doi:10.4149/neo_2009_05_448

Estrogen-dependent Regulation of PPAR-γ Signaling on Collagen Biosynthesis in Adenocarcinoma Endometrial Cells

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Received 21 November, 2008

The link between estrogen and metabolic developmental factors of endometrial carcinoma is well established. PPAR- γ, (an important modulator of metabolism) and estrogen receptor belong to a family of nuclear hormone receptors that were shown to interact with each other. The interaction may affect transcriptional activity of these transcription factors. The anti-diabetic troglitazone (TGZ) is well known PPAR- γ ligand. The effect of troglitazone-induced PPAR- γ activation on estrogen-dependent stimulation of collagen biosynthesis was studied in the Ishikawa endometrial adenocarcinoma cell line. We have found that the presence of estrogen activity in growth medium (1nM) augmented collagen biosynthesis in the cells. An addition of PPAR- γ agonists, as troglitazone or clofibrat to the growth medium induced inhibition of collagen biosynthesis. The inhibition was effective only when estrogen receptor was stimulated, since removal of estrogen receptor by ICI 182- 780-dependent degradation did not affect collagen biosynthesis. The mechanism of the inhibition was found at the level of NF-kB (known inhibitor of collagen gene expression) and MAPK signaling. PPAR- γ ligands stimulated expression of NF-kB, while they inhibited expression of p-38 but not ERK1/ERK2. The data document for the first time that inhibitory effect of PPAR-γ ligands on collagen biosynthesis in endometrial adenocarcinoma cells requires functional estrogen receptor.

Key words: PPAR- γ, PPAR- γ agonists, collagen, estrogen, Ishikawa cells

Endometrial carcinoma is one of the most common gynecological malignancies. In younger, perimenopausal and early postmenopausal women endometrial adenocarcinoma is considered to be an estrogen dependent disease [1]. In older women the malignancy is not related to estrogens. Although the etiology of endometrial carcinoma is still controversial the link between estrogen and metabolic factors (as an excessive fat consumption, overweight, hypertension and insulin resistance) in endometrial carcinogenesis has been known for many years. Estrogen dependency of endometrial carcinoma is fully established by epidemiological data and the therapeutic efficacy of progestagen and aromatase inhibitor therapy [2]. Estrogens profoundly influence biology of normal endometrial cells and neoplastic cells by regulation of basic cellular processes as proliferation, apoptosis and differentiation. The exact role of metabolic disturbances in the development of endometrial carcinoma is not known. The specific metabolic pathways found in obesity and diabetes may contribute to increase in circulating concentration of bioavailable estrogen from extraglandular source of aromatization of androgens [3].

Estrogens are well known stimulators of collagen biosynthesis and cell growth in several cell types [4, 5, 6]. Collagens are not only structural tissue proteins but they play also important role as a signaling proteins, acting through integrin class of surface adhesion receptors [7]. The interaction between cells and collagen can regulate cellular gene expression, differentiation, growth and plays an important role in tumorigenicity and invasiveness [8, 9]. Therefore any alterations in collagen metabolism may influence cell growth and metabolism.

Estrogens are involved in regulation of many physiologic and pathologic processes. Their activity is regulated by multiple factors [10]. Peroxisome proliferator-activated receptor-γ (PPAR- γ) may represent one of them since it has been reported that estrogen receptor is capable of interacting with PPAR- γ [11] PPAR- γ belongs to a family of nuclear hormone receptors that include estrogen and thyroid hormone receptors [12]. They are known as a ligand-activated transcription factors. Among specific PPAR- γ agonists are natural lipophilic agents, mainly arachidonic acid metabolites, and

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polyunsaturated fatty acids [13] as well as synthetic thiazolidinediones (TZD), the new class of anti-diabetic drugs that improve insulin sensitivity in type 2 diabetes [14, 15]. They are represented by troglitazone, rosiglitazone, pioglitazone and ciglitazone. These, as well as natural agonists, when bound to PPAR- γ activate its association with retinoid X receptor (RXR). Then the complex is binding to specific recognition sites of target genes and regulates its expression [16].

