Original Article

A comparison of peroxisome proliferator-activated receptor-α agonist and antagonist on human umbilical vein endothelial cells angiogenesis

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Abstract

Background: There are controversial reports about the antiangiogenic effects of peroxisome proliferator-activated receptor α (PPARα). In the current study, we compared the effects of PPARα agonist and antagonist on human umbilical vein endothelial cells (HUVECs) angiogenesis with matrigel assay.

Materials and Methods: HUVECs (1×10^5 cells/well) treated with PPARα agonist (fenofibrate) and antagonist (GW6471) were cultured on matrigel for 24 h. Treated cells were stained with calcein and investigated by fluorescent microscopy. The obtained images were also analyzed by AngioQuant software. Finally, the data were analyzed using SPSS 15 software, Kruskal-Wallis and one way ANOVA.

Results: Statistical analysis showed that fenofibrate significantly inhibit the tube formation (size, length, junction) ($P < 0.05$) but there was a trend to increased angiogenesis in GW6471 treated group ($P > 0.05$).

Conclusion: These results showed that PPARα agonist is effective in suppression of angiogenesis. Further studies are needed to confirm these results in *in vivo* studies.

Key Words: Angiogenesis, human umbilical vein endothelial cells, peroxisome proliferator-activated receptor

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INTRODUCTION

Angiogenesis occurs during the development and vascular remodeling as a controlled series of events leading to neovascularization,[1] which plays a key role in a variety of physiological processes and in the pathological development and progression of various diseases.[2,3]

Antiangiogenic therapy is considered as an efficient strategy for controlling the growth and metastasis of solid tumors, as well as for other diseases involving pathological angiogenesis.[4-7]

A great deal of effort is now being devoted to the development of new drugs that will control pathological angiogenesis.[8] As a well-known transcription factor, PPARα regulates the expression of genes known to be involved in the energy metabolism, cellular

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proliferation, and angiogenesis.[9]

So, in addition to its proven lipid modifying effects, fenofibrate also exhibits several metabolic and pleiotropic properties.[10]

PPARα is expressed in human aortic endothelial cells, carotid artery endothelial cells, and human umbilical vein endothelial cells.[11-14]

The observation that PPARα is expressed by endothelial cells together with the finding that PPARα ligands regulate cell growth, survival, migration, and invasion[15,16] prompted investigators to determine whether these receptors play a role in the pathophysiology of angiogenesis.[17,18] However, the functions of PPARα in endothelial cells, mainly in terms of angiogenesis, are only just beginning to be understood.

PPARα ligands can inhibit endothelial cell proliferation and migration and induce endothelial cell apoptosis *in vitro*.[19-21] In addition, fenofibrate reduces adventitial angiogenesis and inflammation in a porcine model[22] and decreases VEGF levels in patients with hyperlipidemia and atherosclerosis.[23]

Despite the reported anti angiogenic effects of PPARα ligands several other studies have shown endothelial protective and angiogenic effects of these ligands. For example, Biscetti *et al*. have shown PPARα agonists induced neoangiogenesis through a VEGF dependent mechanism.[24]

According to the purpose of the present study, we used selective synthetic PPARα agonist and antagonist and tested their potential ability to stimulate angiogenesis in a well-established *in vitro* matrigel assay.

MATERIALS AND METHODS

Cell culture

The human umbilical vein endothelial cells (HUVECs) (National Cell bank of Iran affiliated to Pasteur Institute, Tehran, Iran) were cultured in endothelial basal medium supplemented with 1% antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) and 10% fetal calf serum until the third passage before experiments were performed. All the cell culture material were from Gibco, USA. Cells were grown to confluence at 37°C in 5% CO2.

Angiogenesis assays

The tube formation assay was performed on 24-well plates coated with 100 μl of Matrigel Basement Membrane Matrix (invitrogen, USA) and polymerized for 30 min at 37°C. PPARα activators (fenofibrate) and PPARα inhibitors (GW6471) were purchased from Sigma (Sigma-Aldrich Chemicals, St. Louis, MO) and dissolved in DMSO. The cells (1×10^5 cells/well) in 4 groups with different treatments which include: Group 1, VEGF165 (1 ng/ml-recombinant human) as a positive control; Group 2, dimethyl sulfoxide (DMSO) 10% as a negative control; Group 3, fenofibrate (50 (μmol/L)) and Group 4, GW6471 (50 (μmol/L)). The treated cells plated on to a layer of matrigel for 24 h. Then the cells were stained with a cell-permeable dye (Calcein, acetoxymethyl ester) to make the network more visible. At the end, final dye concentration was 2 μg/mL.

Finally, the center of each well was photographed with a Nikon camera attached to a fluorescent microscope. Fluorescence images were quantified using the AngioQuant v1.33 software (The Math Works, Natick, MA) to quantitate the extent of tubule formation (lengths, sizes, and number of junctions) in each replicate well.

