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Effects of 15d-PGJ₂ on VEGF-induced angiogenic activities and expression of VEGF receptors in endothelial cells

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Abstract

15-deoxy-^{12,14}Δ-prostaglandin-J₂ (15d-PGJ₂) upregulates expression of vascular endothelial growth factor (VEGF), but may inhibit angiogenesis. We found that 15d-PGJ₂ (1–10 μM) attenuated all VEGF-induced angiogenic activities in human umbilical vein endothelial cells (HUVEC). It blocked almost completely cell proliferation, potently reduced migration, assembly into tube-like network on matrigel, and growth of capillaries into collagen gel. 15d-PGJ₂ inhibited expression of VEGFR-1 and VEGFR-2 receptors both at mRNA and protein levels. This inhibition, however, was transient (observed after 6–12 h, but not after 24 h) and weak (20–30%), and could not fully explain inhibition of response to VEGF. Accordingly, proliferation was inhibited when 15d-PGJ₂ was added 24 h after VEGF or in cells stimulated with basic fibroblast growth factor. Interestingly, 15d-PGJ₂ decreased activities of c-jun and c-myc in HUVEC and overexpression of c-myc attenuated its antiproliferative effects. This suggests that inhibition of this transcription factor by 15d-PGJ₂ contributes to decrease in angiogenic response.

Introduction

Angiogenesis, a formation of new capillaries from the preexisting vessels, is characterized by a complex morphogenic cascade of events during which the quiescent, resting endothelial cells become activated to proteolytically degrade their underlying extracellular matrix, directionally migrate toward the angiogenic stimulus, proliferate and align into new three-dimensional capillary networks [1].

15-deoxy-^{12,14}Δ-prostaglandin-J₂ (15d-PGJ₂) is a natural ligand of peroxisome proliferator-activated receptor-γ (PPARγ) [2,3], a transcription factor of nuclear receptor superfamily. PPARγ mediates also therapeutic effects of thiazolidinediones (TZDs), the insulin-sensitizing compounds approved for the treatment of insulin-resistance in type II diabetes in humans [3,4]. Because diabetes represents a disease with numerous vascular injuries, it is very important to understand how activation of PPARγ influences angiogenesis.

Several elegant studies strongly suggest the involvement of PPARγ pathway in regulation of blood vessel formation. The most convincing proof of PPARγ importance is finding that inactivation of PPARγ gene or the ablation of PPAR binding protein (PBP) gene, a

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coactivator of PPAR γ , result in embryonic lethality secondary to defective placental vascularization [5,6,7]. This implicates that PPAR γ is necessary for a proper angiogenesis.

It has been evidenced that PPAR γ is an active transcription factor in the vessel wall, being expressed both in vascular smooth muscle cells [8,9] and endothelium [10,11,12]. In endothelial cells, ligands of PPAR γ influence expression of several genes involved in angiogenesis, including upregulation of interleukin-8 (IL-8) [12], matrix metalloproteinase-1 (MMP-1) [13], and plasminogen activator inhibitor-1 (PAI-1) [11], or inhibition of endothelin-1 [14] and urokinase plasminogen activator (uPA) [10,13].

Importantly, we and others have demonstrated that activators of PPAR γ upregulate expression of vascular endothelial growth factor-A (VEGF), one of the major proangiogenic mediators. Treatment of cells with 15d-PGJ₂ or TZDs increased production of VEGF in macrophages [9,15], VSMC [8,9], coronary endothelial cells [16], and microvascular endothelial cells [12]. This upregulation was associated with activation of PPAR γ transcription factor and mediated by increased VEGF promoter activity [9].

In spite of the upregulation of VEGF synthesis, several in vitro studies convincingly demonstrated that both TZDs and 15d-PGJ₂ inhibit angiogenesis. For example, ligands of PPAR γ decreased proliferation of endothelial cells and reduced their assembly into the tube-like structures [10,17-19]. Moreover, intravenous injection of TZDs markedly inhibited choroidal or retinal neovascularization in mice, rats and monkeys [20-22]. Similarly, in mice with rhabdomyosarcoma and glioblastoma the antitumor effect of TZD resulted from a reduction in tumor microvessel density and a decrease in endothelial cell proliferation [23].

Mechanisms underlying the antiangiogenic effects of PPAR γ ligands, especially 15d-PGJ₂, are still not fully clarified. The most common believe is that the inhibition of VEGF-induced angiogenesis is caused by downregulation of VEGF receptors. This opinion is based mostly on the analysis of expression of VEGFR-1 and VEGFR-2 mRNAs in three-dimensional cultures of endothelial cells embedded in collagen gels [10]. Data obtained in other experimental settings are, however, scarce and inconsistent. Therefore, the aim of our study was to check which angiogenic activities induced by VEGF in endothelial cells are modulated by 15d-PGJ₂ and determine the expression of VEGF receptors using quantitative methods both at mRNA and protein levels. The obtained results suggest that down-regulation of VEGF receptors, although can contribute to the observed inhibitory effects, is not a major mechanism responsible for anti-angiogenic potential of 15d-PGJ₂.

