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Effect of Prostaglandin-J_2 on VEGF Synthesis Depends on the Induction of Heme Oxygenase-1

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ABSTRACT

Heme oxygenase-1 (HO-1) is an inducible enzyme that degrades heme to carbon monoxide, iron ions, and biliverdin. Its expression can be induced by 15-deoxy-$\Delta^{12,14}$prostaglandin-J_2 (15d-PGJ_2), a natural ligand of peroxisome proliferator-activated receptor-γ transcription factor. In macrophages and vascular smooth muscle cells, 15d-PGJ_2 up-regulates the expression of vascular endothelial growth factor (VEGF), a fundamental regulator of angiogenesis. Here we investigated the involvement of HO-1 in the 15d-PGJ_2-mediated regulation of VEGF production by human microvascular endothelial cells (HMEC-1). Resting HMEC-1 released ~20 pg/ml VEGF protein after 24 h of incubation. Treatment of cells with 15d-PGJ_2 (1–10 μ*M*) significantly and dose-dependently increased the VEGF promoter activity, mRNA expression, and protein secretion. In the same cells, 15d-PGJ_2 potently induced the expression of HO-1 protein that correlated with HO-1 promoter activity. Activation of HO-1 with hemin or ectopic overexpression of HO-1 in HMEC-1 perfectly mimicked the effect of 15d-PGJ_2 and led to increased VEGF production. Importantly, the inhibition of the HO-1 pathway by tin protoporphyrin-IX significantly reduced the stimulatory effect of 15d-PGJ_2 on VEGF synthesis. Thus, we postulate that the up-regulation of VEGF expression in response to 15d-PGJ_2 in HMEC-1 is mediated by the activation of HO-1. *Antioxid. Redox Signal.* 4, 577–585.

INTRODUCTION

PROSTAGLANDIN-D_2 and its derivative 15-deoxy-$\Delta^{12,14}$-prostaglandin-J_2 (15d-PGJ_2) are major products of arachidonic acid metabolism in macrophages and mastocytes under immunological challenge (40). During inflammation, they are the most abundantly produced prostaglandins within the vessel wall (15). They have been shown to cause a variety of biologic effects, including induction of vasodilatation, inhibition of platelet aggregation, decrease in inflammatory response, and cessation of cell growth and differentiation (15, 23).

15d-PGJ_2, formed spontaneously in biologic fluids by dehydration and isomerization of prostaglandin-D_2, is the most potent natural ligand of peroxisome proliferator-activated receptor-γ (PPARγ) (23, 40). PPARγ transcription factor is expressed mainly in adipose tissue (31), but it is also present in the vessel wall in endothelium, vascular smooth muscle cells (VSMC), and infiltrating macrophages, especially within atherosclerotic plaques (14, 18, 27, 30). Recently, we demonstrated that PPARγ is also an active transcription factor in human microvascular endothelial cells (HMEC-1) (19).

A growing body of evidence has shown that ligands of PPARγ, apart from the induction of adipocyte maturation and fatty acid deposition (23, 31), are also involved in the modulation of inflammatory response and angiogenesis (32, 41). One of the genes being up-regulated in the course of

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inflammation is vascular endothelial growth factor (VEGF). VEGF is produced by many types of cells in response to hypoxia, hypoglycemia, or proinflammatory cytokines (12). It is a specific endothelial cell mitogen and survival agent, which induces vascular permeability and angiogenesis during tumor growth, wound healing, age-related macular degeneration, rheumatoid arthritis, diabetic retinopathy, or collateral formation in ischemic tissue (12).

Several recent reports have demonstrated that treatment of cells with 15d-PGJ_2 can induce the expression of VEGF protein in macrophages (2, 18), VSMC (18, 42), and coronary endothelial cells (14). We showed that the up-regulation of VEGF synthesis in VSMC and macrophages is related to the activation of PPARγ transcription factor and mediated by increased VEGF promoter activity (18). The VEGF promoter, however, does not seem to contain the consensus sequence of PPAR responsive element, suggesting that PPARγ ligands up-regulate the VEGF expression indirectly (14, 18).

It has been shown that PPARγ ligands increase the expression of the heme oxygenase-1 (HO-1) gene in many cell types (5, 17, 22) including endothelium (24). HO-1 is a stress-inducible enzyme, which catalyzes the oxidation of heme to biologically active molecules: carbon monoxide, iron, and biliverdin, the latter being converted further to bilirubin (29). Numerous studies have demonstrated that HO-1 may play a protective role in the vessel wall by attenuation of inflammatory reaction, reduction of oxidative stress, or inhibition of endothelial cell apoptosis (3, 7, 36). Our preliminary study suggested that HO-1 activation can also augment the synthesis of VEGF in VSMC (9), which led us to speculate that HO-1 activity might contribute to the VEGF up-regulation (18).

