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IMPACT OF THE OPERATION CIGLITAZONE ON PPAR ACTIVATION AND APOPTOSIS PROCESS IN MODELS OF GASTROINTESTINAL CANCER IN VITRO

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Abstract:

Background: *Peroxisome proliferator-activated receptors (PPAR-γ) are nuclear transcription factors which affect the stimulation of glucose and lipid metabolism, modulation of inflammation, tissue sensitivity to insulin, immune response, cell proliferation and differentiation. Current research on PPAR-γ receptors is contradictory.*
A significant part of the research suggests that these receptors may be targeted for anti-cancer therapy and have anti-inflammatory properties. Other analyzes speculate on the role of PPAR-γ in promoting cancer. It is therefore important that further studies help to better understand the role of PPAR-γ receptors, which may be relevant in the context of public health and cancer therapy
Methods: *The purpose of the study was to determine the effect of ciglitazone (10 μM) on expression of PPAR-γ receptors. In addition, it was investigated whether action on PPAR-γ nuclear receptors with a specific ligand concentration (10 μM of ciglitazone) could lead to increased expression of apoptotic protein (Bcl-2, PKB/Akt) in gastric cancer cells (PANC-1, HT-29) in models In vitro. The effects of ciglitazone were tested in HT-29 and PANC-1 cell lines by MTT growth test (tetrazolium growth assay) for 48 hours post-treatment. Investigation of the relationship between ciglitazone and PPARγ in the context of apoptosis was investigated at the protein level by Western Blot analysis.*
Results: *The results obtained reflect the trend in the publication. In the conducted studies, it was observed that at 10 and 20 μM concentration of ciglitazone affects the growth of the investigated cell lines (PANC-1, HT-29). In addition, studies have shown that this drug increases the activity of PPAR-γ receptors and may affect the kinase gene Akt and Bcl-2 through the receptors themselves.*
Conclusion: *The studies show that treatment of cancer cells 10 μM ciglitazone for a certain time affects the upregulation of anti-apoptotic proteins. What may suggest that certain types of ligand does not result in inhibition of the process of carcinogenesis? Therefore, studies on the effect of PPAR-γ receptors and their ligands on intestinal tumors should be conducted.*

Keywords:- *Peroxisome proliferator-activated receptor γ (PPAR-γ), ciglitazone, apoptosis, gastrointestinal cancer*

List of abbreviations:
PPARs - peroxisome proliferator activated receptors
PPAR-γ - peroxisome proliferator activated receptors γ
PPAR-α - peroxisome proliferator activated receptors α
PPAR-β - peroxisome proliferator activated receptors β
PPAR- δ - peroxisome proliferator activated receptors δ
TZDs - thiazolidinedione's
RXR - retinoid receptor
MTT - tetrazolium-based growth assay
PANC-1 - human pancreatic adenocarcinoma epithelial cell line
HT-29 - human epithelial colorectal adenocarcinoma cell line
Bcl-2 - anti-apoptotic Bcl-2 proteins
PKB/Akt – protein kinase B
DMSO - dimethyl sulfoxide

1. INTRODUCTION:

Peroxisome proliferator activated receptors (PPARs) are transducer proteins belonging to the nuclear receptor superfamily [15]. The first PPAR receptors were discovered in 1990 in the liver cells and termed PPAR. Since then they cloned and characterized by three types of receptors – α, β/δ, γ [6, 15]. In the human PPAR-α, PPAR-β / δ and PPAR-γ are encoded by genes located, respectively, at 22, 6 and 3 chromosome. Genes of receptors PPAR are highly expressed in organs whose cells contain many mitochondria: liver, renal cortex, the intestinal mucosa and the heart, to a lesser extent in cells of other organs [19].

PPARs are responsible for the metabolism of fatty acids, adipogenesis and processes proliferation and differentiation of cells [1, 20]. PPAR-γ also responsible for the maintenance of glucose homeostasis and are involved in the regulation of inflammation, cardiovascular diseases and cancer [4, 7, 13, 16].

PPAR-γ receptors be able to bind various ligands. Activators of nuclear receptors are compounds of natural or synthetic origin [2]. The first synthetic PPAR agonists are drugs sensitizing the peripheral tissues to insulin, known as thiazolidinediones (TZDs) [4, 5].

These compounds include ciglitazone, pioglitazone, roziglitazone and other compounds prostaglandins, arachidonic acid and fatty acids or their derivatives activate PPAR-γ receptors [3, 9,10]. Nuclear receptors are ligand dependent. Full transactivation of these receptors require dimerization receptor PPAR to a retinoid receptor (RXR), which has the effect of inducing the expression of target genes [2, 11, 21].