Methods

Reagents. 15d-PGJ₂, troglitazone and ciglitazone were obtained from Biomol and T0070907 from Cayman. L-glutamine, carboxymethylcellulose, and polybrane were purchased from Sigma. FCS was procured from PromoCell. CytoTox-96 assay, pSV β gal plasmid, Total RNA Extraction Kit, Reverse Transcription System, and PCR Core System were obtained from Promega. QuantiTect™ SYBR® Green RT-PCR kit was purchased from Qiagen. Human recombinant VEGF₁₆₅, human recombinant basic fibroblast growth factor (bFGF), TiterTACS Apoptosis ELISA Kit, anti-VEGFR-2 and anti-VEGFR-1 polyclonal antibodies, were obtained from R&D Systems. ELISA kit for soluble form of VEGFR-1 receptor from RealiaTech. The cell proliferation ELISA were obtained from Roche Diagnostic. Total Protein Test Kit was procured from Biorad. Quantitative Cell Migration Assay and In Vitro Angiogenesis Kit were purchased from Chemicon. All others reagents were obtained from Gibco BRL.

Cell culture conditions. Human umbilical vein endothelial cells (HUVEC) were freshly isolated from umbilical veins of newborn babies by collagenase digestion. Cells were incubated in M-199 medium supplemented with FCS (20%), endothelial cell growth supplement (ECGS), HEPES, heparin, L-glutamine, and antibiotics. Experiments were performed in cell cultures at second, third or fourth passages.

Measurement of sVEGFR-1 concentration. To measure the release of sVEGFR-1 protein endothelial cells were seeded into 24-well plates and grown to full confluence. Then, fresh medium was introduced and supplemented with 15d-PGJ₂ at doses 1-10 μ M. Culture media were collected after the designed times for and concentrations of sVEGFR-1 were measured using ELISA.

Isolation of RNA. To measure the expression of VEGF receptors at mRNA levels cells were seeded into 6 well plates. Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform (GTC) extraction [24] using 600 μ L of GTC. Procedure was performed according to vendor's protocol. After isolation, RNA was dissolved in 30 μ L of RNase-free H₂O.

Real time PCR. Amounts of mRNAs for VEGFR-1 and VEGFR-2 were assessed by the one step qRT-PCR performed in the DNA Engine OPTICON™ (MJ Research, USA) using the QuantiTect™ SYBR® Green RT-PCR kit as recommended by the manufacturer. Each reaction contained 25 μ L of 2x QuantiTect SYBR Green RT-PCR Master Mix, 0.5 μ M of forward and reverse primers, 0.5 μ L of QuantiTect RT Mix, 50 ng of total RNA and RNase-free water to 50 μ L. Reactions were performed in the low-profile Multiplate™ 96-well unskirted microplates capped with optical clarity caps. The thermal cycling parameters were as follows: 30 minutes at 50°C for reverse transcription, 15 minutes at 95°C for deactivation reverse transcriptase and activation HotStarTaq DNA Polymerase, and then 50 cycles at 94°C for 15 seconds, and 60°C for 30 seconds, and 72°C for 30 seconds for the melting, annealing, and extending phases, respectively. Quantitative RT-PCR was performed in triplicate for each primer set. The amount of VEGFR-1 and VEGFR-2 mRNAs was standardized to the β -actin mRNA expression. Sequences of primers used were as follows: 5'-ATC ATG AAT GTT TCC CTG CAA and 5'-TGA CAG TCT AAA GTG GTG GAA CTG (for VEGFR-1), 5'-GGA GGA CGA AGG CCT CTA CA and 5'-AAC GGT CCG TAG GAT GAT GA (for VEGFR-2), 5'-TGC ACC ACC AAC TGC TTA and 5'-CAG CGG AAC CGC TCA TTG CCA ATG G (for β -actin). Each product of RT-PCR reaction was examined by melting temperature profile. The optimum temperature was that which gave the maximum reading for specific product when the non-specific product could no longer be detected. RT-PCR products were run on 6% polyacrylamide gels and visualised with silver salts. To further confirm the product identity, they were sequenced with a BigDye Terminator V2.0 Cycle Sequencing Kit (Applied Biosystems, USA) using automated sequencer (ABI PRISM™ 310 Genetic Analyzer, Applied Biosystems, USA).

