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Opposite effects of prostaglandin-J_2 on VEGF in normoxia and hypoxia: role of HIF-1

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Abstract

The vascular endothelial growth factor (VEGF) is produced in response to hypoxia or inflammatory cytokines. In normoxia VEGF synthesis is upregulated by 15-deoxy-$\Delta^{12,14}$-prostaglandin-J_2 (15d-PGJ_2) via induction of heme oxygenase-1 (HO-1). Here we compared the influence of 15d-PGJ_2 on VEGF expression in human microvascular endothelial cells in normoxia (~20% O_2) and hypoxia (~2% O_2). Regardless of the oxygen concentration, 15d-PGJ_2 inhibited activity of hypoxia inducible factor-1 (HIF-1), the major hypoxic regulator of VEGF. However, in normoxic conditions 15d-PGJ_2 (1–10 μM) activated the VEGF promoter and increased synthesis of the VEGF protein. Concomitantly, it strongly induced expression of HO-1. In contrast, in hypoxia, 15d-PGJ_2 decreased VEGF promoter activity and reduced VEGF release by 50%. Inhibition of HO-1 activity additionally attenuated VEGF synthesis in hypoxia. We conclude that induction of HO-1 by 15d-PGJ_2 results in augmentation of VEGF synthesis in normoxia. In hypoxia, however, the stimulatory effect of HO-1 is outweighed by 15d-PGJ_2-mediated inhibition of the HIF-1 pathway.

Keywords: Peroxisome proliferator-activated receptor-γ; Prostaglandin-J_2; Endothelial cells; Hypoxia; Normoxia; Vascular endothelial growth factor; Hypoxia inducible factor-1; Heme oxygenase-1

The vascular endothelial growth factor (VEGF) is a specific endothelial cell mitogen and survival agent, which induces angiogenesis in ischemic or inflamed tissues, e.g., during tumor growth, wound healing, rheumatoid arthritis or diabetic retinopathy. It is generated by many cell types in response to some growth factors or proinflammatory cytokines, but the strongest inducer of its expression is hypoxia [1].

Prostaglandin-D_2 (PGD_2) and its derivative 15-deoxy-$\Delta^{12,14}$-prostaglandin-J_2 (15d-PGJ_2) are the most abundantly produced prostaglandins within the vessel wall [2]. They cause a variety of biologic effects, including induction of vasodilatation, inhibition of platelet aggregation, a decrease in inflammatory response, and a cessation of cell growth [2]. 15d-PGJ_2 is best known as a natural ligand of the peroxisome proliferator-activated receptor-γ (PPARγ) transcription factor [3,4]. It is also one of the most potent inducers of heme oxygenase-1 (HO-1) [5], a stress-inducible enzyme which catalyzes the oxidation of heme to biologically active molecules: carbon monoxide (CO), iron, and biliverdin [6].

Several reports have demonstrated that 15d-PGJ_2 and other PPARγ activators can induce expression of the VEGF protein in macrophages [7,8], vascular smooth muscle cells (VSMC) [8–10], and endothelial cells [5,11]. The upregulation of VEGF is accompanied by activation of PPARγ and results from increased transcription rate [8]. However, the VEGF promoter does not seem to include the consensus sequence of the PPAR responsive element, suggesting that PPARγ ligands augment VEGF expression indirectly or through different pathway(s) [8,11].

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Recently, we have shown that upregulation of VEGF production by human microvascular endothelial cells (HMEC-1) treated with 15d-PGJ_2 can be mimicked by the induction of HO-1 expression [5] and these changes in VEGF levels are proportional to CO production [12]. Accordingly, stimulatory effect of 15d-PGJ_2 can be reversed by HO-1 competitive inhibitor, tin protoporphyrin (SnPPIX) or by CO scavenger hemoglobin [12]. This suggests that in HMEC-1, 15d-PGJ_2 upregulates VEGF synthesis through the induction of HO-1 and increased synthesis of CO.

All earlier experiments designed to clarify the role of 15d-PGJ_2 in regulating VEGF expression were performed under normoxic conditions, in an atmosphere of ~20% oxygen. However, in mammalian organs O_2 concentration ranges from 12% to 0.5%, with ~14% in arterial blood, ~10% in the myocardium, and less than or equal to 6% in most other organs. Exceptionally low oxygen values have been detected in solid tumors and in ischemic tissues [13]. Recently, 15d-PGJ_2 has been proposed as a protective compound in myocardium subjected to ischemia–reperfusion injury [14]. It has been also suggested as an antitumor agent due to its strong antiproliferative action [15]. Therefore, it appears crucial to investigate the effect of 15d-PGJ_2 not only in normoxia but also under hypoxic conditions.

The aim of our study was to compare the influence of 15d-PGJ_2 on the expression of VEGF in microvascular endothelial cells cultured in normoxia and hypoxia. We found that 15d-PGJ_2 has the opposite effect on VEGF synthesis under normoxic and hypoxic conditions and that these activities are mediated by different signal transduction pathways.

Materials and methods

Reagents. 15d-PGJ_2, ciglitazone, and troglitazone were obtained from Biomol. Tin protoporphyrin-IX (SnPPIX) was purchased from Porphyrin Products. Fetal calf serum (FCS) was procured from PromoCell. CytoTox-96 assay, Total RNA Extraction Kit, Reverse Transcription System, PCR Core System, and Luciferase Assay Reagents were obtained from Promega. The Maxiprep QIAfilter Plasmid Isolation Kit and the SuperFect transfection reagent were purchased from Qiagen. The ELISA kit for human VEGF was obtained from R & D Systems. Rabbit polyclonal antibodies recognizing human HO-1 and goat anti-rabbit monoclonal antibodies conjugated with biotin were purchased from Stressgen. Alkaline phosphatase conjugated with streptavidin is from Dako. The TransAM HIF-1 Transcription Factor Assay Kit is from Active Motif. All others reagents were procured from Sigma.