In the present study, we sought to investigate whether HO-1 might regulate VEGF expression in microvascular endothelial cells stimulated with 15d-PGJ_2.

MATERIALS AND METHODS

Reagents

15d-PGJ_2 was obtained from Biomol; L-glutamine, epidermal growth factor, and hydrocortisone were purchased from Sigma; fetal calf serum (FCS) was procured from PromoCell and pcDNA3.1+ expression plasmid from Invitrogen. Tfx-50 reagents, CytoTox-96 assay, SV Total RNA Extraction Kit, Reverse Transcription System, PCR Core System, control pSVβgal plasmid, β-galactosidase assay reagents, and luciferase assay reagents were obtained from Promega, and Maxiprep QIAfilter Plasmid Isolation Kit was purchased from Qiagen. Enzyme-linked immunosorbent assay (ELISA) kits for human VEGF and interleukin-8 (IL-8) proteins were obtained from R&D Systems. All others reagents were procured from GIBCO.

Cell culture and incubation experiments

HMEC-1 were purchased from Centers for Disease Control and Prevention (Atlanta, GA, U.S.A.) and cultured in Dulbecco's modified Eagle medium (DMEM) F-12 medium containing 10% FCS, L-glutamine (2 m*M*), epidermal growth factor (10 ng/ml), hydrocortisone (1 μg/ml), penicillin (100 U/ml), and streptomycin (10 μg/ml). Cells were placed into 24-well plates and grown to full confluence. Then fresh medium was introduced and supplemented with 15d-PGJ_2 or ciglitazone, at 1–10 μ*M*. After 24 h, media were collected for determining VEGF or IL-8 protein concentrations and lactate dehydrogenase (LDH) activities. The adherent cells were washed twice with cold phosphate-buffered saline (PBS) and subjected to total RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR) analysis. In another set of experiments, the cells were treated for 2 h with hemin (10 μ*M*), followed by a 6-h incubation period with fresh medium without hemin. Then medium was changed again, and the concentrations of VEGF and IL-8 proteins were measured 24 h later. To inhibit HO-1 activity, cells were preincubated for 1 h with tin protoporphyrin-IX (SnPPIX; 10 μ*M*). To investigate HO-1 mRNA stability, some cells were treated with lipopolysaccharide (LPS; 100 ng/ml) and then stimulated with 15d-PGJ_2 (10 μ*M*) in the presence of actinomycin D (1 μg/ml) for 0.5, 1, 2, 4, 6, 8, 10, and 12 h.

NIH 3T3 cells (ATCC, CRL-1658) were cultured in high-glucose DMEM medium supplemented with 5% FCS. These easily transfectable cells were used for some experiments.

RT-PCR

Total RNA was isolated from the cells by acid guanidinium thiocyanate–phenol–chloroform extraction (4). RT-PCR reactions were performed on 100 ng of total RNA with primers recognizing VEGF (5′-CAC CGC CTC GGC TTG TCA CAT and 5′-CTG CTG TCT TGG GTG CAT TGG), HO-1 (5′-CTT TCA GAA GGG TCA GGT GTC C and 5′GTG GAG ACG CTT TAC GTA GTG C), and β-actin (5′-AGC GGG AAA TCG TGC GTG and 5′-CAG GGT ACA TGG TGG TGC). RT was carried out for 1 h at 42°C using AMV reverse transcriptase, according to 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. The product length for the $VEGF_{121}$ was 431 bp, for $VEGF_{165}$ 563 bp, for HO-1 250 bp, and for β-actin 310 bp.

Western blotting

Confluent HMEC-1 were incubated for 24 h 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 of PBS with 1% Triton X-100. The protein extracts were used for western blotting as described elsewhere (32).

Transient transfection assay

Rat HO-1 cDNA (kindly provided by Dr. Mahin Maines) was cloned into pcDNA3.1+ expression plasmid. The resulting construct (pcDNA-HO1), as well as a control plasmid pSVβgal (bacterial β-galactosidase gene under the control of an SV40 promoter) and reporter plasmids containing a human VEGF promoter or a mouse HO-1 promoter were amplified in HB-101 *E. 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 200 μl of serum-free medium mixed with 0.5 μg of plasmid DNA and 1.5 μl of Tfx-50 liposomes per well. After 1 h, the cells were washed and overlaid with routine culture medium. Media from cells transfected with expression plasmids were collected after a 48-h incubation period for determination of VEGF concentrations.

To assess the effect of HO-1 overexpression on activity of the VEGF promoter, NIH 3T3 cells were co-transfected with a reporter plasmid pGL2, harboring the firefly luciferase cDNA regulated by the human VEGF promoter (+54 to −2,279; construct was kindly provided by Dr. Hideo Kimura) and with a pcDNA-HO1 expression plasmid or with pSVβgal control construct.