Analysis of VEGFR-2 expression by flow cytometry. To determine the surface expression of VEGFR-2 protein on HUVEC, cells were washed twice with Hepes-buffered saline solution, treated with collagenase, washed once with PBS, and resuspended at 10⁶ cells/ml in FACS buffer (1% (w/v) bovine serum albumin, 0.03% (w/v) NaN₃, 25 mM Hepes in PBS, pH 7.4). Cell staining was performed using phycoerythrin (PE)-conjugated polyclonal mouse antibodies against human VEGFR-2. Appropriate mouse IgG isotype controls were used to determine the levels of background staining. Approximately 10,000 cells/sample were analyzed using an Epics XL flow cytometer equipped with System II software (Beckman Coulter, Inc., Fullerton, CA).

Proliferation assay. Experiments were performed in HUVEC cultured in media with 10% FCS but without growth factors. Cells were incubated with VEGF₁₆₅ (30 ng/ml) or bFGF (30 ng/ml) in the presence or absence of PPAR γ ligands. After a 48 h incubation period, BrdU was added for 2 h and proliferation was measured using BrdU incorporation assay, performed according to vendor's protocol.

Migration assay. Experiments were performed in HUVEC cultured in medium without FCS and ECGS, but supplemented with 5% BSA. Migration was tested using a modified Boyden chambers (diameter of pores 8 μ m) coated with vitronectin. The assay was carried out 24 h after stimulation with VEGF₁₆₅ (30 ng/ml), according to the manufacturer's protocol.

Morphogenesis. Experiments were conducted using medium containing 10% FCS but without ECGS. HUVEC were seeded on ECMatrixTM (a solid gel basement proteins from Engelbreth Holm-Swarm mouse tumor) and assayed according to the vendor's instruction. Tube formation was inspected under an inverted microscope 24 h after the beginning of the experiment.

Capillary sprouting. Experiments were performed according to procedure described by Korff and Augustin [25] in medium with 10% FCS, but without ECGF. Shortly: in order to generate endothelial cell spheroids, 750 cells were suspended in culture medium containing 0.25% (w/v) carboxymethylcellulose, and seeded in non-adherent round bottom 96-well plates. During 24 h all suspended cells contributed to the formation of a single spheroid. These spheroids were harvested and embedded in collagen gels, prepared by mixing acidic collagen extract of rat tails with 10x DMEM medium and 0.1 N NaOH. Under these conditions spheroids formed capillary-like sprouts, which were inspected and measured after 24 h, using digitized imaging system connected to an inverted microscope.

Cell viability assays. Cell viability was assessed by colorimetric measurement of LDH release or by colorimetric TUNEL TiterTacs assay according to vendors' protocols.

TransAM ELISA. Bindings of nuclear cell extracts to oligonucleotides containing consensus sequences for Mef-2, STAT-1, c-jun, and c-myc were assayed using TransAM ELISA, according to vendor's protocol.

Overexpression of c-myc with retroviral vectors. Plasmid pBabe-puro containing cDNA of c-myc was kindly gifted by Dr. Piotr Sicinski (Boston, USA). Retrovirus production was based on a transient transfection system of the Phoenix-Ampho packaging cell line (established and generously supplied by Dr. Garry Nolan, Stanford, USA) according to procedure described earlier [26]. HUVECs at the confluence ~70% were transduced with retroviral vectors harboring c-myc or control GFP and then incubated for 24 h. Efficacy of transduction was about 30% as estimated by visualization of GFP using fluorescent microscope. Then cells were seeded into 96-well plate and subjected to proliferation assay.

Statistical analysis. All experiments were performed in duplicates and were repeated 3-7 times. Data are presented as mean $\pm$ SD. Statistical evaluation was done with Student's t-test (for two samples) or with ANOVA followed by Tukey test (for more than two samples). Differences were accepted as statistically significant at $P < 0.05$.

Results

Effect of 15d-PGJ₂ on endothelial cell proliferation. All experiments were performed in HUVEC, as these cells do not release detectable quantities of endogenous VEGF and display a low spontaneous angiogenesis, but respond well to exogenous stimulation [19].

Treatment of HUVEC with human recombinant VEGF₁₆₅ protein (30 ng/ml, 48 h) potently induced cell proliferation. This stimulatory effect was very strongly and concentration-dependently attenuated by treatment of cells with 1-10 μ M 15d-PGJ₂ (Fig. 1a). The highest dose used blocked completely the mitogenic response to VEGF, leading to the proliferation rate significantly lower than in intact cells incubated without VEGF.