Cell culture and incubation experiments. HMEC-1 were purchased from Centers for Disease Control and Prevention (Atlanta) and cultured in a DMEM F-12 medium containing 10% FCS, L-glutamine (2 mM), EGF (10 ng/ml), hydrocortisone (1 μg/ml), penicillin (100 U/ml), and streptomycin (10 μg/ml). Cells were placed in 24-well plates and grown to full confluence. Then, a fresh medium was introduced, supplemented with 15d-PGJ_2, troglitazone or ciglitazone (all at doses of 1–10 μM) and cells were transferred to a standard incubator or to a hypoxia chamber. After 24 h, media were collected for the purpose of determining VEGF protein concentrations and LDH activities. The adherent cells were washed twice with cold PBS and subjected to total RNA isolation and RT-PCR analysis or to protein isolation followed by Western blotting to assess HO-1 expression. To inhibit HO-1 activity, cells were preincubated for 30 min with SnPPIX (10 μM) and then cultured in the presence of SnPPIX throughout all the experiments. SnPPIX is a competitive inhibitor, which increases HO-1 mRNA and protein expression, but decreases HO enzymatic activity by approximately 70% [16].

Incubation of cells in hypoxic conditions. Hypoxia was created using a Modular Incubator Chamber (Billups-Rothenberg, Del Mar, CA, USA) as previously described [17]. In short: prior to incubation, the medium was deoxygenated using a vacuum pomp for 1 h. Cells were treated according to experimental protocols and then placed in a hypoxia chamber. Immediately, a gas mixture (5% CO_2 and 95% N_2) was flushed through the chamber for 20 min. The chamber was then tightly sealed and placed at 37 °C in a standard incubator for 3 or 24 h. Measurement of pO_2 with a gas analyzer demonstrated that the O_2 concentration reached a nadir of 2% and remained constant during the experiment.

RT-PCR. Total RNA was isolated from the cells by acid guanidinium thiocyanate–phenol–chloroform extraction [18]. Reverse transcription was carried out on 1 μg of total RNA for 1 h at 42 °C using random primers and AMV reverse transcriptase, according to the vendor's instruction. Then, PCR with *Taq* DNA polymerase was performed for 35 cycles using the following protocol: 95 °C for 40 s, 58 °C for 40 s, and 72 °C for 50 s. PCR products were analyzed by electrophoresis in 2% agarose gels. PCRs were carried out using primers for VEGF (5′-CAC CGC CTC GGC TTG TCA CAT and 5′-CTG CTG TCT TGG GTG CAT TGG) or for the GAPDH housekeeping gene (5′-CGT ATT GGG CGC CTG GTC ACC and 5′-GGG ATG ATG TTC TGG AGA GCC C). The product length for the $VEGF_{121}$ was 431 bp, for $VEGF_{165}$—563 bp, and for GAPDH—587 bp.

Western blotting. Confluent HMEC-1 were incubated for 24 h in normoxia or hypoxia in the presence or absence of 15d-PGJ_2. Then, cells were washed twice with cold PBS without Ca^{2+} and Mg^{2+}, scraped, centrifuged, and resuspended in 400 μl PBS with 1% Triton X-100. The protein extracts were used for Western as described elsewhere [17].

Transient transfection assay. To assess the effect of 15d-PGJ_2 on activity of the VEGF promoter, HMEC-1 were transfected with a reporter plasmid pGL2, harboring the firefly luciferase cDNA regulated by the human full-length VEGF promoter (+54 to −2279) or by a HRE fragment of this promoter (−1014 to −903). Both constructs were kindly provided by Dr. Hideo Kimura (Japan) [19]. In some experiments we used a luciferase reporter plasmid (kindly gifted by Dr. Steven McKnight, Dallas, USA) driven by a promoter containing three optimal NPAS2/BMAL1 recognition sites [20]. These sequences differ from HRE by substitution of one nucleotide (CACGTGC instead of TACGTGC) and were used as a negative control to confirm specificity of effects of 15d-PGJ_2 on HRE. Plasmids were amplified in HB-101 *Escherichia coli* bacteria and isolated on maxiprep columns. The quality of the DNA was assessed by spectrophotometry and by electrophoresis in 1% agarose gel.

HMEC-1 grown to 80% confluence were transfected in 24-well plates using 0.5 μg plasmid DNA and 2.5 μl SuperFect Reagent per well, according to the vendor's protocol. After transfection, cells were exposed to 10 μM 15d-PGJ_2 in normoxia or hypoxia for 24 h. Then, they were collected and the cell lysates were assayed for luciferase activity according to the manufacturer's instruction.

Measurement of HIF-1 activity. Confluent HMEC-1 were incubated with or without 15d-PGJ_2 (10 μM) in normoxia or hypoxia for 3 h. The binding of nuclear cell extracts to oligonucleotides containing HRE was assayed using TransAM ELISA, according to the vendor's protocol. To confirm the specificity of the assay employed, some measurements were performed in the presence of wild type (5′-GAT

CGC CCT ACG TGC TGT CTC AGA TC) or mutated (5′-GAT CGC CCT AAA AGC TGT CTC AGA TC) hypoxia inducible factor-1 (HIF-1) consensus oligonucleotides.

Measurement of VEGF protein concentration. Concentration of VEGF protein in the culture media was quantified using the sandwich ELISA, following the manufacturer's instructions.

Cell viability assay. Cell viability was assessed by colorimetric measurement of LDH release.

Statistical analysis. All experiments were performed in duplicate and repeated 2–4 times. Data are presented as means ± SD. Statistical evaluation was done with Student's *t* test or with ANOVA followed by the Tukey test. Differences were accepted as statistically significant at $P < 0.05$.

Results

Effect of 15d-PGJ$_2$ on VEGF synthesis

After 24 h incubation, confluent, resting HMEC-1 released approximately 20 pg/ml VEGF protein, as measured by ELISA. Similar as in our previous study [5] treatment of the cells with 15d-PGJ$_2$ (1–10 μM) significantly and dose-dependently increased VEGF secretion (Fig. 1A).