HO-1 promoter activity was measured in HMEC-1 or NIH 3T3 cells transfected with pHO15luc, containing the luciferase cDNA driven by a full-length mouse HO-1 promoter (construct was a kind gift from Dr. Jawed Alam). Cells transfected with pGL2-VEGF or with pHO15luc were exposed to 15d-PGJ$_2$ 24 h after transfection. Forty eight hours later, the cells were collected and lysed and lysates were assayed for luciferase activity.

Measurement of VEGF and IL-8 protein concentrations

Concentrations of VEGF and IL-8 proteins in the culture media were quantified using the sandwich ELISAs, following the manufacturer's instructions.

Measurement of VEGF mRNA concentrations

Concentrations of VEGF mRNA were measured by ELISA performed on 2.5 μg of total RNA using Quantikine mRNA Base Kit, and Probe and Calibrator Kit for human VEGF. As an additional control for the amount of total RNA, the concentration of constitutively expressed glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was measured using a kit specific for human GAPDH. Before analysis, the samples were diluted 1:9.

Cell viability assay

Cell viability was assessed by colorimetric measurement of LDH release.

Statistical analysis

All experiments were performed in duplicates and were repeated three to five times. Data are presented as means ± SD. Statistical evaluation was done with Student's *t* test or with ANOVA followed by Tukey test. Differences were accepted as statistically significant at $p < 0.05$.

RESULTS

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

After 24 h of incubation, confluent, resting HMEC-1 released ~20 pg/ml of VEGF protein, as measured by ELISA. Treatment of the cells with 15d-PGJ$_2$ (1–10 μ*M*), a naturally occurring ligand of PPARγ transcription factor, significantly and dose-dependently increased VEGF secretion (Fig. 1A). At the doses used, 15d-PGJ$_2$ was not toxic to HMEC-1, as assayed by LDH-release test (data not shown).

The expression of VEGF mRNA in HMEC-1 was confirmed by RT-PCR analysis. The presence of mRNAs for VEGF$_{121}$ and VEGF$_{165}$ isoforms was regularly detected, and RT-PCR signals were stronger in the cells incubated with 15d-PGJ$_2$ (Fig. 1B, inset). To confirm the induction of VEGF mRNA expression by 15d-PGJ$_2$, the quantity of mRNA was measured using the colorimetric mRNA-ELISA system. In unstimulated HMEC-1, the concentration of VEGF mRNA in cell lysates was 20.1 ± 0.82 amol/ml. Incubation with 10 μ*M* 15d-PGJ$_2$ led to a twofold increase in VEGF mRNA accumulation when compared with untreated cells (Fig. 1B).

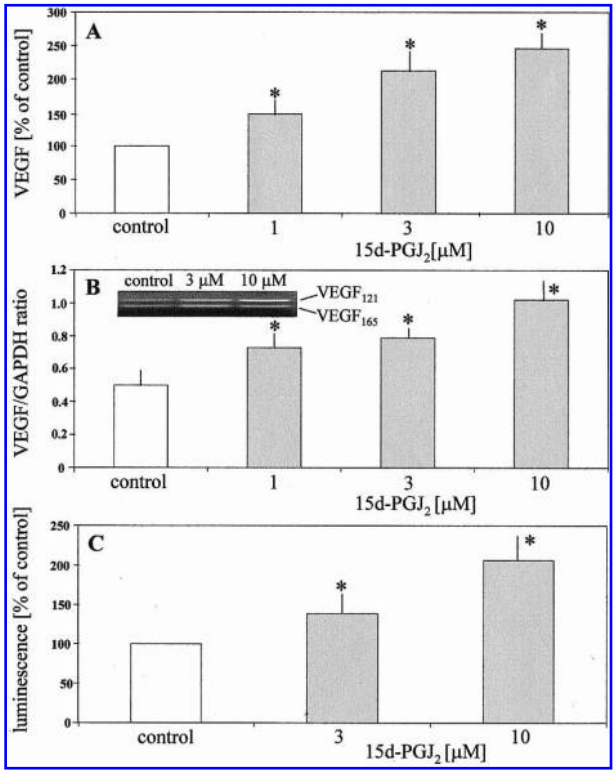

FIG. 1. **(A)** Effect of 15d-PGJ$_2$ on the release of VEGF protein from HMEC-1 after 24 h of incubation. Each column represents the mean ± SD of five independent experiments performed in duplicate, shown as percentage of control value. **(B)** Effect of 15d-PGJ$_2$ on the expression of VEGF mRNA, shown as VEGF/GAPDH ratio (mRNA ELISA). Each column represents the mean ± SD of two independent experiments made in duplicate. **Inset:** Representative of three RT-PCR results, showing the increased VEGF mRNA expression in cells treated with 15d-PGJ$_2$ (3 and 10 μ*M*). **(C)** Effect of 15d-PGJ$_2$ (3 and 10 μ*M*) on the VEGF promoter activity (luciferase assay). HMEC-1 were transfected with a reporter plasmid containing the luciferase gene regulated by the full-length, human VEGF promoter. Each column represents the mean ± SD of three independent experiments performed in duplicate, shown as percentage of control value. *$p < 0.05$, in comparison with control (ANOVA followed by Tukey test).