In the same conditions, supplementation of cells with 10 μ M troglitazone or ciglitazone, more potent and specific ligands of PPAR γ , resulted in much weaker, approximately 40% decrease in mitogenic response of HUVEC. The stronger anti-proliferative effect of 15d-PGJ₂ than troglitazone or ciglitazone indicates the contribution of PPAR γ -independent pathways in inhibition of HUVEC proliferation. Noteworthy, 15d-PGJ₂ almost completely blocked incorporation of BrdU in endothelial cells stimulated by bFGF (Fig. 1b). The rate of reduction was equally strong as in the case of VEGF, suggesting that 15d-PGJ₂ is a general inhibitor of proliferation, regardless of mitogen used. Again, influences of ciglitazone and troglitazone were much weaker and did not exceed 30% of control values (Fig. 1b).

Importantly, in our cell culture conditions, the observed inhibitory effects of 15d-PGJ₂ were not related with any cytotoxicity, as checked by measuring LDH content in culture medium (data not shown). Similarly, treatment of HUVEC with 15d-PGJ₂ did not induce significant level of apoptosis (Fig. 2).

Effect of 15d-PGJ₂ on endothelial cell migration. Migration of HUVEC was tested using modified Boyden chambers and quantified spectrophotometrically, after staining the cells with crystal violet. Basal migration was very low, near the detection threshold of the assay used. Addition of VEGF₁₆₅ protein (30 ng/ml, 24 h) strongly increased cell migration. 15d-PGJ₂ (at the dose of 10 μ M) diminished the VEGF-induced cell migration by about 50% (Fig. 3).

Effect of 15d-PGJ₂ on endothelial cell morphogenesis. The most popular model of angiogenesis in vitro is a tube-formation assay, in which the endothelial cells seeded on Matrigel, align to form a network of cell cords. This multistep process involves cell adhesion, migration, differentiation and growth, reflecting some morphogenetic properties of endothelial cells in vivo [27].

In our experiments HUVEC seeded on the top of ECMatrix in the absence of VEGF remained round and separated (Fig. 4A). If VEGF₁₆₅ protein was added (30 ng/ml, 24 h) the endothelial cells rapidly aligned and formed hollow tube-like structures (Fig. 4B). In the presence of 10 μ M 15d-PGJ₂ (Fig. 4C) the tube assembly was disturbed, tubes were shorter, less netted, and some cells remained separated. The quantification of three experiments indicates that 15d-PGJ₂ inhibits formation of tubes by approximately 70% (Fig. 4D).

Effect of 15d-PGJ₂ on capillary sprouting. In addition to the tube-formation assay, we employed another angiogenic test, using HUVEC spheroids of defined cell number (750 cells/spheroid) embedded in collagen. Cells originating from the core of spheroids invaded the gel and formed a complex network of capillary-like structures. This process is different than the alignment of large number of separated endothelial cells into tubular structures and appears to be better in vitro representation of the angiogenic invasion as it occurs in vivo [25].

We found that cells incubated without growth factors had a very low level of spontaneous angiogenesis (Fig. 5A). They were, however, readily responsive to VEGF. Addition of VEGF to HUVEC spheroids induced a strong capillary sprouting after 24 h (Fig. 5B). This stimulation was almost completely blocked by 10 μ M 15d-PGJ₂ (Fig. 5C). Although

number of sprouts outgrowing from one spheroid was not affected in the cultures incubated with PPAR γ ligand, the capillary length was significantly reduced (Fig. 5D).

Effect of 15d-PGJ₂ on VEGF receptors. Because 15d-PGJ₂ inhibits all VEGF activities tested, one can suppose that it may modulate expression of VEGF-receptors. To investigate this possibility we checked the surface expression and VEGFR-2 protein, the major receptor mediating the angiogenic activity of VEGF. By means of FACScan analysis we found that treatment of cells with 15d-PGJ₂ for 6-12 h decreased expression of VEGFR-2 protein by approximately 30% (Fig. 6A). The expression of the receptor returned to the control level after 24 h, and later on it was even significantly increased when compared with control HUVEC.

Using the same technique we were not able to detect reproducible levels of surface expression of VEGFR-1 protein on the HUVEC cells. Therefore, we focused on measuring the amount of soluble form of VEGFR-1 (sVEGF-R1) in culture media. Using ELISA we demonstrated that treatment of HUVEC with 15d-PGJ₂ concentration-dependently reduced the release of sVEGF-R1 up to 30% after 24 h (Fig. 6B). Statistically significant attenuation could be observed already after 12 h (Fig. 6C).

The inhibitory effect of 15d-PGJ₂ on VEGF receptors was also observed at the mRNA levels (Fig. 7A,B). As demonstrated by a real-time PCR analysis, both VEGF-R1 and VEGF-R2 mRNAs were decreased by about 20% at 6 h after stimulation. Then the levels of expression returned to the control values.