It is well established that at least in fibroblasts, agonists of PPAR- γ evokes antiproliferative activity, reduces collagen synthesis and secretion and decreases metalloproteinase-9 (MMP-9) activity [17]. PPAR-gamma is expressed in many types of cancer and it is well established that activation of the receptor by either natural or pharmacologic ligands may inhibit cell proliferation and induce apoptosis [12]. It suggests that knowledge of the mechanism of PPAR-gamma activation in cancer cells may contribute to novel approaches for pharmacotherapy of neoplastic diseases.

The current study was therefore undertaken to characterize the effect of troglitazone-induced PPAR- γ activation on estrogen-dependent stimulation of collagen biosynthesis in the Ishikawa endometrial adenocarcinoma cell line.

Materials and methods

Materials. L-glycyl-L-proline, L-proline, and Dulbecco`s modified Eagle's medium with or without phenol red (DMEM), bacterial collagenase, sodium bicarbonate, penicillin, streptomycin, fetal bovine serum (FBS) or controlled process serum replacement I, (CPSR1, Sigma Chemical Co.) used in cell culture, Dulbecco's phosphate buffered saline (DPBS), 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate reagent (BCIP/NBT), monoclonal (mouse) anti- phosphor - MAPK (p-38, ERKs) antibody Anti–Rabbit IgG antibody, Anti–Mouse IgG antibody, were purchased from Sigma Chemicals as were most other chemicals used. Polyclonal (rabbit) Anti - NF-κB antibody, polyclonal (rabbit) collagen type I antibody were obtained from Santa Cruz Biotechnology, INC. Troglitazone and Clofibrat were purchased from Alexis Biochemicals. ICI 182,780 was received from Tocris Bioscience.

Nitrocellulose membrane (0.2 μm), sodium dodecylsulphate (SDS), polyacrylamide, molecular weight standards and Coomassie Briliant Blue R-250 were received from Bio-Rad Laboratories, USA. L-5(^{3}H) proline (28 Ci/mmol) was purchased from Amersham, UK.

Ishikawa cultures. Endometrial carcinoma Ishikawa cells were maintained in DMEM without phenol red supplemented with 10% CPSR1, 50 μg/ml penicillin, 50 ug/ml streptomycin at 37^{0}C in a 5% CO_2 incubator. Cells were cultured in Costar flasks and subconfluent cells were detached with 0.05% trypsin, 0.02% EDTA in calcium-free phosphate buffered saline, counted in hemocytometers and inoculated at 5 x 10^5 cells per well of 6-well plates (Nunc) in 2 ml of growth medium (DMEM without phenol red with 10% CPSR1). Cells reached about 80% of confluency at day 2 after plating and in most cases such cells were used for the assays.

Western blot analysis. Slab SDS/PAGE was used, according to the method of Laemmli [18]. After SDS-PAGE, the gels were allowed to equilibrate for 5 min. in 25 mM Tris, 0.2 M glycine in 20% (v/v) methanol. The proteins were transfered to 0.2 μm pore-sized nitrocellulose at 100 mA for 1 hour by using a LKB 2117 Multiphor II electrophoresis unit. The nitrocellulose was incubated with: polyclonal antibody against human Collagen type I at concentration 1:1,000; monoclonal anti-MAPK antibody (p38 and ERK1/ERK/2) at concentration 1:1,000 and polyclonal anti- NFkB antibody at concentration 1:500 in 5% dried milk in Tris buffered saline with Tween 20 (TBS-T) (20 mmol/l Tris-HCl buffer, pH 7.4, containing 150 mmol/l NaCl and 0.05% Tween 20) for 1 hour. In order to analyze Collagen and NFkB, anti-Rabbit IgG (whole molecule) alkaline phosphatase conjugate was added at concentration 1:5,000 in TBS-T, in order to analyze MAP-kinases second antibody-alkaline phosphatase conjugated, anti-Mouse IgG (whole molecule) was added at concentration 1:5000 in TBS-T and incubated for 30 min while slowly shaking. Then nitrocellulose was washed with TBS-T (5 x 5 min) and submitted to 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate reagent (BCIP/NBT).