In hypoxia, production of VEGF by resting HMEC-1 was about sixfold higher than in normoxia (125.9 ± 3.77 pg/ml VEGF protein after a 24 h incubation period). Interestingly, in contrast to the effect observed in normoxic conditions, treatment of HMEC-1 cultured in hypoxia with the same doses of 15d-PGJ$_2$ decreased synthesis of VEGF in a concentration-dependent manner (Fig. 1B).

Analogous results were obtained using RT-PCR analysis (Fig. 1C). Expressions of two isoforms of VEGF mRNAs (VEGF$_{121}$ and VEGF$_{165}$) were stronger in hypoxia than in normoxia. 15d-PGJ$_2$ (10 μM) augmented signals from VEGF mRNAs in normoxia, but attenuated them in hypoxia. At the doses used, 15d-PGJ$_2$ did not influence HMEC-1 viability either in normoxia or in hypoxia, as assayed by LDH-release test (data not shown). Thus, effects of 15d-PGJ$_2$ cannot be explained by its cytotoxicity.

Similar changes, i.e., the upregulation of VEGF in normoxia and its down-regulation in hypoxia, we also found in HMEC-1 treated with specific PPARγ ligands, ciglitazone, and troglitazone (1–10 μM). Their influence, however, was much weaker compared to 15d-PGJ$_2$. In normoxia, they stimulated VEGF synthesis by 55% (15d-PGJ$_2$—150%), whereas in hypoxia, they inhibited VEGF generation by 16% (15d-PGJ$_2$—50.7%) (data not shown).

Effect of HO-1 inhibition on VEGF synthesis

15d-PGJ$_2$ is known to be one of the strongest inducers of HO-1 expression. As we have shown using Western blot analysis, 15d-PGJ$_2$ potently upregulates

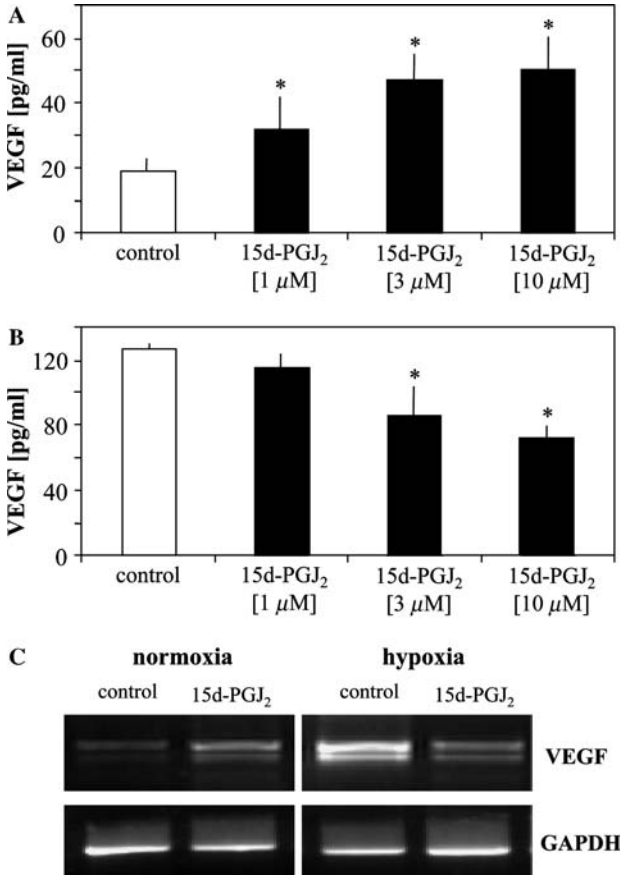

Fig. 1. Effect of 15d-PGJ$_2$ on the release of VEGF protein from HMEC-1 after 24 h incubation in normoxia (A) and hypoxia (B) measured by ELISA. The mean of four independent experiments. *$P < 0.05$ in comparison to the control. (C) Representative of four RT-PCR results, showing the expression of VEGF mRNA in HMEC-1 treated with 15d-PGJ$_2$ (10 μM) in normoxia and hypoxia. Two isoforms of VEGF mRNA are visible (VEGF$_{121}$—the lower band; VEGF$_{165}$—the upper band), GAPDH is shown as a housekeeping gene.

synthesis of the HO-1 protein in HMEC-1 cultured both in normoxic and hypoxic conditions (Fig. 2A). We have previously demonstrated that activation of HO-1 mediates (at least in part) the stimulatory effect of 15d-PGJ$_2$ on VEGF synthesis in normoxia [11]. Therefore, we decided to assess the contribution of HO-1 in regulating VEGF synthesis in response to 15d-PGJ$_2$ in normoxic and hypoxic conditions. With this aim in mind we pretreated cells for 30 min before adding 15d-PGJ$_2$ with a competitive inhibitor of HO-1, SnPPIX (10 μM). As shown in Fig. 2B, SnPPIX reduces the 15d-PGJ$_2$-modulated synthesis of VEGF both in normoxia and hypoxia.

Effect of 15d-PGJ$_2$ on the VEGF promoter

To investigate whether 15d-PGJ$_2$ regulates VEGF transcription, HMEC-1 were transfected with reporter plasmid pGL2-VEGF containing luciferase cDNA under control of a full-length human VEGF promoter.