It seems that influence on the VEGF receptors may contribute to antiangiogenic potential of 15d-PGJ₂, but possibly it plays only a minor role in the observed effects. In some experiments we supplemented the HUVEC with 15d-PGJ₂ (10 μ M) at 1 h before, 1 h after or 24 h after stimulation with VEGF (Fig. 7C). The obtained results demonstrated that 1 h exposure to 15d-PGJ₂ before stimulation with VEGF inhibits proliferation more effectively than adding 15d-PGJ₂ at 1 h after VEGF. However, supplementation of cells with 15d-PGJ₂ at 1 h after or even 24 h after mitogenic stimulation still very potently inhibited proliferation of HUVEC. This suggests that although downregulating the VEGF receptors and/or disturbed signal transduction may contribute to inhibition of mitogenesis, the major effect of 15d-PGJ₂ is exerted rather on the level of cell cycle regulators.

Effect of 15d-PGJ₂ on MAP-kinase regulated transcription factors. We checked the influence of 15d-PGJ₂ on binding activity of several transcription factors (STAT-1, Mef-2, c-jun, and c-myc) regulated by MAP kinases in response to mitogenic stimulation. To this aim we isolated nuclear proteins from HUVEC treated for 1 h with 15d-PGJ₂ (1-10 μ M). Then, the nuclear extracts were assayed using TransAM ELISA system. We did not find any effect of 15d-PGJ₂ on STAT-1 and Mef-1 (data not shown). In contrast, 15d-PGJ₂ significantly and concentration-dependently decreased binding activity of c-myc (Fig. 8A) and c-jun (Fig. 8B), two transcription factors involved in regulation of response to VEGF and bFGF. In both cases, inhibition was very potent and at the highest dose of 15d-PGJ₂ (10 μ M) binding activity of transcription factors did not exceed 40% of control values.

Interestingly, the inhibitory effect was equally strong in HUVEC incubated with PPAR γ inhibitor T0070907 (10 μ M). Moreover, rosiglitazone (10 μ M), a potent and specific PPAR γ ligand, did not affect the c-jun and c-myc activities (Fig. 8). This indicates that influence of 15d-PGJ₂ on these transcription factors is independent of PPAR γ pathway.

Effect of 15d-PGJ₂ on proliferation of cells overexpressing c-myc. As c-myc is considered as a one of crucial transcription factors in regulation of cell proliferation we decided to check the effect of c-myc overexpression on action of 15d-PGJ₂. To this aim we

employed retroviral vectors harboring c-myc, whereas control cells were left untreated or transduced with viruses bearing GFP. After transduction HUVEC were cultured for 24 h to obtain the expression of proteins of interest and then the same numbers of cells were seeded on 96-well plate and stimulated with VEGF in the presence or absence of 15d-PGJ₂. As shown in Fig. 9, 15d-PGJ₂ potently and concentration-dependently inhibited proliferation of the intact or GFP-transduced cells. In contrast, if HUVEC were transduced with c-myc, 15d-PGJ₂ exerted only moderate effect, which could be observed only at the highest dose of compound used (10 μM). This observation suggests that inhibition of c-myc may contribute to antiproliferative and antiangiogenic potential of 15d-PGJ₂.

Discussion

Angiogenesis involves proliferation, migration, and differentiation of endothelial cells followed by formation of capillaries. All these activities can be induced in vitro by supplementation of endothelial cell cultures with VEGF, a major mediator of neovascularization [1].

We tested how the proangiogenic effects of VEGF are modified by 15d-PGJ₂, a natural ligand of PPARγ and potent inducer of HO-1. The most important findings of our study are: i) 15d-PGJ₂ strongly inhibits all VEGF-induced angiogenic activities in HUVEC, namely proliferation, migration, assembly into tube-like network on the matrigel, and ingrowth of capillaries into the collagen gel; ii) 15d-PGJ₂ inhibits expression of VEGF receptors, both VEGFR-1 and VEGFR-2. This effect, however, is transient and relatively weak, and cannot fully explain reduction of response to VEGF; iii) 15d-PGJ₂ strongly and PPARγ-independently inhibits activities of c-jun and c-myc, the transcription factors involved in regulation of cell cycle; iv) overexpression of c-myc protects cells from antiproliferative activity of 15d-PGJ₂.

Growing body of evidence indicates that agonists of PPARγ can efficiently influence neovascularization. This control can result from a regulation of both stimulators and inhibitors of angiogenesis. Thus, it has been shown that PPARγ ligands downregulate vascular endothelial growth factor-C (VEGF-C), leptin and tumor necrosis factor-α (TNFα), which are proangiogenic compounds [28,29], whereas upregulate the antiangiogenic maspin, and CD36, the receptor for thrombospondin [30-32]. On the other hand, a target gene for PPARγ, termed PGAR (PPARγ angiopoietin-related gene) and selectively localized to highly vascularized tissues, exerts a protective function in endothelium [33].