Collagen synthesis Incorporation of radioactive precursor into proteins was measured after labeling confluent cells in serum-free medium for 24 h with the 5-[^{3}H] proline (5 μCi/ml, 28 Ci/mmol). Incorporation of label into collagen was determined by digesting proteins with purified C. histolyticum collagenase according to the method of Peterkofsky [19]. Results are shown as combined values for cell plus medium fractions.

Statistical analysis**.** In experiments presented on Fig.1 and Fig. 2, the mean values for six assays ± standard deviations (SD) were calculated. The results were submitted to statistical analysis using the Student`s "t" test, accepting $p < 0.05$, as significant.

Results

It is well known that estrogens stimulate collagen metabolism [4, 5, 6]. In cultured cells, phenol red contained in medium mimics activity of estrogens [20]. Therefore, in the experiments we used Ishikawa endometrial adenocarcinoma (EA) cells cultured in medium without phenol red, containing 10% CPSR1 with or without 1nM estradiol to test the effect of activated estrogen receptor on collagen biosynthesis. In order to evaluate the effect of PPAR-γ agonist on estrogen – dependent stimulation of collagen biosynthesis we used troglitazone as well as clofibrat that activate also other subtypes of PPARs. Collagen biosynthesis in the cells was measured by 5[^{3}H] - proline incorporation into proteins susceptible to the action of bacterial collagenase. It was found that both troglitazone and clofibrat inhibit collagen biosynthesis in EA cells in a dose dependent manner (Fig.1A and B). In case of troglitazone, IC