Statistical analysis

The experiments were performed in duplicate and replicated three times. At last, data was analyzed using the software SPSS 15 tests in one way ANOVA and Kruskal-Wallis analysis. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Effect of PPARα agonist (fenofibrate) on angiogenesis

To verify the anti-angiogenic activity of fenofibrate, the cells treatment started at the time of seeding HUVEC on to matrigel, and endothelial cells tube formation was observed over a period of time. Fenofibrate significantly suppressed the formation of tube-like structures ($P < 0.05$) [Figure 1].

Our results showed that fenofibrate significantly decreased the size [Figure 2], length [Figure 3] and junction of tubes [Figure 4] compared to negative and positive control groups ($P < 0.05$).

Effect of PPARα antagonist (GW6471) angiogenesis

Our result have shown that GW6471 had increasing effects on tubes size [Figure 2], length [Figure 3] and number of junction [Figure 4] than negative control group, but did not significantly. Compared with positive control tube formation was decreased but it was not significant.

DISCUSSION

The ability of endothelial cells to form capillary tubes is a specialized function of this cell type.[25]

PPARα, PPARβ/δ, and PPARγ are expressed in endothelial cells, where they regulate cell proliferation,

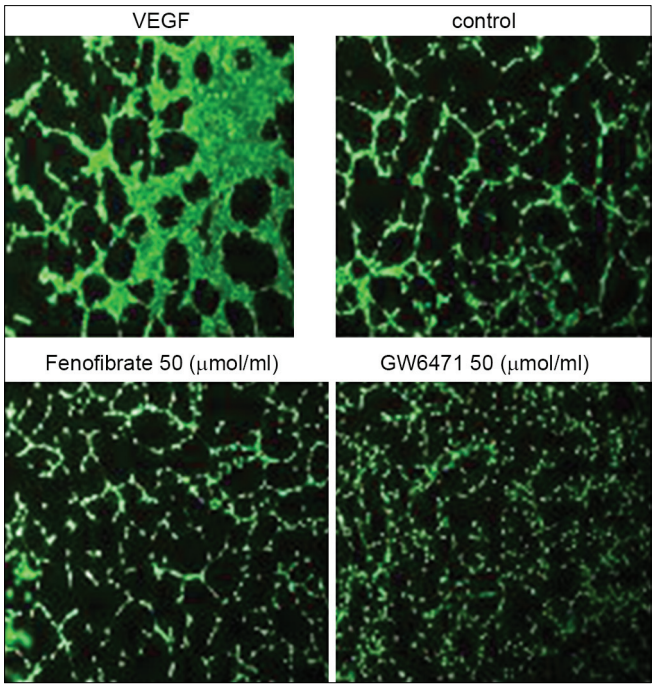

Figure 1: The effect of PPARα agonist (fenofi brate) and antagonis (GW6471) on tube formation of HUVECs. HUVECs were plated on a well coated with 100 μl of Matrigel basement membrane matrix. After they were treated for 24 h, the cells were dyed and photographed with a Nikon camera attached to a fluorescent microscope at ×10 magnification

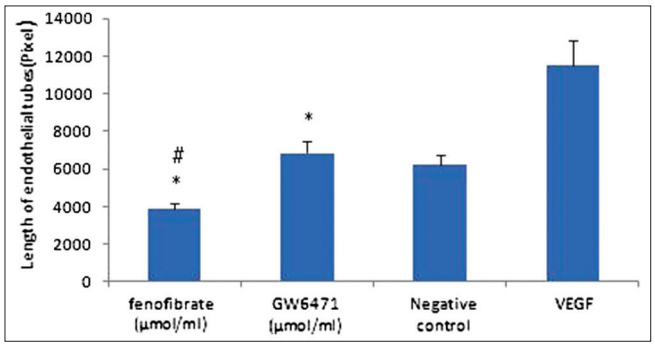

Figure 3: The effect of fenofi brate and GW6471 on angiogenesis by HUVECs. Quantitative analysis of mean tube length from three independent experiments are shown.*Signifi cantly different from positive control group. #Significantly different from negative control group

angiogenesis, inflammation, thrombosis, and coagulation.[11,26]

It has been shown that PPARα ligands inhibit endothelial cell proliferation and migration and induce endothelial cell apoptosis *in vitro*.[19-21] Due to the pleiotropic effects of fibrates, much of the evidence of their effect on angiogenesis is derived from animal models.[22]

Extrapolation from these findings to make a convincing conclusion is hampered by the shortage of data.