Antiangiogenic potential of PPARγ agonists has been described in earlier reports, where troglitazone and rosiglitazone, specific ligands of TZD family inhibited endothelial cell proliferation, migration, and differentiation into tube-like structures in response to VEGF [10,20,21]. Also in our previous experiments proangiogenic effects of VEGF were reduced by another TZD compound, ciglitazone. It attenuated the VEGF-induced HUVEC growth, migration, and alignment into the tube network [19].

Noteworthy, effects of TZD, the more potent activators of PPARγ are weaker than those observed for 15d-PGJ₂. The biggest difference concerns VEGF-induced proliferation, which is reduced by troglitazone or ciglitazone by approximately 40%, while 15d-PGJ₂ at the same dose (10 μM) ceases it almost completely. This suggests the involvement of PPARγ independent pathways in antiangiogenic activities of 15d-PGJ₂. Comparison of the present results with those obtained for ciglitazone in our earlier experiments demonstrates that also formation and growth of capillaries are inhibited by 15d-PGJ₂ more strongly than by TZDs. In contrast, migration of endothelial cells is decreased by approximately 50% both by ciglitazone and 15d-PGJ₂ [19, and this study].

Mechanisms underlying the inhibitory effects of 15d-PGJ₂ and other agonists of PPAR γ are not fully understood. Some early observations suggested that inhibition was caused by down-regulated expression of VEGF receptors in endothelial cells [10]. In accordance with this thesis, also retinal pigment epithelial cells (RPE) stimulated with VEGF were inhibited by the PPAR γ ligands [21]. RPE cells are one of the few normal cell types, other than endothelial cells, that express high-affinity VEGF receptors and respond to VEGF as a mitogen and chemotactic agent [34].

VEGFR-1 (Flt-1) and VEGFR-2 (Flk/KDR) are two structurally related tyrosine kinase receptors, which act as transducers to signal endothelial cell proliferation, migration, differentiation, and morphogenesis [35-38]. Xin and coworkers (1999) shown that in endothelial cells grown in three-dimensional collagen gels and stimulated with a mixture of growth factors 15d-PGJ₂ can down-regulate mRNA of both VEGF receptors. As measured by real-time PCR the expression of VEGFR-1 was unchanged after a 4 h incubation period, but reduced by approximately 60% after 24 h. Expression of VEGFR-2 was influenced even stronger and decreased by about 60% both after 4 and 24 h. Very similar data were obtained by the same authors when HUVEC were grown on the collagen-coated surface with growth factor stimulation [10].

Further papers, however, described contradictory results. Meissner and colleagues (2004) postulated that expression of VEGFR-2 in endothelial cells is inhibited by ligands of PPAR α transcription factor, but not by activators of PPAR γ . They were not able to find any significant effects even at the very high dose of 15d-PGJ₂ (25 μ mol/L), in spite of testing several time-points (4, 16 and 24 h). Moreover, VEGFR-1 expression levels were largely unchanged by treatment with either PPAR α or PPAR γ ligands. Thus it was concluded that antiangiogenic properties of PPAR γ activators were mediated primarily by their capacity to control cell cycling rather than by suppressing endothelial VEGFR-2 expression [38].

Furthermore, recently reported results of transactivation and electrophoretic mobility shift assays demonstrated that PPAR γ itself increased VEGFR-2 transcription rate in retinal capillary endothelial cells, although there is no consensus PPAR response element in the VEGFR-2 gene promoter [39]. This effect was caused by physical interaction of PPAR γ protein with Sp1 and Sp3 transcription factors leading to enhanced binding of Sp1 to the target DNA sequence. However, in the same cells 15d-PGJ₂, a PPAR γ ligand, dose-dependently suppressed binding of VEGFR-2 promoter region with Sp1 and Sp3, leading to an inhibition of VEGFR-2 gene expression. Northern-blotting for VEGFR-2 mRNA shown strong inhibition after 4 h and only very weak effects after 10 and 24 h of treatment with 15d-PGJ₂ [39]. Thus it was postulated that PPAR γ enhances VEGFR-2 gene expression at the transcriptional level in the absence of its ligands, PPAR γ activators, however, suppress VEGFR-2 gene expression.