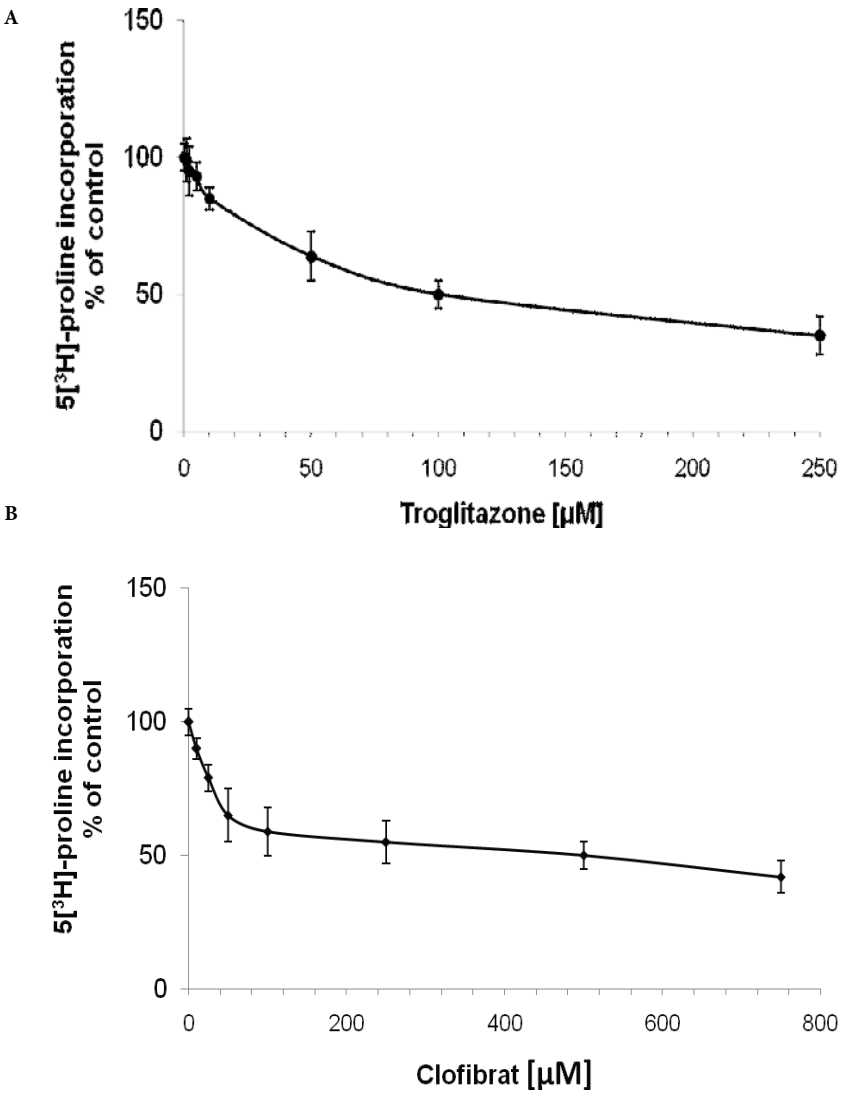

Fig. 1. Effect of different concentration of troglitazone and clofibrat on collagen biosynthesis in subconfluent endometrial adenocarcinoma cells cultured for 24 hours in DMEM without phenol red with 10% CPSR1 supplemented with 1nM estradiol.

50 for the process was found at 100μM while in case of clofibrat at 500 μM. As can be seen from Fig. 2, 1nM estradiol is a potent stimulator of collagen biosynthesis in EA cells. About 70% increase in radioactive proline incorporation into collagen was observed in the cells treated for 24 hours with medium containing 1nM estradiol, compared to control. An addition of PPAR-γ agonists, troglitazone at 100 μM or clofibrat at 500 μM to the estradiol containing medium, inhibited collagen biosynthesis by about 3 fold. The effect was not observed in medium without estrogen.

The role of estrogen receptor stimulation in PPAR-γ-dependent inhibition of collagen biosynthesis was proved by experiments showing that degradation of estrogen receptor by ICI 182,780 abolished inhibitory effect of troglitazone or clofibrat on collagen biosynthesis. Similar data were obtained using Western blot analysis for α1 subunit of type I collagen (Fig.3A). The experiment suggests that the collagen biosynthesis in EA cells is regulated through estrogen and PPAR- γ receptors.

Since IGF-I receptor as well as β1integrin are involved in regulation of collagen biosynthesis, we decided to evaluate the expression of both receptors in studied cells in the same conditions as those described for collagen biosynthesis assay. No effect of the estrogen or PPAR- γ agonists on the receptor expressions were found (data not shown).

Interestingly, we have found that in the presence of estrogen, PPAR- γ agonists (troglitazone and clofibrat) induced