The function of these receptors in the intestinal epithelium were not been fully explained. Current research suggests that these receptors may be an attractive target for cancer therapy. There are studies which demonstrate the antiproliferative activity and inhibit angiogenesis, which result in slowing the progression of the cancer [1].

Current reports also indicate that activation of PPAR receptors promotes the development of colon cancer tumors in mice APC/min [5]. These data indicate an increased level of PPAR-γ in polyps in APC/min in mice and is contrary to the tests demonstrating these receptors as an attribute of antitumor activity in the gastrointestinal tract [8, 17].

1. Materials and methods

Tissue culture

Human pancreatic adenocarcinoma epithelial cell line PANC-1 obtained from the European Collection of Animal Cell Cultures and human epithelial colorectal adenocarcinoma cell line HT-29 obtained from American Type Culture Collection. Cell lines were routinely in Roswell Park Memorial Institute (RPMI) 1640 culture medium (Life Technologies, Inc., Paisley, UK) containing 10% heat-inactivated fetal bovine serum (FBS; Sigma, Poole, UK) at 37ºC in 95% air, 5% CO2 and humidified atmosphere.

Reagents

The cell lines were treated with 10 μM ciglitazone (Sigma-Aldrich, USA).

Tetrazolium-based growth assay (MTT)

Cells were plated out in 100μl aliquots into the middle 60 wells (1x104 cells/well) of a 96well tissue culture plate and incubation for 24 hours at 37°C and 5% CO2. Cells were then treated with different concentrations of ciglitazone (1-20 μM). After 72 hours of incubation, to each well added 50 μl of methyl thiazoyl tetrazolium (MTT) and incubated for four hours. The contents of the wells is removed and loaded 75 μl of MTT dissolved in DMSO (dimethyl sulfoxide) to the cell. Measuring the optical density measured at a wavelength of 550 nm.

Western Blot analysis

Cells were lysed in phosphatate-buffered saline containing 1% Triton X-100 (Sigma Chemical Company, St. Louis, MO), 1μM phenylmethylsulfonyl fluoride (Sigma), 100 μM iodoacetoamide and 1 μM ethylenediamine tetraaceti-acid (Sigma). Lysates were frozen at – 80 ºC for 15 minutes and centrifuged at 10 000 g for 10 minutes. Protein concentration in the supernatant was measured using the spectrophotometer NanoDrop 2000 (Thermo Scientific). Total cellular protein (20-30 μg) was separated by electophoresis on a 12,5% sodium dodecyl sulfate-polyacrylamide gel and transferred onto Immobilon membrane (Millipore, Bedford, MA). After blocking, the membranes was incubated with the antibodies indicated as follows: PPAR-γ monoclonal antibody (Thermo Scientific) Bcl-2 monoclonal antibody (Sigma), Phospho Akt monoclonal antibody (Thermo Scientific). Next membranes washed and were then probed with secondary antibody conjugated horseradish peroxidase (Sigma) for 30 minutes. In the same way actin controls were performed. Chemiluminescent visualisation and protein detection were carried out using the enhanced chemiluminescence kit (Amersham, Little Chalfont, UK). Quantitation of the protein was carried out using the Chemi Genius 2 Biolmaging System (Syngene, Cambridge, UK) in conjunction with the GeneTools image analysis software (Syngene).

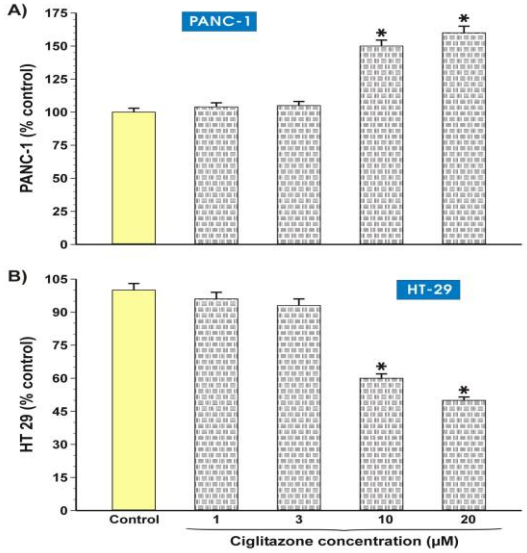
Statistics For evaluation of in vitro data non-parametric Mann-Whitney U test was used.