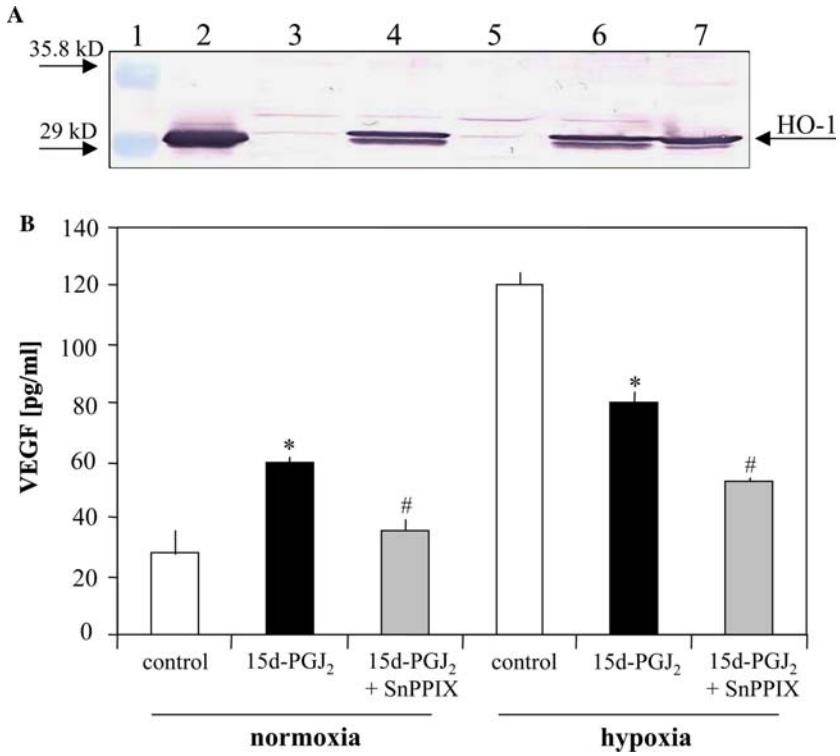

Fig. 2. (A) Western blot showing the effect of 15d-PGJ_2 (10 μM) on the expression of the HO-1 protein from HMEC-1 cultured in normoxia and hypoxia (one of the four similar experiments). Lanes: 1—marker; 2—HO-1 standard; 3—normoxia, control; 4—normoxia, 15d-PGJ_2 (10 μM); 5—hypoxia, control; 6—hypoxia, 15d-PGJ_2 (10 μM); and 7—positive control (proteins isolated from cells stably transfected with HO-1 cDNA). (B) Effect of SnPPIX (10 μM), an inhibitor of HO-1, on 15d-PGJ_2-regulated release of the VEGF protein from HMEC-1 incubated in normoxia and hypoxia (measured by ELISA). One of the two similar experiments. $^{*}P < 0.05$ in comparison to control, $^{\#}P < 0.05$ in comparison to 15d-PGJ_2 treatment.

After transfection, some cells were supplemented with 15d-PGJ_2 (10 μM) and cultured for 24 h in normoxic or hypoxic conditions. In the resting HMEC-1, hypoxia potently induced the VEGF transcription rate, as determined by a luciferase assay (Fig. 3A). 15d-PGJ_2 increased the activity of the VEGF promoter in normoxia, but decreased it by about 50% in hypoxic conditions (Fig. 3A). The extent of changes in the promoter activity was quantitatively similar to that of protein synthesis (Figs. 1A and B), suggesting that 15d-PGJ_2 modulates VEGF expression primarily at the gene transcription level. Modulation of the VEGF promoter by troglitazone or ciglitazone was statistically insignificant (data not shown).

Since in hypoxic conditions VEGF expression is regulated primarily by HIF-1 [21] we measured the effect of 15d-PGJ_2 on the activity of hypoxia responsive element (HRE), a consensus sequence for HIF-1. To this aim HMEC-1 were transfected with pGL2-HRE reporter plasmid, with luciferase cDNA regulated by HRE. Like a full-length promoter, HRE in resting cells was potently induced by hypoxia. Importantly, treatment of HMEC-1 with 15d-PGJ_2 significantly decreased HRE activity under hypoxic as well as under normoxic conditions (Fig. 3B).

To confirm that this effect was specific for HRE, some cells were transfected with a reporter plasmid driven by NRE, a modified HRE sequence (CACGTGC instead of TACGTGC). NRE, a recognition site for the NPAS2/BMAL1 heterodimer [20], was induced by hypoxia, but was not influenced by 15d-PGJ_2 (Fig. 3C). This observation indicates that the inhibitory effect of 15d-PGJ_2 is specific to HIF-1.

Effect of 15d-PGJ_2 on HIF-1 activity

Finally, we measured the activity of the HIF-1 protein in nuclear extracts isolated from HMEC-1 incubated for 3 h in normoxic or hypoxic conditions in the presence or absence of 15d-PGJ_2 (10 μM). The isolated extracts were assayed using the ELISA format to measure a specific binding to HRE oligonucleotides, attached to a 96-well plate. In accordance with the results of the reporter assay, these experiments showed higher HIF-1 activity in cells cultured in hypoxia and confirmed the inhibitory effect of 15d-PGJ_2 both in normoxia and hypoxia (Fig. 4A). Additionally, to exclude the possibility of unspecific binding of proteins from nuclear extracts to the oligonucleotides used, we repeated the measurement of HIF-1 activity from