Our present study confirms that 15d-PGJ₂ reduces expression of VEGFR-2 both at the mRNA and protein levels. Very similar effects we observed also for VEGFR-1, a receptor acting rather as a sink for VEGF. Worth mentioning, we did not find any significant influence of specific PPAR γ ligands, ciglitazone and troglitazone (data not shown). Additionally, we noticed that changes in mRNA levels in cells treated with 15d-PGJ₂ only roughly reflected the modulation of protein expression. This could be caused by different sensitivity and precision of the methods used (real-time PCR and FACScan analysis), but it might suggest as well that 15d-PGJ₂ influences a protein translation or stability.

It must be stressed, however, that in our hands the inhibition of VEGF receptors was relatively weak (approximately 30%) and short-lasting. So, already 24 h after treatment with 15d-PGJ₂ we were not able to see any differences between treated and untreated cells either

at mRNA or protein levels, and after 48 h the expression of VEGFR-2 was even higher than in unstimulated cells. Moreover, supplementation of HUVEC with 15d-PGJ₂ at 24 h after stimulation with VEGF still produced very potent inhibition of cell proliferation, suggesting that VEGF receptor is not the crucial target in anti-mitogenic action of 15d-PGJ₂. Finally, 15d-PGJ₂ decreased very efficiently proliferation of HUVEC induced by another mitogen, bFGF.

Taken together, the decreased expression of VEGF receptors cannot be a major explanation of the antiangiogenic activity of 15d-PGJ₂. Accordingly, it has been reported that both 15d-PGJ₂ and TZDs exert potent antiproliferative effects on many normal and malignant cell types, which do not express VEGF receptors and do not respond to VEGF [40-42]. Therefore, it seems that 15d-PGJ₂ can influence some fundamental pathways involved in regulation of mitogenesis induced by numerous stimuli.

It has been suggested that antiproliferative effects of 15d-PGJ₂ in ECV304 cells (described as endothelial, but being in fact the bladder carcinoma cells), Jurkat T cells and Raji B cells were associated with decreased expression of AP-1 or c-myc [43,44]. It seems that the same relationship may also occur in endothelium. We demonstrated that 15d-PGJ₂ inhibits DNA-binding activity of c-Jun (this study) as well as AP-1 in HUVEC [45]. Similarly, 15d-PGJ₂ was proposed to act, independently of PPAR γ activation, by decreased AP-1 activation in mesangial cells and chondrocytes [46,47]. These observations are in agreement with finding that 15d-PGJ₂ can covalently modify c-Jun protein at cysteine 269 within the DNA binding domain and directly inhibit the DNA binding activity of AP-1. In addition, 15d-PGJ₂ was shown to promote the oligomerization of a fraction of c-Jun through the formation of intermolecular disulfide bonds or 15d-PGJ₂-bonded dimers [48]. Thus, 15d-PGJ₂ can decrease AP-1-DNA binding even in the presence of unaffected or increased nuclear levels of c-Jun protein [46,48,49]. Attenuation of AP-1 activity in HUVEC in response to 15d-PGJ₂ is accompanied by decreased expression of endothelial nitric oxide synthase (eNOS) [45]. Because eNOS-derived NO is important permissive molecule in all VEGF-induced angiogenic activities [50], one can suppose that decrease in eNOS expression may play a role in antiangiogenic effects of 15d-PGJ₂.

Finally, it was reported that 15d-PGJ₂ can regulate the expression of c-myc [44,49]. This proto-oncogene, whose activation in VEGF-treated dermal endothelium was shown to induce angiogenic phenotype, is involved in the regulation of cell proliferation, differentiation, and apoptosis [51]. However, the influence of 15d-PGJ₂ on c-myc may vary in different cell types and experimental settings. Thus, 15d-PGJ₂ caused marked suppression of c-myc mRNA expression in HT-29 human colon cancer cells [49] or in T and B lymphocytes [44], whereas no effect was detected in HL-60 human leukemic cells [52]. We found that 15d-PGJ₂ potently and concentration-dependently decreases DNA binding of c-myc in HUVEC. This effect appears independent of PPAR γ , as it is not mimicked by rosiglitazone and is not changed in cells subjected to inhibition of this nuclear receptor. Our results suggest also that reduction of c-myc activity may be an important mediator in antiangiogenic effects of 15d-PGJ₂. This suggestion is supported by results of experiments showing that overexpression of c-myc strongly attenuated the 15d-PGJ₂-induced inhibition of HUVEC proliferation.

In summary, our data show that despite augmenting the expression of VEGF, 15d-PGJ₂ exerts strong antiangiogenic effects by inhibition of VEGF activity revealed by decreased proliferation and migration of endothelial cells and by reduced formation of capillaries. Although 15d-PGJ₂ decreases the expression of VEGF receptors, this influence is relatively weak and transient. Our data indicate that more important may be an inhibition of some transcription factors involved in regulation of angiogenesis, especially c-myc oncogene.