2. Results

The effect of the treatment cell ciglitazone

The experiment is a continuation of the research already carried out. The results obtained reflect the trend in the publication. [14]. The effect of ciglitazone was tested in the cell lines HT-29 and PANC-1 by means of the MTT assay 48 hours after treatment (Fig. 1). It was observed that the effect of ciglitazone at concentration of 10 and 20 μM has the effect of growth of the cell line PANC-1 ($p<0,01$, non-parametric Mann-Whitney U test, significant indicated by) (Fig. 1A). However, in the case of cell line HT-29 it was observed a decrease of cell growth after 48 hours of treatment 10 and 20 μM ciglitazone ($p<0,01$, non-parametric Mann-Whitney U test, significant indicated by) (Fig. 1B). The obtained data suggest that the effect of the treatment ciglitazone with through PPAR receptors may depend on the type of cancer cells.

Fig. 1 Growth effects after 48 hours treatment of ciglitazone in the PANC-1 and HT-29 cell lines.
(A) After treatment with ciglitazone at a concentration of 10 and 20 μM in PANC-1 cell lines, intensive cell growth occurred. The results were observed using the tetrazolium-based growth assay - MTT; ($p<0,01$ non parametric Mann-Whitney U test, significant indicated by). **(B)** After treatment with ciglitazone at a concentration of 10 and 20 μM, cell growth was reduced in the HT-29 cell line. The results were observed using the tetrazolium-based growth assay - MTT; ($p<0,01$ non parametric Mann-Whitney U test, significant indiated by).



Examination of the relationship between ciglitazone and PPAR-γ receptors in the context of apoptosis

Western Blot analysis revealed expression of the 55 kDa protein corresponding to the size of PPAR-γ (Fig. 2B). It was observed that after treatment with 10 μM ciglitazone expression of PPAR-γ increases with time ligand stimulation. A similar effect was observed in both the tumor cell lines. The strongest expression ($p<0,001$) was observed after 90 minutes stimulation ciglitazone compared to untreated control (Fig. 2E).

Our research has shown that ciglitazone increases PPAR-γ activity and affects apoptosis. Treatment with 10μM ciglitazone alone resulted in a increase in ($p<0.001$ after 90 min) PKB/Akt and Bcl-2 gene expression in comparison to the control group (Fig. 2 F,G; Fig. 3 F,G). This effect was observed using Western-Blot analysis in PANC-1 (Fig. 2 C, D) and HT-29 (Fig. 3 C,D) cell lines. These results suggest that in the tumor cells of PANC-1 and HT-29, ciglitazone exerts an influence on the Akt kinase gene and Bcl-2 via PPAR-γ.

Fig. 2 Effect of stimulation ciglitazone (10μM) on PPAR-γ, Bcl-2 and PKB/Akt on PANC-1 cell lines.
(A-D) Results of the study on effects ciglitazone treatment by Western Blot analysis on PANC-1 cell lysates. **(E-G)** Results of expression level for PPARγ receptors, PKB/Akt and Bcl-2 proteins. In the studied cell lines, expression increased at a specific incubation time ($p <0.001$ after 90 min) with ciglitazone for PPARγ, PKB/Akt and Bcl-2 as compared to the control group. Negative controls were lysates of cells not treated with ciglitazone.

