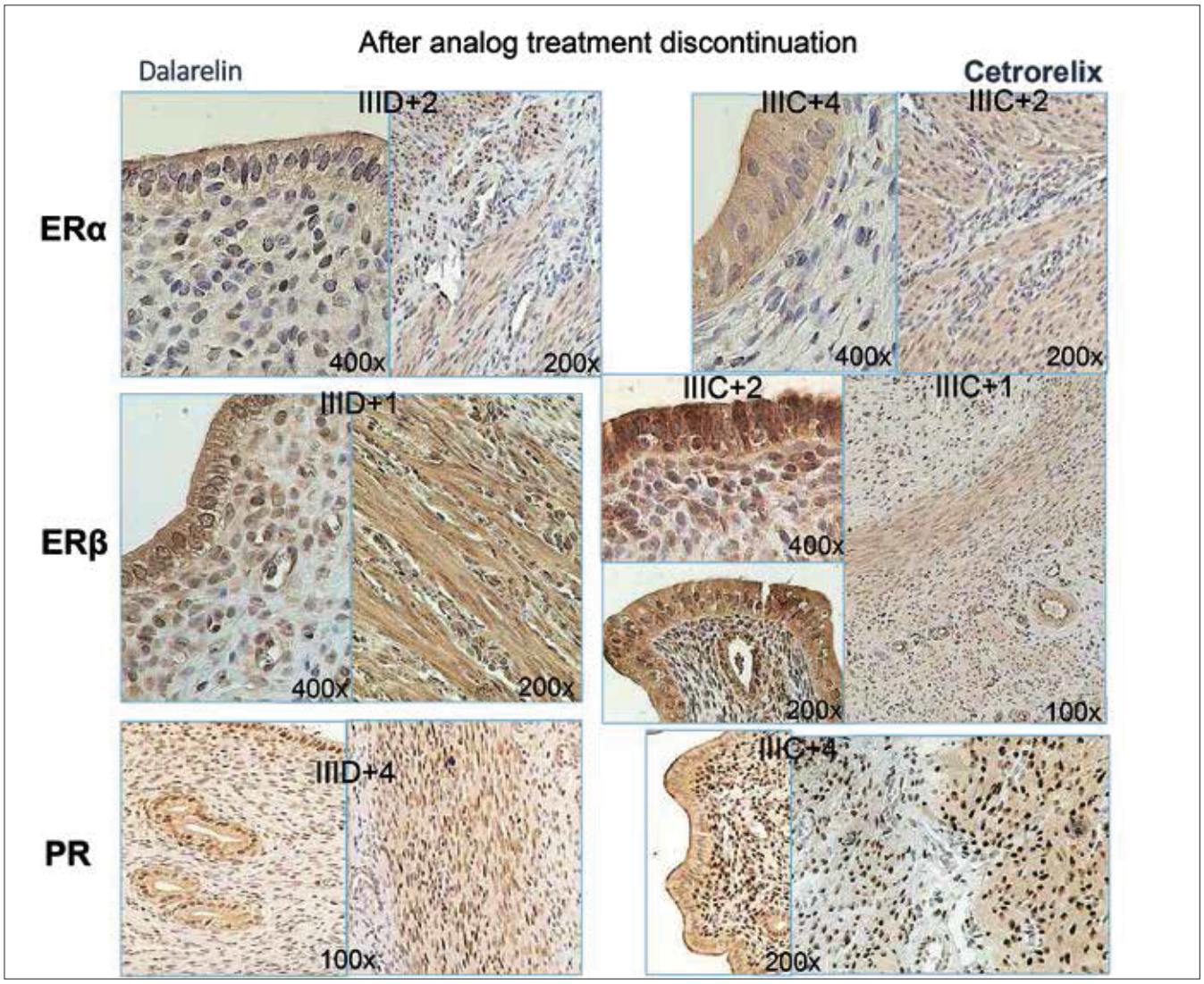

Figure 4. ERα, ERβ and PR immunolocalization in the uterus of rats after one (III+1), two (III+2) and four (III+4) weeks following the three month of dalarelin (D) and cetrorelix (C) treatment duration. Microscope magnification is also shown.

The role of ER and PR receptors in various uterine tissues is not identical. The cells of the lining epithelium do not proliferate in response to estradiol alone but growth factors paracrinally released by endometrial stromal cells, where the ERα is activated. The mechanism of increased PR expression – one of the classic estrogen targets – is similar in their case [52, 55]. In turn, progesterone antagonizes the mitogenic effect of estradiol with two strategies: 1) direct mechanism – by affecting PR in the lining epithelium; 2) indirect mechanism – through paracrinal action on the stromal PR. Only stromal PR is responsible for inhibition of the uterine mass growth [56, 57]. Mice with selectively suppressed ERα or PR expression only in the uterine epithelium are infertile, what is the consequence of abnormal implantation and disrupted decidual transformation [55,57]. Furthermore, epithelial ERα may directly stimulate lactoferrin expression and generate anti-apoptotic signals in cells [55]. Direct PR actions in the epithelium include induction of Areg (Amphiregulin – cytokine belonging to the epidermal growth factor family), Gata1 (GATA family) and Cyp26a1 (P450 cytochrome connected with metabolism of retinoic acid) [57]. The role of ER in the development of endometrial cancer and uterine myomas has been widely recognized [4, 7].