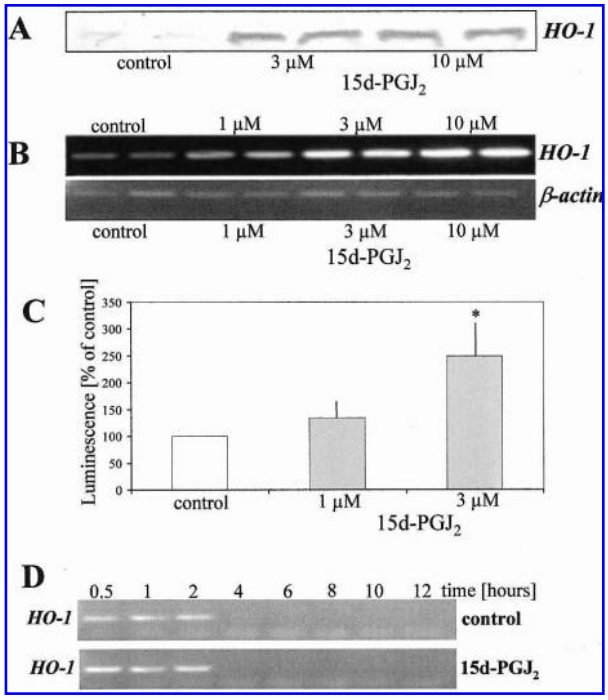

FIG. 2. **(A)** Effect of 15d-PGJ_2 on the expression of HO-1 protein (western blotting). One of two similar experiments. **(B)** Effect of 15d-PGJ_2 on the expression of HO-1 mRNA (RT-PCR). One of two similar experiments. **(C)** Effect of 15d-PGJ_2 on the HO-1 promoter activity (luciferase assay). NIH 3T3 cells were transfected with a reporter plasmid containing the luciferase gene regulated by a full-length mouse *ho-1* promoter. Each column represents the mean ± SD of three independent experiments performed in duplicate, shown as percentage of control value. $*p < 0.05$, in comparison with control (ANOVA). **(D)** No effect of 15d-PGJ_2 (10 μ*M*) on the HO-1 mRNA stability. HMEC-1 were activated with LPS to induce HO-1 mRNA and then were supplemented with actinomycin D (1 μg/ml) with or without 15d-PGJ_2 (10 μ*M*). One of two similar RT-PCR analyses.

Finally, to investigate whether 15d-PGJ_2 is able to induce VEGF transcription, HMEC-1 cells were transfected with reporter plasmid under control of a full-length human VEGF promoter. Treatment of such transfectants with 15d-PGJ_2 (3 and 10 μ*M*) significantly and dose-dependently increased the activity of the VEGF promoter, as assayed by luciferase activity (Fig. 1C). The level of induction of VEGF promoter activity was quantitatively similar to that of mRNA accumulation, suggesting that the augmentation of VEGF expression by 15d-PGJ_2 is regulated primarily at the level of gene transcription.

Effect of 15d-PGJ_2 on HO-1 expression

It has been reported that 15d-PGJ_2 stimulates the expression of HO-1 in several cell types (5, 17, 22, 24). To determine whether the same is true for HMEC-1, we visualized the level of HO-1 protein by western blotting. As shown in Fig. 2A, the HO-1 protein was weakly expressed in unstimulated HMEC-1, but its abundance was potently augmented in the cells treated with 15d-PGJ_2. Similarly, the low basal expression of HO-1 mRNA was very strongly and dose-dependently increased in the presence of 15d-PGJ_2 (Fig. 2B).

In a next step, we aimed at determining whether the elevated expression of HO-1 mRNA results from increased transcription or from mRNA stabilization. Experiments performed on the cells transfected with a reporter plasmid showed that 15d-PGJ_2 induces the activity of the mouse *ho-1* gene promoter (Fig. 2C). This result implies that the augmentation of HO-1 expression induced by 15d-PGJ_2 is at least partly regulated at the transcriptional level.