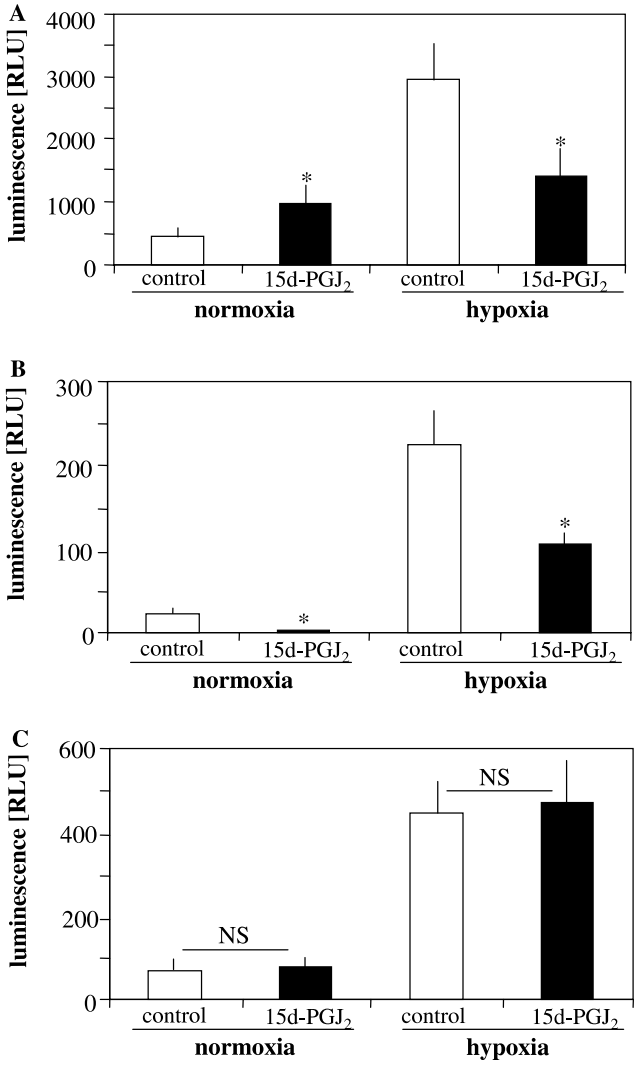

Fig. 3. Effect of 15d-PGJ_2 (10 μM) on the activity of a full-length VEGF promoter (A) HRE sequence of the human VEGF promoter (B) and on NRE sequence (C) in HMEC-1 cultured in normoxia and hypoxia. One of the four similar experiments. The cells were transfected with reporter plasmids (containing a luciferase cDNA regulated by a full-length human VEGF promoter, its HRE fragment or NRE sequence) and subjected to luciferase assay 24 h after transfer. $^{*}P < 0.05$ in comparison to control.

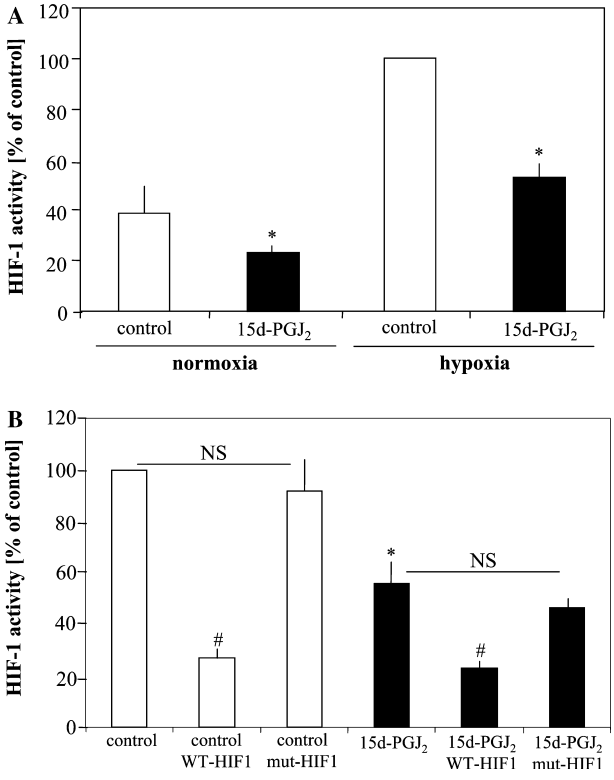

Fig. 4. (A) Effect of 15d-PGJ_2 (10 μM) on the activity of HIF-1 in HMEC-1 cultured in hypoxia and normoxia. Nuclear proteins were isolated from HMEC-1 after 3 h incubation of cells with 15d-PGJ_2 and analyzed using TransAM ELISA. (B) Effect of 15d-PGJ_2 on the HIF-1 activity in hypoxia—test for assay specificity. Analyses were performed in the presence of wild-type HIF-1 (WT-HIF1) or mutated HIF-1 (mut-HIF1) oligonucleotides. Data are presented as percentage of the control value (control: untreated cells in hypoxia). The mean of three (A) or two (B) experiments. $^{*}P < 0.05$ in comparison to control, $^{\#}P < 0.05$ in comparison to cells untreated with WT-HIF1.

HMEC-1 incubated in hypoxia (both from the control and 15d-PGJ_2 stimulated cells) in the presence of competitors (20 pmol/well): HIF-1 wild-type consensus oligonucleotides or HIF-1 mutated consensus oligonucleotides. As shown in Fig. 4B, reaction was potently inhibited by wild-type, but not by mutated sequences. This confirms that the results obtained, presented in Fig. 3A, are specific to HIF1.

Discussion

We have demonstrated here that 15d-PGJ_2 influences the expression of VEGF by HMEC-1 in the opposite way in normoxia and hypoxia. Under normoxic conditions, 15d-PGJ_2 at concentrations of 1–10 μM significantly upregulates VEGF promoter activity and VEGF protein synthesis. In contrast, in the case of hypoxia, 15d-PGJ_2 at the same doses strongly reduces VEGF promoter activity and decreases VEGF protein generation by 50%. Interestingly, transcription of the reporter gene regulated by a fragment of the VEGF promoter containing the HRE sequence, as well as the binding of HIF-1 to HRE, is inhibited by 15d-PGJ_2 both under normoxic and hypoxic conditions.

The effects of 15d-PGJ_2 are usually considered to be mediated by the activation of the PPARγ transcription factor. It is well known that ligands of PPARγ can induce expression of VEGF in many cell types, e.g., in the RT4 papillary tumor cell line, macrophages, VSMC, and in arterial or microvascular endothelial cells [5,8,9,11,22]. The upregulation is observed both at mRNA and protein levels [8,11] and is dependent on activation of VEGF transcription [5,8]. PPARγ activators probably only have an indirect influence, as the

VEGF promoter does not contain a PPAR response element [11,22].

We have already shown that PPARγ is expressed in HMEC-1 and can be activated by its specific ligands: TZDs (troglitazone and ciglitazone) and 15d-PGJ_2 [23]. In accordance with earlier reports [3] we have found that 15d-PGJ_2 is a much weaker PPARγ activator than TZDs [23]. The upregulation of VEGF synthesis in HMEC-1 in response to 15d-PGJ_2 was, however, much stronger compared to ciglitazone or troglitazone. It indicates that, in addition to the possible involvement of PPARγ transcription factor, some PPAR-independent pathways can be involved in regulation of VEGF by 15d-PGJ_2 in these cells.