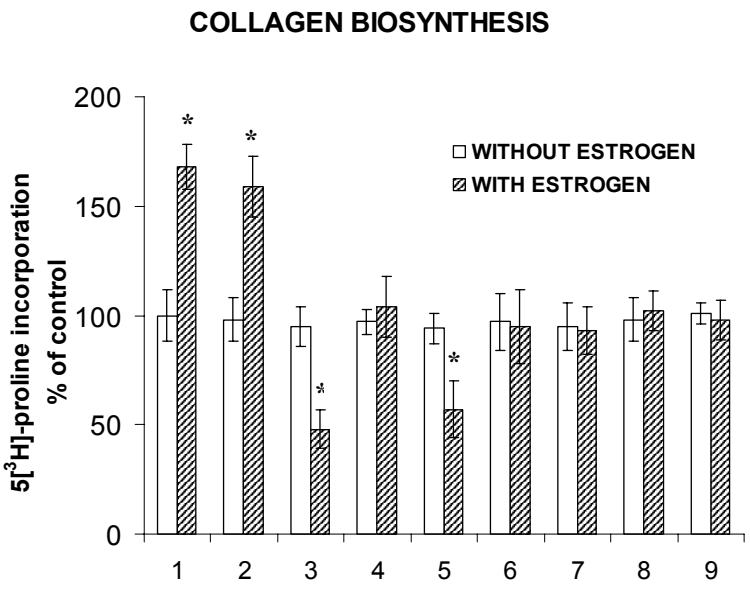

Fig. 2. Collagen biosynthesis in subconfluent endometrial adenocarcinoma cells (1) cultured in the absence (open bars) or presence (closed bars) of 1 nM estrogen in DMEM without phenol red with 10% CPSR1 and submitted for 24 hours to 10 μM troglitazone (2), 100 μM troglitazone (3), 50 μM clofibrat (4), 500 μM clofibrat (5), 30 μM MK 886 (6), 100 nM ICI (7), 100 nM ICI + 100 μM troglitazone (8) and 100 nM ICI + 500 μM clofibrat (9).

expression of NF-kB, well known transcriptional inhibitor of collagen gene expression (Fig.3B). The effect was not observed in the absence of estrogen in the medium (not shown) or in conditions in which estrogen receptor was degraded by ICI 182,780 (Fig.3B, lane 8 and 9).

In order to establish whether MAP-kinases (ERK_1/ERK_2 and p-38) are involved in PPAR- γ-dependent functions, western blot analysis was performed. As can be seen in Fig. 3C in the presence of estrogen in the medium, PPAR- γ ligands induced inhibition of p-38 MAP kinase, while they had no effect on the expression of ERK_1/ERK_2 kinases (Fig.3D). In both cases the effect was only observed in the presence of estrogen.

Discussion

Estrogens are known to stimulate the growth of normal and transformed epithelial cells, including endometrial epithelial cells [21]. The mechanism of their action involves interaction with estrogen receptor that after binding of ligand is targeted to the nucleus as an transcription factor [22]. Several studies documented increased risk of endometrial cancer in patients with elevated circulating levels of estrogens [23, 24, 25]. However, activation of estrogen receptor is regulated not only by its ligands but also by number of factors, including kinases, phosphatases and growth factors [26]. For instance, in endometrial cancer cells p38 mitogen-activated protein kinase (MAPK) by signaling phosphorylates estrogen receptor α, promote its nuclear localization [27].

Despite the fact that estrogens play an important role in the promotion and development of female epithelial-derived cancer [3], it has been postulated that estrogen receptor (ER) positive cancer cells are poorly metastatic compared to ER negative ones [28]. The mechanism of the regulatory role of estrogens in cancer cell growth and metastasis however is not understood. Although in this report we do not study growth and metastasis of Ishikawa cells, the approach to the understanding the effect of estrogens on the processes is focused on the metabolism of main extracellular matrix (ECM) protein, collagen.

The ability of tumor cells to grow and metastasize appear to correlate with how such cells interact with ECM proteins [29]. An important component of this process is the ability of the tumor cells to attach to, migrate on and invade through tissue barriers [30]. It has been shown that most cells (normal and neoplastic) recognize many extracellular proteins using specific cell surface receptors, integrins [31]. Adherent interactions between integrins and ECM proteins including collagens [32, 33, 34], play an important role in embryonic morphogenesis [35, 36] gene expression [33, 37], tumorigenicity and invasiveness [9].