To investigate the effect of 15d-PGJ_2 on HO-1 mRNA stability, HMEC-1 were preincubated with LPS (100 ng/ml) for 4 h to induce HO-1 expression, and then transcription was terminated by addition of actinomycin D in the presence or absence of 15d-PGJ_2 (10 μ*M*). Using RT-PCR analysis, we were able to detect HO-1 mRNA in cells incubated with actinomycin D for 30 min, 1 h, and 2 h. Longer incubation periods resulted in the lack of HO-1 mRNA signal from cells cultured with as well as without 15d-PGJ_2 (Fig. 2D). This observation suggests that 15d-PGJ_2 does not measurably influence the HO-1 mRNA stability.

Effect of HO-1 activation or overexpression on the VEGF synthesis

Our preliminary studies suggested that activation of HO-1 may increase the synthesis of VEGF in VSMC (9). Incubation of HMEC-1 with 15d-PGJ_2 up-regulated the expressions of both HO-1 and VEGF. Therefore we wondered whether the effect of 15d-PGJ_2 on VEGF generation in HMEC-1 may be mimicked by activation of HO-1.

Incubation of cells with hemin (10 μ*M*), a HO-1 inducer, augmented the synthesis of VEGF. This stimulatory effect was completely blocked in the presence of SnPPIX (10 μ*M*), a well characterized HO-1 inhibitor (Fig. 3). Importantly, copper protoporphyrin-IX (CuPPIX, 10 μ*M*), a heme analogue that does not appreciably influence HO-1 activity, did not show any significant effect (Fig. 3). These results suggest

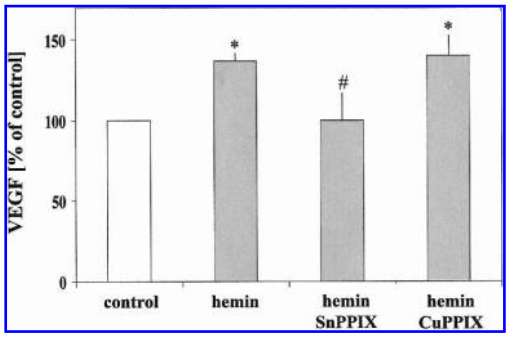

FIG. 3. Effect of modulation of HO-1 activity on the generation of VEGF. HMEC-1 were incubated with hemin (10 μ*M*; HO-1 activator), SnPPIX (10 μ*M*; protoporphyrin that inhibits HO-1 activity), or CuPPIX (10 μ*M*; control protoporphyrin, which does not influence HO-1 activity). Each column represents the mean ± SD of four independent experiments performed in duplicate, shown as percentage of control value. $*p < 0.05$, in comparison with control; $\#p < 0.05$, in comparison with hemin treatment (Student's *t* test).

that the HO-1 pathway may be involved in the regulation of VEGF expression, and that the activation of HO-1 may stimulate VEGF synthesis.

To confirm this suggestion, we transfected HMEC-1 with an expression plasmid containing the rat HO-1 cDNA driven by the SV40 promoter. The gene transfer efficacy was ~20%, as assessed in cells transfected with the control pSVβgal plasmid and incubated with X-gal substrate (data not shown). Transfection of HMEC-1 with pSVβgal did not change significantly the generation of VEGF, as measured by ELISA (Fig. 4A). In contrast, overexpression of HO-1 elevated the secretion of VEGF protein into the culture media. Similarly, pcDNA-HO1 transfection led to a twofold increase in accumulation of VEGF mRNA in cell lysates, as determined using mRNA-ELISA (Fig. 4B). Finally, overexpression of HO-1 resulted in activation of the VEGF promoter, as demonstrated in NIH 3T3 cells co-transfected with pcDNA-HO1 expression plasmid and pGL2-VEGF reporter construct (Fig. 4C). Taken together, these experiments showed that the up-regulation of HO-1 may imitate some activities of 15d-PGJ$_2$, leading to the stimulation of VEGF synthesis.