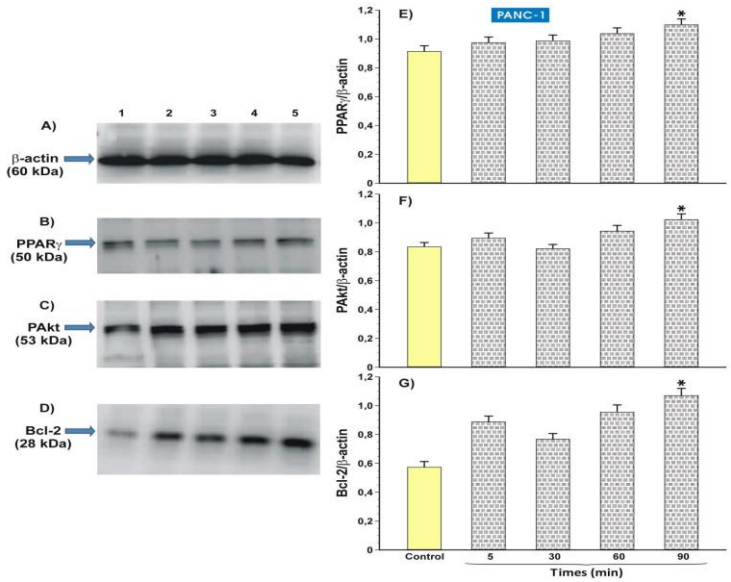
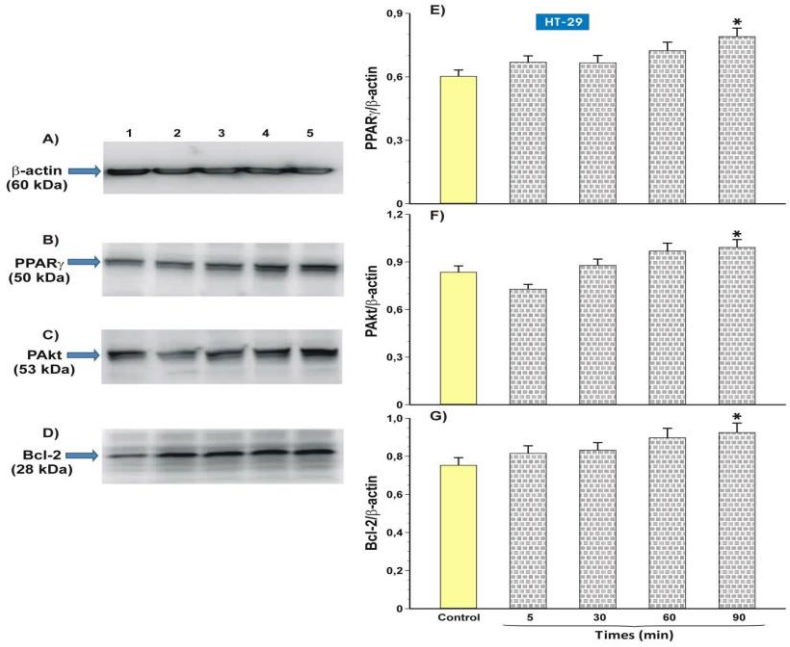


Fig. 3 Effect of stimulation ciglitazone (10µM) on PPAR-γ, Bcl-2 and PKB/Akt on HT29 cell lines.

(A-D) Results of the study on effects ciglitazone treatment by Western Blot analysis on HT-29 cell lysates. **(E-G)** Results of expression level for PPAR-γ receptors, PKB/Akt and Bcl-2 proteins. In the studied cell lines, expression increased at a specific incubation time ($p < 0.001$ after 90 min) with ciglitazone for PPAR-γ, PKB/Akt and Bcl-2 as compared to the control group. Negative controls were lysates of cells not treated with ciglitazone.



3. Discussion

The development of cancer depends on the activation of certain signaling pathways and the control of cell growth. Recent studies have shown that PPAR-γ receptors exhibit antiproliferative activity. At the same time, the number of reports on the role of PPAR-γ in the promotion of cancer in the large intestine is increasing [14]. Coexistence of chronic inflammatory processes can significantly contribute to hyper proliferative epithelial growth within the large intestine. The resulting changes may be the cause of cancer development. Because PPAR-γ affects cell proliferation and inflammation is important in the pathogenesis of colon cancer [6, 12, 14].

In research conducted the tumor cells are observed increase in expression of PPAR-γ, which confirms that these receptors may be a marker of neoplastic process. There are studies indicating that they contribute to the tumor growth. The groups of Lefebvre et al. and Saez et al. have shown that treatment with PPAR-γ ligands enhances colon carcinogenesis in APC/Min mice [8, 17]. There are many reports suggesting that PPARs are one of the attributes of antitumor activity [13]. PPAR-γ ligands are silencing the proliferation of colon cancer regardless of apoptosis, suggesting that the activation of these receptors induces a cellular program that significantly lowers the level of epithelial proliferation. Thus, the interaction of PPAR-γ with a specific ligand is necessary to inhibit tumor progression [5]. Ligands of nuclear receptors are therefore used in studies to suppress tumor progression at various stages of its development, as observed by reversing

the malignant phenotype in in vitro cell cultures. Sarraf et al. have demonstrated that troglitazone and rosiglitazone inhibit the growth of human colorectal cancer cells [18]. The different results suggest that the molecular mechanisms underlying the suppression of the process of carcinogenesis using PPAR-γ have not yet been fully explained [12].

The purpose of the study was to investigate whether the effect on nuclear receptors with a specific ligand concentration (ciglitazone) could lead to an increase in apoptosis protein expression in gastrointestinal cancer cells in in vitro models. The studies show that treatment of cancer cells 10 μM ciglitazone for a certain time affects the upregulation of antiapoptotic proteins. What may suggest that certain types of ligand does not result in inhibition of the process of carcinogenesis? Therefore, studies on the effect of PPAR-γ receptors and their ligands on intestinal tumors should be conducted.

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