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Fig. 1. Effect of 15d-PGJ₂, ciglitazone and troglitazone on proliferation of HUVEC in response to VEGF (**A**) or bFGF (**B**). Cells were treated with mitogens for 48 h and then the proliferation was measured using BrdU incorporation assay. Data are presented as percentage of the reference value (reference - cells stimulated with mitogen alone). * - P<0.05 in comparison with control, untreated cells; # - P<0.05 in comparison with cells stimulated with mitogen alone.

Fig. 2. Lack of effect of 15d-PGJ₂ on apoptosis of HUVEC. Cells were incubated in the presence of VEGF (30 ng/ml) and 10% FCS with or without 15d-PGJ₂ for 48 h and then apoptosis was measured using colorimetric TUNEL assay.

Fig. 3.

Effect of 15d-PGJ₂ (10 μ M) on VEGF-induced migration of HUVEC. Cells were assayed 24 h after stimulation. Amounts of cells which migrated through modified Boyden chambers were assessed by staining cells with crystal violet, extraction of the stain and measurement of absorbance. * - $P < 0.05$ in comparison with control, untreated cells; # - $P < 0.05$ in comparison with cells stimulated with VEGF alone.

Fig. 4.

Effect of 15d-PGJ₂ (10 μ M) on formation of tubules on matrigel. Cells were assayed 24 h after stimulation with VEGF. **A, B, C** - representative pictures showing HUVEC on matrigel. **D** - quantitative presentation of measurements of length of tubules expressed in arbitrary units. * - $P < 0.05$ in comparison with control, untreated cells; # - $P < 0.05$ in comparison with cells stimulated with VEGF alone.

Fig. 5. Effect of 15d-PGJ₂ (10 μ M) on sprouting of capillaries from endothelial spheroids embedded in collagen gel. Cells were assayed 24 h after stimulation with VEGF. **A, B, C** - representative pictures showing HUVEC spheroids. **D** - quantitative presentation of measurements of length of capillaries expressed in arbitrary units. Data are presented as percentage of the reference value (reference - cells stimulated with VEGF alone). * - $P < 0.05$ in comparison with control, untreated cells; # - $P < 0.05$ in comparison with cells stimulated with VEGF alone

Fig. 6.

A - Effect of 15d-PGJ₂ (10 μM) on surface expression of VEGF-R2 protein in HUVEC after 6, 12, 24 and 48 h incubation periods. **B** - Effect of different doses of 15d-PGJ₂ on release of sVEGF-R1 from HUVEC into culture media after a 24 h incubation period. **C** - Effect of 15d-PGJ₂ (10 μM) on release of sVEGF-R1 from HUVEC into culture media at different time-points. Surface expression of VEGF-R2 was measured using flow cytometer. Concentrations of sVEGF-R1a in culture med were determined by ELISA. Data are presented as percentage of the control value (control - untreated cells). * - $P < 0.05$ in comparison with control.

Fig. 7.

Effect of 15d-PGJ₂ (10 μ M) on expression of mRNA of VEGF receptors in HUVEC after 6, 12, 24 and 48 h incubation periods. **A** - VEGFR-1; **B** - VEGFR-2. Expression was measured using real-time PCR. Data are normalized to the expression of β -actin housekeeping gene and presented as percentage of the control value (control - untreated cells). **C** - Effect of 15d-PGJ₂ added at different time points on VEGF-induced proliferation of HUVEC. Proliferation was measured using BrdU incorporation assay after a 48 h incubation. Data are presented as percentage of the reference value (reference - cells stimulated with VEGF alone). * - $P < 0.05$ in comparison with control, untreated cells; # - $P < 0.05$ in comparison

with cells stimulated with mitogen alone; % - $P < 0.05$ in comparison with cells exposed to 15d-PGJ₂ 1 h before adding VEGF.

Fig. 8.

Effect of 15d-PGJ₂ and rosiglitazone on activity of c-myc (**A**) and c-jun (**B**) after an 1 h incubation period. Some cells were treated with PPAR γ inhibitor T0070907 (10 μ M). Binding activities of the transcription factors in extracts of nuclear proteins were measured using TransAM ELISA. Data are presented as percentage of the control value (control - untreated cells). * - $P < 0.05$ in comparison with control.

Fig. 9.

Effect of 15d-PGJ₂ on proliferation of HUVEC. Some cells were transduced with retroviral vectors harboring control GFP or c-myc. Cells were incubated with VEGF (30 ng/ml) with or without 15d-PGJ₂ for 48 h and then the proliferation was measured using BrdU incorporation assay. Data are presented as percentage of control value (control - cells incubated without 15d-PGJ₂). * - $P < 0.05$ in comparison with control.