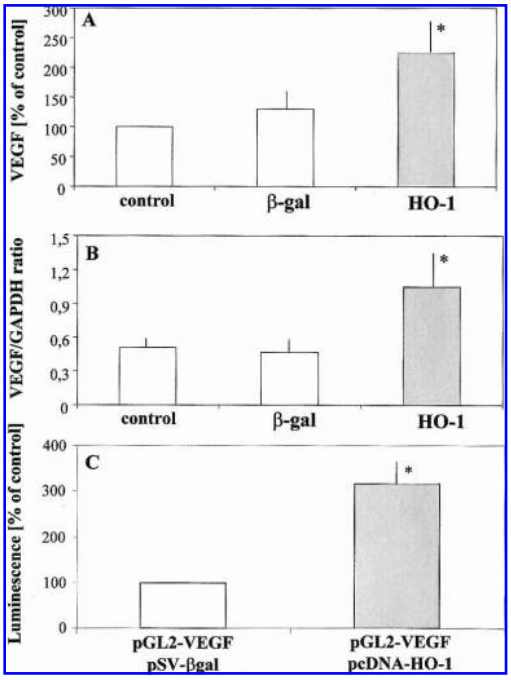

FIG. 4. **(A)** Effect of overexpression of HO-1 on the synthesis of VEGF protein by HMEC-1. Cells were transfected with control plasmid (pSVβgal) or with expression plasmid harboring the rat HO-1 cDNA. VEGF was measured by ELISA after a 48-h incubation period. Each column represents the mean ± SD of three independent experiments performed in duplicate, shown as percentage of control value. **(B)** Effect of overexpression of HO-1 on the expression of VEGF mRNA by HMEC-1. Concentrations of mRNAs were measured by mRNA ELISA after a 48-h incubation period. Each column represents the mean ± range of two independent experiments performed in duplicate, shown as VEGF/GAPDH ratio. **(C)** Effect of overexpression of HO-1 on the activity of VEGF promoter. NIH 3T3 cells were co-transfected with 0.25 μg of pGL2-VEGF plasmid and 0.25 μg of pSVβgal or 0.25 μg of pcDNA-HO1 plasmid. Luciferase activity was determined in cellular extract 48 h after transfection. *$p < 0.05$, in comparison with control (Student's t test).

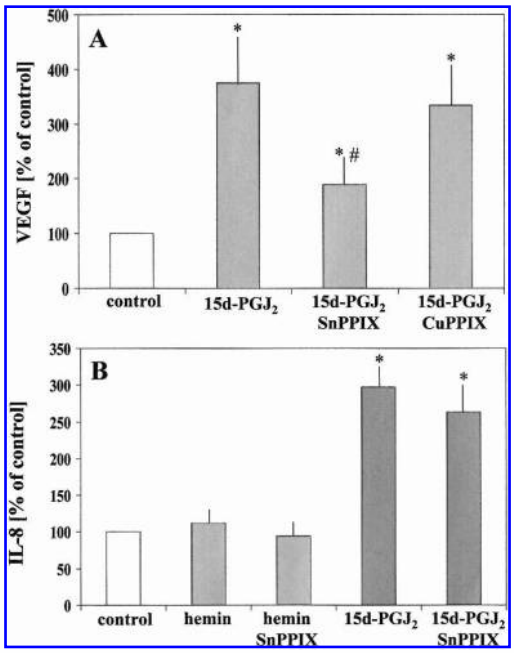

FIG. 5. **(A)** Effect of inhibitors of the HO-1 pathway on the 15d-PGJ$_2$-augmented production of VEGF. HMEC-1 were incubated with 10 μ*M* 15d-PGJ$_2$ and supplemented with SnPPIX (10 μ*M*) or CuPPIX (10 μ*M*). Each column represents the mean ± SD of four independent experiments performed in duplicate, shown as percentage of control value. **(B)** No effect of modulation of HO-1 activity on the 15d-PGJ$_2$-augmented production of IL-8 protein. HMEC-1 were incubated with 10 μ*M* 15d-PGJ$_2$ and supplemented with hemin, SnPPIX, or CuPPIX (all at 10 μ*M*). Each column represents the mean ± SD of three independent experiments performed in duplicate, shown as percentage of control value. *$p < 0.05$, in comparison with control; #$p < 0.05$, in comparison with 15d-PGJ$_2$ treatment (Student's t test).

Effect of HO-1 pathway inhibitors on the 15d-PGJ$_2$-induced VEGF synthesis

Because the effect of 15d-PGJ$_2$ on VEGF synthesis was mimicked by HO-1 activation or overexpression, we investigated whether inhibition of the HO-1 pathway modifies 15d-PGJ$_2$-mediated VEGF up-regulation.

Similar to the hemin-induced augmentation of VEGF synthesis (Fig. 3), the 15d-PGJ$_2$-exerted up-regulation of VEGF gene was potently reduced by SnPPIX (10 μ*M*). The control protoporphyrin, CuPPIX, did not show a significant influence (Fig. 5). This result implies a pivotal role for HO-1 activation in mediating the effect of 15d-PGJ$_2$ on VEGF gene regulation. Unlike the hemin-induced augmentation, which was blocked completely, the 15d-PGJ$_2$-mediated VEGF induction was inhibited in the presence of SnPPIX on average by 67% (Fig. 5A). That may suggest the involvement of additional,

HO-1-independent pathway(s) in 15d-PGJ_2-induced expression VEGF.