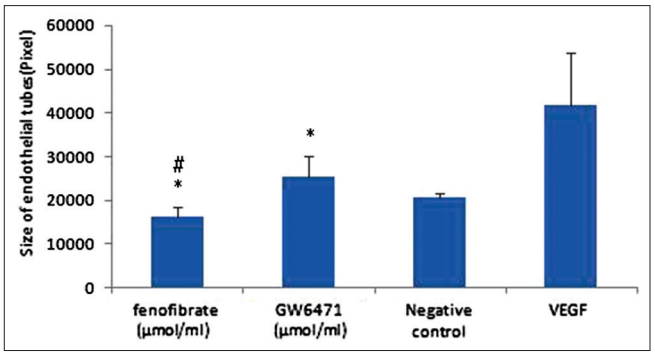

Figure 2: The effect of fenofibrate and GW6471 on *in vitro* angiogenesis by HUVECs. Quantitative analysis of mean tube size from three independent experiments are shown.*Significantly different from positive control group. #Significantly different from negative control group

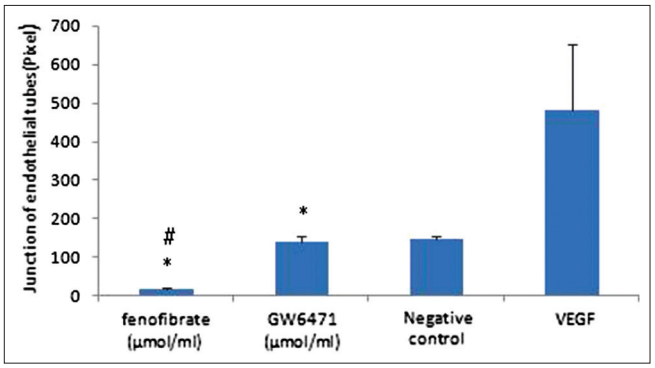

Figure 4: The effect of fenofi brate and GW6471 on in vitro angiogenesis by HUVECs. Quantitative analysis of mean tube number of junction from three independent experiments are shown.*Significantly different from positive control group. #Significantly different from negative control group

As endothelial cells express PPAR alpha, we have examined its effects on tube formation. Here, we report that fenofibrate reduced tube size, length and number of junction. Recent studies using immortalized human dermal microvascular endothelial cells show that the PPARα ligand fenofibrate inhibits the endothelial cell proliferation, migration, and tube formation (on a fibrin matrix) *in vitro* and angiogenesis *in vivo*.[20] Fenofibrate acts by disrupting the formation of the actin cytoskeleton and inhibits bFGF-induced Akt activation and cyclooxygenase 2 (COX-2) gene expression.[20] Furthermore, PPARα modulates nitric oxide synthase (NOS)-induced NO production. PPARα agonists enhance NOS expression and NO release.

Consistent with our result that PPARα ligands might act as potent direct and/or indirect antiangiogenic factors, Panigrahy *et al*. have shown a potent antiangiogenic role of fenofibrate through suppression of VEGF-mediated endothelial cell proliferation.[27] Moreover, fenofibrate prevent VEGF-mediated endothelial cell migration

by inhibiting Akt phosphorylation[19] and also prevents endothelial cell proliferation by inhibiting cyclooxygenase-2 expression.[20] Finally, PPARα agonists were found to inhibit endothelial VEGFR2 expression by preventing Sp1-dependent promoter binding and transactivation.[21]

However, despite of the above mentioned finding, Meissner, M. *et al.*, have shown that HUVEC treatment with PPARα agonist (fenofibrate or pirinixic acid) downregulated expression of VEGF receptor 2.[21]

Another molecular mechanism by which fenofibrate inhibits angiogenesis may be through inhibition of Akt activation. As the angiogenesis inhibition was also observed with WY-14,643, another PPAR-alpha activator, the activation of PPAR-alpha by fenofibrate may be involved in its anti-angiogenic activity. However, a molecular mechanism independent of PPAR-alpha has also to be considered.[21]

We have shown that there was a trend toward increased angiogenesis in GW6471 treated cells. Treatment with fenofibrate concomitantly with this PPARα antagonist led to activation of adenosine monophosphate-activated protein kinase (AMPK) and a consequent increase in VEGF messenger RNA (mRNA) expression, which evidently involved a PPARα-independent mechanism. Overall, AMP kinase activation is considered to have a protective effect on the endothelium.[28]

Interestingly, it has been shown that fenofibrate acts on human retinal endothelial cells through a PPARα independent pathway, as demonstrated by increased cell survival and decreased apoptosis in the simultaneous presence of fenofibrate and the PPARα antagonist MK886.66.

AMPK can influence a number of signaling cascades including increased NO bioavailability, reduced free radical generation, and the activation of angiogenic factors.[29]

CONCLUSION

We used selective synthetic agonists and antagonist of PPARα and demonstrated that the stimulation of PPARα results in the activation of an anti-angiogenic process *in vitro*. Lack of significant increased angiogenic response in GW6471 treated group may suggest that anti-angiogenic effect of fenofibrate does not occur through direct stimulation of endothelial cells but is instead related to PPARα independent pathways. These findings may shed some light to understand the biological effects of drugs that stimulate the activity of PPARα with potentially important implications for the management of several angiogenesis dependent diseases such as type 2 diabetes and cancers.

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