In our study, we proved differential ERα and ERβ expression in uterine epithelial, glandular, stromal and smooth muscle cells in intact rats at proestrus. Furthermore, we demonstrated ERβ expression and lack of ERα expression in endothelial cells of endometrium and myometrium in rats. Our findings are consistent with reports of other authors [3,11-13, 15]. Our immunohistochemical images from the control group are very similar to findings reported by Chen et al., in their study on mice [22].

We found that, similarly to morphologic changes occurring during GnRH administration, the profile of ER and PR receptor expression is also determined in the first month of analog injection. Prosecretory changes in the lining and glandular endothelium of a uterus, caused by dalarelin, were accompanied by increased nuclear expression (activation) of ERα. The activation, with simultaneous lack of ERα receptor activation in the endometrial stroma, did not induce typical pro-proliferative changes. Demonstration of features characteristic for early prosecretory changes in the epithelial cells may be associated with

PR activation in the uterine epithelium, resulting in the inhibition of epithelial proliferation [56, 57]. In turn, activation of PR expression by cetrorelix did not cause pro-secretory, but rather pro-proliferative changes in the epithelium. Morphological effects in the uterine epithelium indicated lack of changes typical for progesterone action. Pro-proliferative changes in the lining and glandular epithelium caused by cetrorelix were connected with unaltered expression of the epithelial ERα. Increased uterine epithelial ERα expression, stimulated by dalarelin, and absence of change after administrating cetrorelix, were accompanied by its decrease in stromal and smooth muscle cells due to the action of both analogs. Thus, dissimilarity of ERα expression in uterine epithelia is the main difference between the consequences of GnRHR agonist and antagonist actions, what might be connected with morphological differences and dissimilar mechanisms of desensitization. The relationship may take the form of an indirect regulation, stemming from decreased concentrations of sex hormones accompanying the process of desensitization of the pituitary gland, and/or a direct effect of analogs on the local hormonal axis in the uterus [58].

Different ERα epithelial expression caused by the agonist and antagonist may be a result of functional interaction of ERα with ERβ and PR receptors. The changes in ERβ and PR were observed after administration of both analogs, namely: elevated PR expression in uterine epithelia, unchanged PR expression in stromal and muscle cells, and diminished ERβ expression in all layers of the uterine wall. Most of these changes were reversible after treatment discontinuation. The similarities between the effect of cetrorelix as compared to dalarelin indicate that expression of stromal ERβ may be key in epithelial PR expression. If the changes are to be linked with the mechanism of estrogen signaling, PR activation in the epithelia may turn out to be more the result of suppressed ERβ in the epithelia, stroma or smooth muscle cells, than the epithelial activation of ERα. Inhibition of the expression of stromal ERα attests to lack of potential stromal ERα-epithelial PR paracrine interactions. However, diminished ERβ expression has been known to be strongly related to increased PR expression [20, 55]. Thus, long-term administration of both, dalarelin and cetrorelix resulted in reversible changes in the location and expression of ERβ and PR receptors, and in a similar manner for both analogs. Therefore, it cannot be excluded that diversified effect of ERα expression after administration of agonist and antagonist is caused by dissimilar efficacy of the applied analog dose, and that the effect of ERα induction might be achieved after larger doses of cetrorelix. Silvestri and Fraser used another GnRH antagonist – tevarelix, at a single dose of 12 mg/kg, and reported similar findings regarding ER nuclear expression in the uterine epithelia and endometrial stroma of monkeys, but demonstrated diminished PR expression [11].

The mechanism of regulation of ER expression, both by physiologic factors and drugs, remains to be fully elucidated. Reports on decreased ERα and ERβ expression in the uterus after ovariectomy [11], as well as their increased expression, especially of ERα [3], are conflicting. Numerous clinical trials to apply gonadoliberin analogs aim to decrease the size of uterine leiomyomas, where the primary expression of ERα and PR is greater than in normal uterine smooth muscle cells [34, 59]. ERα expression in healthy uterine muscularis does not change after long-term treatment [34, 60], so the expression of ERα in leiomyomas tissues may be the predictor of response to GnRH analogs [61]. Diminished PR expression, both in the leiomyomas as well as the surrounding tissues, was observed after 2- or 3-month therapy with goserelin [34, 62]. The literature often reports increased ERα [27, 63] and PR [63] expression after, for example, tryptorelin or goserelin. That phenomenon might be partially explained by the effect of the applied doses on the decrease in ligand concentration (endogenous hormone), resulting in compensatory up-regulation of the specific receptors.

Conclusions

To sum up, during a long-term exposure to low dose of GnRH analogs, a persistent antiovulatory effect can be reached. Desensitization effectiveness resulted in specific histological changes in the ovaries and the uterus, which were stable even for 4 weeks after the last injection of both analogs. These effects were accompanied mainly by reversible, strong activation of epithelial ERα in dalarelin-treated rats and unchanged ERα expression in cetrorelix-treated ones. Both analogs caused disappearance of ERα in the endometrial stromal and myometrial muscle cells, weaker ERβ expression in all uterine tissues, enhanced PR expression in the uterine surface and glandular epithelium, and unchanged PR expression in stromal and muscle cells. Most of these changes were reversible after treatment discontinuation. The time required for ER and PR expression stabilization was shorter than an extended period of time necessary for structural uterine recovery, after treatment discontinuation. These changes should be quantified but, if confirmed, they would indicate that the used agonist and antagonist affect ERα by different mechanisms. However, functional interaction between receptors characterized by the primary role of ERβ, direct analog effect on the local hormonal axes in the uterus, and dose-dependent analog effects, cannot be excluded.

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