Apart from activation of PPARγ, 15d-PGJ_2 is one of the strongest inducers of the expression of the HO-1 enzyme. Induction of HO-1 is independent of PPARγ, but is mediated through stimulation of Nrf-2, a transcription factor which forms heterodimer with the Maf protein and binds to the stress-response element (StRE) in the HO-1 promoter [24].

HO-1 oxidizes heme to biologically active molecules: CO, iron ions, and biliverdin, the latter being converted in most tissues to bilirubin. Among HO-1 products, CO is an important cellular messenger, with the signaling functions resembling those of nitric oxide [25]. The HO-1 pathway is involved in regulating VEGF expression. In earlier experiments, we have shown that activation or overexpression of HO-1, followed by generation of CO, leads to the upregulation of VEGF synthesis in VSMC and in microvascular endothelium [5,16,26]. The stimulatory impact of HO-1 overexpression on VEGF production has very recently been corroborated using in vivo models [27,28].

In the present study, we have shown that 15d-PGJ_2 strongly induces HO-1 expression by HMEC-1 both in normoxia and hypoxia. In experiments performed in normoxic conditions we confirmed our earlier observations [5,12] that augmentation of VEGF synthesis by HMEC-1 in response to 15d-PGJ_2 is probably mediated by induction of HO-1. Pretreatment of cells with SnPPIX, a competitive inhibitor of HO-1, significantly reduced the stimulatory effect of 15d-PGJ_2.

In the case of hypoxia, however, in spite of HO-1 induction, synthesis of the VEGF protein was reduced in the presence of 15d-PGJ_2. Interestingly, pretreatment of HMEC-1 with SnPPIX in hypoxic conditions additively strengthened the inhibitory influence of 15d-PGJ_2 on VEGF production. Thus, it seems that HO-1 induction can upregulate generation of VEGF regardless of the oxygen concentration but, in hypoxic conditions, the stimulatory effect of HO-1 is masked by other pathway(s) regulated by 15d-PGJ_2.

Analysis of the VEGF promoter region reveals several potential binding sites for transcription factors AP-1, AP-2, SP-1, and HIF-1 [19]. In the case of normoxia, the basal and cytokine-enhanced VEGF expression is mediated mainly by the SP-1 protein, which interacts with four SP-1 binding sequences located in the proximity of the transcription start site [29]. In contrast, the most important transcription factor responsible for hypoxia-induced generation of VEGF is HIF-1 [21].

In the present study, we showed for the first time that treating cells with 15d-PGJ_2 reduces HIF-1 activity. This was illustrated by two independent methods: (i) measuring luciferase activity in HMEC-1 transfected with a pGL2-HRE plasmid harboring the reporter gene driven by an HRE-containing fragment of a VEGF promoter and (ii) measuring the binding of an HIF-1 heterodimer from HMEC-1 nuclear extracts to HRE oligonucleotides. This inhibitory effect does not seem to be characteristic for all prostaglandins, since, e.g., prostaglandin E_2 was found to upregulate HIF-1 activity, thereby augmenting the expression of VEGF [30].

15d-PGJ_2 downregulates HIF-1 both in normoxia and hypoxia. Under hypoxic conditions it inhibits the activity of a full-length VEGF promoter and reduces the transcription of VEGF mRNA. In contrast, in normoxia, when HIF-1 is not a major factor regulating VEGF transcription, 15d-PGJ_2-mediated inhibition of HIF-1 was outweighed by stimulation of other, still unidentified transcription factor(s). As a result, under normoxic conditions, the full-length VEGF promoter activity was higher and transcription of VEGF mRNA was stronger in the presence of 15d-PGJ_2 than in untreated cells.

The mechanism underlying inhibited HIF-1 activity through 15d-PGJ_2 is not known. As was demonstrated in the experiments performed in cardiomyocytes, PPARγ pathway is not directly involved in regulating the HIF-1α [31]. On the other hand, however, 15d-PGJ_2 (but not TZDs) induces interaction of the PPARγ protein with the p300 co-activator [32]. It was postulated that competition for p300 is one of the mechanisms through which PPARγ regulates the activities of other transcription factors, namely NFκb, AP-1, and STATs [33]. As the transactivation activity of HIF-1 requires recruiting the p300/CREB-binding protein [34], we can speculate that 15d-PGJ_2-induced interaction of PPARγ and p300 could play a role in inhibition of HIF-1.

15d-PGJ_2 is regarded as a very potent blocker of cell proliferation, influencing the very basic regulators of the cell cycle, namely p21 and cyclin D [35]. By suppressing the proliferation of endothelium it acts as an efficient inhibitor of angiogenesis [36]. Also in our hands 15d-PGJ_2 strongly and dose-dependently reduced proliferation of endothelial cells both in normoxic and hypoxic conditions (data not shown).

Because of its anti-mitogenic potential, 15d-PGJ_2 was implicated as a possible agent in cancer therapy [15,37]. In this case, however, increased VEGF synthesis in response to 15d-PGJ_2 in normoxia, as reported in

earlier studies, could be a disadvantage. VEGF is a fundamental regulator of angiogenesis regulating multiple endothelial cell functions, including mitogenesis, morphogenesis, vascular permeability, or the production of vasoactive molecules [1] and its upregulation is a bad predictive biomarker in cancer patients (e.g., [38]).

Our present study indicates, however, that in hypoxia, 15d-PGJ_2 is an inhibitor of VEGF production. Such conditions are detected in most tumors, where O_2 concentrations are usually lower than 0.5% [39]. Thus, the inhibition of VEGF synthesis might be an additional benefit of 15d-PGJ_2 treatment.

In summary, we demonstrated that 15d-PGJ_2 induces HO-1 expression in HMEC-1 both in normoxia and hypoxia. Under normoxic conditions, this induction results in upregulation of VEGF synthesis. In the case of hypoxia, however, the stimulatory effect of HO-1 is masked by 15d-PGJ_2-mediated inhibition of the HIF-1 pathway, which leads to decrease in VEGF generation. It appears that the results obtained in hypoxic conditions may better reflect the situation in vivo and may shed light on the role played by 15d-PGJ_2 in ischemic tissues.