No effect of SnPPIX on the 15d-PGJ_2-induced IL-8 synthesis

Finally, we wanted to investigate if HO-1 contributes specifically to 15d-PGJ_2-induced synthesis of VEGF or whether it is a general mediator of 15d-PGJ_2 activities in endothelial cells. To this end, we tested the effect of HO-1 inhibitor on the generation of IL-8, another gene strongly induced in HMEC-1 after treatment with 15d-PGJ_2 (24). In contrast to the 15d-PGJ_2-augmented VEGF production, IL-8 synthesis was not influenced by SnPPIX (Fig. 5B). Accordingly, HO-1 activation by hemin did not exert any effect on IL-8 release from HMEC-1 (Fig. 5B), indicating that the induction of IL-8 by 15d-PGJ_2 is independent of the HO-1 pathway. Thus, HO-1 activation mediates only some, but not all, activities of 15d-PGJ_2 in HMEC-1.

DISCUSSION

We showed here that 15d-PGJ_2 significantly and dose-dependently increases the production of VEGF by HMEC-1. The effect of 15d-PGJ_2 could be mimicked by the induction of HO-1 expression. Pharmacological activation of HO-1 as well as its overexpression in HMEC-1 transfected with HO-1 cDNA resulted in the elevated synthesis of VEGF, an effect that was blocked by the HO-1 inhibitor SnPPIX. We revealed that 15d-PGJ_2 is a very potent inducer of HO-1 expression in HMEC-1. The salient finding of our study is that the inhibition of the HO-1 pathway reduces the stimulatory effect of 15d-PGJ_2 on VEGF synthesis. Taken together, it is likely that the up-regulation of VEGF expression in response to 15d-PGJ_2 is mediated by the prior activation of HO-1.

We have reported recently that treatment of HMEC-1 with 15d-PGJ_2 results also in a strong induction of IL-8, a chemokine responsible for chemoattracting and activating neutrophils and T-lymphocytes (19). In the present article, we demonstrated that this effect of 15d-PGJ_2 is not mediated by HO-1 activation. Similarly, Kasai *et al.* showed that the up-regulation of HO-1 was not involved in the augmentation of thyroglobulin production in 15d-PGJ_2-treated thyrocytes (20). Thus, HO-1 is not a general mediator of 15d-PGJ_2 activities, but seems to be involved only in some of them.

Locally produced 15d-PGJ_2 may function as a negative feedback regulator of the inflammatory response (15). As the activation of HO-1 markedly suppresses acute inflammation (36), one can suggest that the HO-1 pathway might contribute to the antiinflammatory effects associated with the 15d-PGJ_2 induction. Accordingly, Colville-Nash and colleagues showed that 15d-PGJ_2 strongly induced the production of HO-1 protein and, concomitantly, reduced the expression and activity of inducible nitric oxide synthase (iNOS) in murine macrophages (5). Zinc protoporphyrin-IX, an HO-1 blocker, significantly reversed the iNOS inhibition, a result that prompted the authors to imply that the antiinflammatory effect of 15d-PGJ_2 was mediated by HO-1 activation (5). On the other hand, in LPS-stimulated glial cells, in addition to suppression of iNOS, 15d-PGJ_2 decreased the synthesis of tumor necrosis factor-α and interleukin-1β (26). Like in macrophages, 15d-PGJ_2 also induced HO-1 expression in glial cells. However, the presence of an HO-1 inhibitor did not alter the reduction of interleukin-1β or tumor necrosis factor-α protein levels, and led to only a partial reversion of nitrite production. These observations suggest that HO-1 up-regulation is not a primary pathway responsible for the antiinflammatory activity of 15d-PGJ_2 (26).

Up-regulation of HO-1 expression may result from both the induction of promoter activity and stabilization of mRNA, although data presented by different authors are not fully consistent. It was reported, for instance, that the induction of HO-1 by nitric oxide depends exclusively on an increased rate of transcription (1, 11, 28), or is mediated mainly through increased mRNA stability (6), or is a consequence of both a higher transcription rate and RNA stabilization (13). We demonstrated that the induction of HO-1 expression in HMEC-1 treated with 15d-PGJ_2 was mediated by the activation of HO-1 promoter, but not by the stabilization of HO-1 mRNA. The increased transcriptional activity of HO-1 promoter in response to another prostaglandin-J_2 derivative, 12d-PGJ_2, was earlier observed in aortic endothelial cells and basophilic leukemia cells (25, 34). Koizumi and colleagues showed that 12d-PGJ_2 induces the expression of the rat HO-1 gene through the phosphorylation of the nuclear proteins, which bind to the specific 12d-PGJ_2 responsive element located in HO-1 promoter (25, 34). However, to our knowledge, these proteins have not been further characterized.