The presence of PPAR receptors in endometrium and endometrial cancer (also in Ishikawa cell line) raised question about their role in cell physiology as well as the endometrial pathology, including cancer. PPARs discovered as a pivotal regulator of adipocyte differentiation play important role in the regulation of expression of myriad genes that regulate energy metabolism, cell differentiation, apoptosis and inflammation in different tissues. Lipid mediators (from fatty acids to

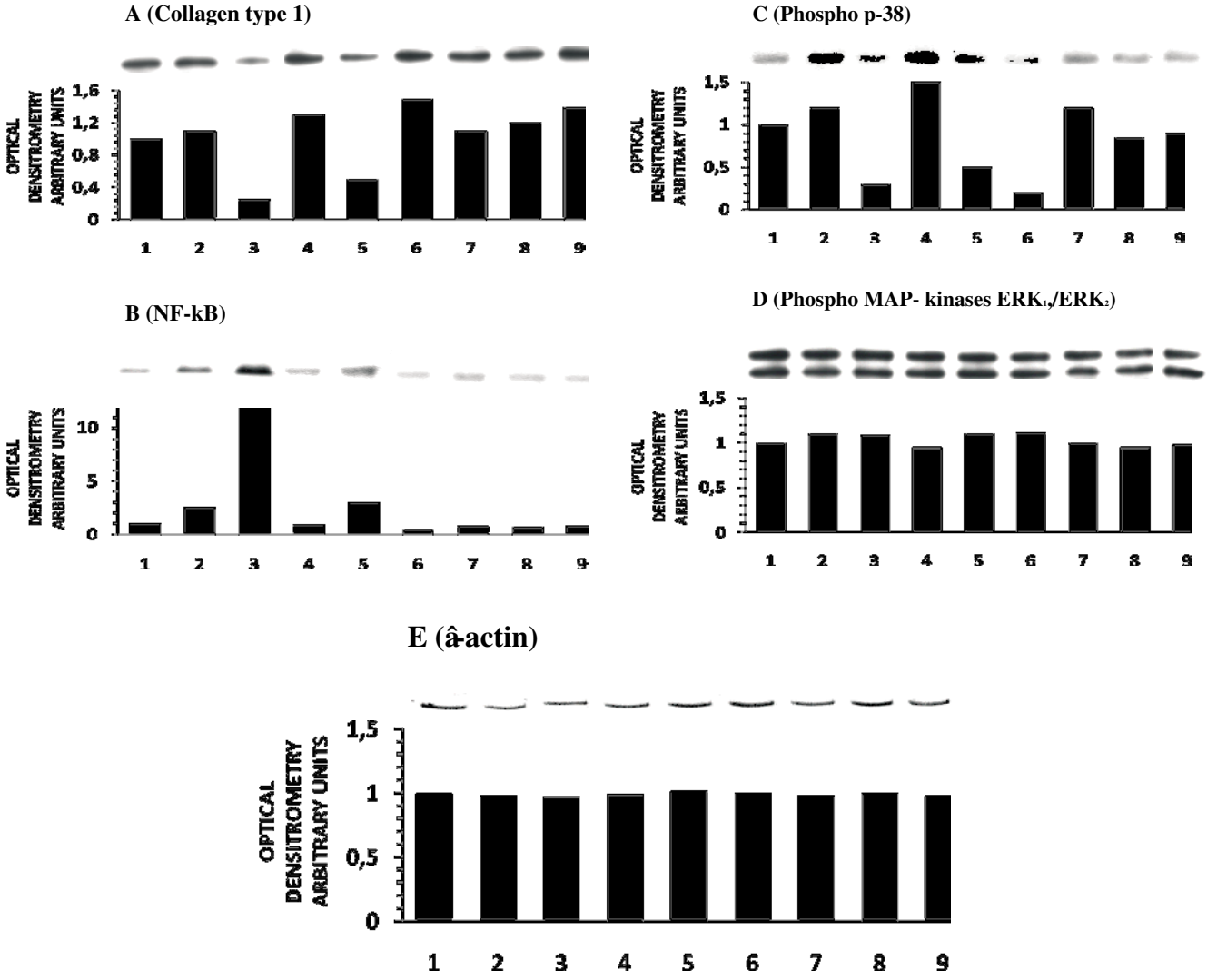

Fig. 3. Western immunoblot analysis for α1 type I collagen (A), NF-kB (B), Phospho -p-38 (C), Phospho - MAP- kinases- ERK_1, ERK_2 (D) and β-actin (E) in subconfluent endometrial adenocarcinoma cells cultured in estrogen supplemented growth medium (1) and submitted for 24 hours to 10 μM troglitazone (2), 100 μM troglitazone (3), 50 μM clofibrat (4), 500 μM clofibrat (5), 30 μM MK 886 (6), 100 nM ICI (7), 100 nM ICI + 100 μM troglitazone (8) and 100 nM ICI + 500 μM clofibrat (9). Samples used for electrophoresis consisted of 20 μg of protein of pooled cell extracts (n = 6). Detection of β- actin was carried out in order to provide the loading control. The intensity of the bands staining was quantified by densitometric analysis.

eicosanoids and related products) can activate PPARs. These findings lead to the suggestion that PPARs may act just as general lipid sensors. It seems that they are responsible for fatty acid-induced effects on gene expression and may provide the link beetwen estrogen and metabolic factors in devolopment of endometrial cancer.

In this study we have found that PPAR- γ and estrogen receptor modulate collagen biosynthesis in Ishikawa cells. Stimulation of estrogen receptor was found to induce collagen biosynthesis, while PPAR- γ agonists, as troglitazone or clofibrat (that also bind PPAR-α) induced inhibition of biosynthesis of this protein. The inhibition was found to be dependent on the activity of estrogen receptor, since removal of estrogen receptor by ICI-dependent degradation abolished inhibitory effect of PPAR- γ agonists on collagen biosynthesis. It seems that the inhibitory action of PPARs is due to γ – subtype, since specific PPAR- γ ligand – troglitazone evoked stronger potency to inhibit collagen