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References

[1] N. Ferrara, K. Houck, L. Jakeman, D.W. Leung, Molecular and biological properties of the vascular endothelial growth factor family of proteins, Endocr. Rev. 13 (1992) 18–32.
[2] S. Ito, S. Narumiya, O. Hayashi, Prostaglandin-D_2: a biochemical perspective, Prostaglandins Leukot. Essent. Fatty Acids 37 (1989) 219–234.
[3] S.A. Klievier, J.M. Lenhard, T.M. Willson, I. Patel, D.C. Morris, J.M. Lehmann, A prostaglandin-J_2 metabolite binds peroxisome proliferator-activated receptor and promotes adipocyte differentiation, Cell 83 (1995) 813–819.
[4] Y. Urade, M. Ujihara, Y. Horiguchi, K. Ikai, O. Hayaishi, The major source of endogenous PGD_2 production is likely antigen-presenting cells, J. Immunol. 143 (1989) 2982–2989.
[5] A. Jozkowicz, I. Huk, A. Nigisch, G. Weigel, F. Weidinger, J. Dulak, Effect of prostaglandin-J_2 on VEGF synthesis depends on the induction of heme oxygenase-1, Antioxidant Redox Signal. 4 (2002) 577–585.
[6] M.D. Maines, The heme oxygenase system: a regulator of second messenger gases, Annu. Rev. Pharmacol. Toxicol. 37 (1997) 517–554.
[7] H. Bamba, S. Ota, A. Kato, C. Kawamoto, K. Fujiwara, Prostaglandins up-regulate vascular endothelial growth factor production through distinct pathways in differentiated U937 cells, Biochem. Biophys. Res. Commun. 273 (2000) 485–491.
[8] A. Jozkowicz, J. Dulak, E. Piatkowska, W. Placha, A. Dembinska-Kiec, Ligands of peroxisome proliferator-activated receptor-γ increase the generation of vascular endothelial growth factor in vascular smooth muscle cells and in macrophages, Acta Biochim. Pol. 47 (2000) 1147–1157.
[9] K. Yamakawa, M. Hosoi, H. Koyama, S. Tanaka, S. Fukumoto, H. Morii, Y. Nishizawa, Peroxisome proliferator-activated receptor-gamma agonists increase vascular endothelial growth factor expression in human vascular smooth muscle cells, Biochem. Biophys. Res. Commun. 271 (2000) 571–574.
[10] J. Dulak, A. Jozkowicz, W. Dichtl, H. Alber, S.P. Schwarzacher, O. Pachinger, F. Weidinger, Vascular endothelial growth factor synthesis in vascular smooth muscle cells is enhanced by 7-ketocholesterol and lysophosphatidylcholine independently on their effect on nitric oxide generation, Atherosclerosis 159 (2001) 325–332.
[11] M. Inoue, H. Itoh, T. Tanaka, T.H. Chun, K. Doi, Y. Fukunaga, N. Sawada, J. Yamshita, K. Masatsugu, T. Saito, S. Sakaguchi, M. Sone, K.I. Yamahara, T. Yurugi, K. Nakao, Oxidized LDL regulates vascular endothelial growth factor expression in human macrophages and endothelial cells through activation of peroxisome proliferator-activated receptor-γ, Arterioscler Thromb. Vasc. Biol. 21 (2001) 560–566.
[12] A. Jozkowicz, I. Huk, A. Nigisch, G. Weigel, W. Dietrich, R. Motterlini, J. Dulak, Heme oxygenase and angiogenic activity of endothelial cells: stimulation by carbon monoxide, inhibition by tin protoporphyrin-IX, Antioxidant Redox Signal. 5 (2003) 155–162.
[13] S. Roy, S. Khanna, A.A. Bickestaff, S.V. Subramanian, M. Atalay, M. Bierl, S. Pendyala, D. Levy, N. Sharma, M. Venojarvi, A. Strauch, C.G. Orosz, C.K. Sen, Oxygen sensing by primary cardiac fibroblasts, Circ. Res. 92 (2003) 264–271.
[14] N.S. Wayman, Y. Hattori, M.C. McDonald, H. Mota-Filipe, S. Cuzzocrea, B. Pisano, P.K. Chatterjee, C. Thiemermann, Ligands of the peroxisome proliferator-activated receptors (PPAR-gamma and PPAR-alpha) reduce myocardial infarct size, FASEB J. 16 (2002) 1027–1040.
[15] K. Hasimoto, R.T. Ethridge, B.M. Evers, Peroxisome proliferator-activated receptor gamma ligand inhibits cell growth and invasion of human pancreatic cancer cells, Int. J. Gastrointest. Cancer 32 (2002) 7–22.
[16] J. Dulak, A. Jozkowicz, R. Foresti, A. Kasza, M. Frick, I. Huk, C.J. Green, O. Pachinger, F. Weidinger, R. Motterlini, Heme oxygenase activity modulates vascular endothelial growth factor synthesis in vascular smooth muscle cells, Antioxidant Redox Signal. 4 (2002) 229–238.
[17] R. Motterlini, R. Foresti, R. Bassi, V. Calabrese, J.E. Clark, C.J. Green, Endothelial heme oxygenase-1 induction by hypoxia: modulation by inducible nitrix oxide synthase (iNOS), J. Biol. Chem. 275 (2000) 13613–13620.
[18] P. Chomczynski, N. Sacchi, Single step method of RNA isolation by guanidinum thiocyanate–phenol–chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[19] H. Kimura, A. Weisz, Y. Karashima, K. Hashimoto, T. Ogura, F. D'Acqusto, R. Addeo, M. Makuuchi, H. Esumi, Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia—inducible factor-1 activity by nitric oxide, Blood 95 (2000) 189–197.
[20] M. Reick, J.A. Garcia, C. Dudley, S.L. McKnight, NPAS2: an analog of clock operative in the mammalian forebrain, Science 293 (2001) 506–509.
[21] Y. Liu, S.R. Cox, T. Morita, S. Kourembanas, Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5′ enhancer, Circ. Res. 77 (1995) 638–643.
[22] S. Fauconnet, I. Lascombe, E. Chabannes, G.L. Adessi, B. Desvergne, W. Wahli, H. Bittard, Differential regulation of vascular endothelial growth factor expression by peroxisome proliferator-activated receptors in bladder cancer cells, J. Biol. Chem. 277 (2002) 23534–23543.