The stimulatory effect of 15d-PGJ_2 on VEGF synthesis in microvascular endothelial cells is consistent with earlier findings. We have previously demonstrated increased VEGF expression in 15d-PGJ_2-treated rodent VSMC and macrophages (18). Similar results were reported from human VSMC (42), macrophages (2), and coronary endothelial cells (14). Expression of VEGF was also augmented by the other naturally occurring PPARγ activators, 9-hydroxyoctadecadienoic acid (9-HODE), 13-HODE, and lysophosphatidylcholine, the components of oxidized low-density lipoprotein (8, 14, 37). However, the mechanism underlying this induction is still not established. In the present experiments, the increased release of VEGF protein from 15d-PGJ_2-treated HMEC-1 was associated with a higher expression of VEGF mRNA and with augmented activity of VEGF promoter, which suggests that the VEGF generation was at least partly regulated at the transcriptional level. These data are in a full agreement with our earlier results demonstrating the activation of VEGF promoter in macrophages and VSMC treated with 15d-PGJ_2 or lysophosphatidylcholine (8, 18). Similarly, incubation of human endothelial cells with 15d-PGJ_2, 9-HODE or 13-HODE led to transcriptional activation of the VEGF promoter (14).

It remains unclear, however, how ligands of PPARγ up-regulate VEGF gene transcription. We (18) and others (14) searched the promoter region of the VEGF gene, but could not find the direct repeat sequence of the PPARγ responsive element. It seems, therefore, that this activation is rather indirect. It was demonstrated that ligands of PPARγ can inhibit the activity of nuclear factor-κB (NFκB), AP-1, and STAT-1 pathways (16, 30, 38, 39), but probably the interactions with these transcription factors do not play an important role in

15d-PGJ$_2$-induced VEGF expression. AP-1 is known to augment the VEGF production, thus its eventual inhibition by 15d-PGJ$_2$ cannot explain the induction of VEGF. NFκB and STAT-1 seem not to be involved in the regulation of the human VEGF gene, although some NFκB-like consensus sequences have been described in the human VEGF promoter (21). Importantly, we have recently shown that NFκB activity is not influenced by 15d-PGJ$_2$ in HMEC-1 (19). Finally, looking for the mechanism of VEGF up-regulation, we decided to investigate the eventual involvement of the HO-1 pathway.

Our preliminary experiments performed on human and rat VSMC suggested that VEGF synthesis is augmented in the presence of hemin, an HO-1 activator, and decreased when the cells are incubated with SnPPIX, an HO-1 inhibitor (9). In the present study, we also demonstrated that in HMEC-1 hemin increases, whereas SnPPIX decreases, VEGF generation. To avoid possible nonspecific effects of hemin and SnPPIX, we transfected the HMEC-1 with HO-1 cDNA. Overexpression of HO-1 led to the transcriptional activation of VEGF promoter followed by the increased generation of VEGF mRNA and protein, which perfectly mimicked the effect exerted by 15d-PGJ$_2$. Keeping in mind that 15d-PGJ$_2$ induces HO-1 expression and that SnPPIX inhibits the 15d-PGJ$_2$-induced synthesis of VEGF, we propose that the up-regulation of VEGF in HMEC-1 treated with 15d-PGJ$_2$ is mediated by HO-1 activation.

The promoter of the human *ho-1* gene contains the regulatory sequences for ETS-1, FLI-1, and ERG, the transcription factors associated with endothelial cell proliferation and differentiation, suggesting the involvement of HO-1 in the regulation of angiogenesis (8). Indeed, it has been shown that HO-1 expression correlated with increased vascular density in human brain tumors (35) and that overexpression of HO-1 in rabbit microvascular endothelial cells resulted in enhanced angiogenic activity (7). In accordance with these findings, we demonstrated the direct effect of HO-1 expression on the synthesis of VEGF. Further investigations are necessary to establish which of the by-products resulting from HO-1 enzymatic pathway is (are) involved in the up-regulation of the VEGF gene.

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ABBREVIATIONS

CuPPIX, copper protoporphyrin-IX; DMEM, Dulbecco's modified Eagle medium; 15d-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin-J$_2$; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HMEC-1, human microvascular endothelial cells; HO-1, heme oxygenase-1; HODE, hydroxyoctadecadienoic acid; IL-8, interleukin-8; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; pcDNA-HO1, expression plasmid containing HO-1 cDNA; NFκB, nuclear factor-κB; PBS, phosphate-buffered saline; pGL2-VEGF, plasmid containing the luciferase cDNA driven by a full-length human VEGF promoter; pHO15luc, plasmid containing the luciferase cDNA driven by a full-length mouse HO-1 promoter; PPARγ, peroxisome proliferator-activated receptor-γ; pSVβgal, expression plasmid containing bacterial β-galactosidase cDNA; RT-PCR, reverse transcription–polymerase chain reaction; SnPPIX, tin protoporphyrin-IX; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cells.

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