biosynthesis than clofibrat. The mechanism of the cross-talk between estrogen and PPAR- γ receptors was found at the level of NF-kB (known inhibitor of collagen gene expression) and MAPK signaling. In our study PPAR- γ ligands were found to stimulate expression of NF-kB, while they inhibited expression of p-38 but not ERK1/ERK2. Both stimulation of NF-kB and inhibition of p 38 MAPK contribute to down regulation of collagen production.

NF-kB is known to be involved in inhibition of collagen gene transcription through binding to both α1 and α2 collagen promoter [38, 39, 40]. NF-κB as transcription factor may regulate also apoptosis, cell proliferation, cell growth arrest, as well as angiogenesis via stimulation of vascular endothelial growth factor expression [41]. On the other hand MAPK p-38 is known as an signaling inductor of collagen production acting through up-regulation of TGFβ1 gene expression [42]. Inhibition of MAPK p-38 by PPAR γ ligands may also affect phosphorylation of estrogen receptor and its translocation to the nucleus [27]. At least in epithelial cells estrogens stimulate collagen biosynthesis [43]. All those activities of PPAR- γ ligands contribute to inhibition of collagen biosynthesis protecting against tissue fibrosis accompanying aging, oxidative stress, high blood glucose level and others [12, 14]. Therefore to those activities of troglitazone and clofibrat an additional activity of inhibition of collagen biosynthesis can be added. Decrease in collagen biosynthesis may also eliminate collagen as a ligand for integrins, that play important role in signaling in endometrium cancer, leading to endothelial cell proliferation. Interestingly all those activities requires participation of estrogen. What is the mechanism for the cross – talk between PPAR- γ and estrogen receptor needs to be resolved. Nevertheless, the data document for the first time that inhibitory effect of PPAR- γ ligands on collagen biosynthesis requires functional estrogen receptor. It also suggest that PPAR-γ may represent therapeutic target in prophylactic and neoplastic disease treatment.

Acknowledgement. This work was supported by Grant No. 2 PO5E 064 28 from the Committee for Scientific Research.

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