[23] A. Jozkowicz, J. Dulak, M. Prager, J. Nanobashvili, A. Nigisch, B. Winter, G. Weigel, I. Huk, Prostaglandin-J_2 induces synthesis of interleukin-8 by endothelial cells in a PPARγ-independent manner, Prostaglandind Other Lipid Mediat. 66 (2001) 165–177.
[24] P. Gong, D. Steward, B. Hu, N. Li, J. Cook, A. Nel, J. Alam, Activation of the mouse heme oxygenase-1 gene by 15-deoxy-Δ12,14-prostaglandin-J2 is mediated by the stress response elements and transcription factor Nrf2, Antioxid Redox Signal. 4 (2002) 249–257.
[25] S.H. Snyder, D.E. Baranano, Heme oxygenase: a font with multiple messengers, Neuropsychopharmacology 25 (2001) 294–298.
[26] J. Dulak, R. Motterlini, I. Huk, O. Pachinger, F. Weidinger, A. Jozkowicz, Carbon monoxide and iron, by products of heme oxygenase, modulate vascular endothelial growth factor synthesis in vascular smooth muscle cells, in: N.G. Abraham (Ed.), Physiology and Pathology of Heme Oxygenase, Kluwer Academic/Plenum Publishers, New York, 2002, pp. 97–107.
[27] D. Kreiser, X. Nguen, R. Wong, D. Seidman, D. Stevenson, S. Quan, N. Abraham, P. Dennery, Heme oxygenase modulates fetal growth in the rat, Lab. Invest. 82 (2002) 687–692.
[28] M. Suzuki, N. Iso, S. Takeshita, K. Tsukamoto, I. Mori, T. Sato, M. Ohno, R. Nagai, N. Ishizaka, Facilitated angiogenesis induced by heme oxygenase-1 gene transfer in a rat model of hindlimb ischemia, Biochem. Biophys. Res. Commun. 302 (2003) 138–143.
[29] M. Ryuto, M. Ono, H. Izumi, S. Yoshida, H.A. Weich, K. Kohna, M. Kuwano, Induction of vascular endothelial growth factor by tumor necrosis factor alpha in human glioma cells. Possible roles of SP-1, J. Biol. Chem. 271 (1996) 28220–28228.
[30] R. Fukuda, B. Kelly, G.L. Semenza, Vascular endothelial growth factor gene expression in colon cancer cells exposed to prostaglandin E2 is mediated by hypoxia-inducible factor 1, Cancer Res. 63 (2003) 2330–2334.
[31] A.J. Belanger, H. Lu, T. Date, L.X. Liu, K.A. Vincent, G.Y. Akita, S.H. Cheng, R.J. Gregory, C. Jiang, Hypoxia up-regulates expression of peroxisome proliferator-activated receptor γ angiopoietin-related gene (PGAR) in cardiomyocytes: role of hypoxia inducible factor-α, J. Mol. Cell. Cardiol. 34 (2002) 765–774.
[32] Y. Kodera, K.I. Takeyama, A. Maruyama, M. Suzawa, Y. Masuhiro, S. Kato, Ligand type-specific interactions of peroxisome proliferator-activated receptor-γ with transcriptional coactivators, J. Biol. Chem. 275 (2000) 33201–33204.
[33] M.E. Greene, J. Pitts, M.A. McCarville, X.S. Wang, J.A. Newport, C. Edelstein, F. Lee, S. Ghosh, S. Chu, PPARγ: observations in the hematopoietic system, Prostaglandins Other Lipid Mediat. 62 (2000) 45–73.
[34] N. Sang, D.P. Stiehl, J. Bohensky, I. Leshchinsky, V. Srinivas, J. Caro, MAPK signaling up-regulates the activity of hypoxia-inducible factors by its effects on p300, J. Biol. Chem. 278 (2003) 14013–14019.
[35] Y. Miwa, T. Sasaguri, H. Inoue, Y. Taba, A. Ishida, T. Abumiya, 15-deoxy-$\Delta^{12,14}$-prostaglandin- J_2 induces G1 arrest and differentiation marker expression in vascular smooth muscle cells, Mol. Pharmacol. 58 (2000) 837–844.
[36] X. Xin, S. Yang, J. Kowalski, M.E. Gerristen, Peroxisome proliferator-activated receptor-γ ligands are potent inhibitors of angiogenesis in vitro and in vivo, J. Biol. Chem. 274 (1999) 9116–9121.
[37] G. Winnet, D. van Hagen, M. Schrey, Prostaglandin J_2 metabolites inhibit aromatase activity by redox-sensitive mechanisms: potential implications for breast cancer therapy, Int. J. Cancer 103 (2003) 600–605.
[38] P. Lissoni, F. Rovelli, F. Malugani, F. Brivio, L. Fumagalli, G.S. Gardani, Changes in circulating VEGF levels in relation to clinical response during chemotherapy for metastatic cancer, Int. J. Biol. Markers 18 (2003) 152–155.
[39] M.C. Krishna, S. English, K. Yamada, J. Yoo, R. Murugesan, N. Devasahayam, J.A. Cook, K. Golman, J.H. Ardenkjaer-Larsen, S. Subramanian, J.B. Mitchell, Overhauser enhanced magnetic resonance imaging for tumor oximetry: coregistration of tumor anatomy and tissue oxygen concentration, Proc. Natl. Acad. Sci. USA 99 (2002) 2216–2221.