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Original Research Communication

15d-PGJ_2 Upregulates Synthesis of IL-8 in Endothelial Cells Through Induction of Oxidative Stress

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Abstract

15-Deoxy-$\Delta^{12,14}$-prostaglandin-J_2 (15d-PGJ_2) is a cyclopentenone prostaglandin regarded as antiinflammatory mediator, which can act through peroxisome proliferator–activated receptor-γ (PPARγ) or through G protein–coupled surface receptors. It has been demonstrated that 15d-PGJ_2 potently increases the generation of interleukin-8 (IL-8) in human microvascular endothelial cells (HMEC-1s); however, the mechanism of this induction is not known. The aim of the study was to find the pathway involved in 15d-PGJ_2–mediated IL-8 stimulation. Our data confirmed that the effect of 15d-PGJ_2 is independent of PPARγ. For the first time, we excluded the activation of G proteins and the contribution of G protein–coupled surface receptors in endothelial cells treated with 15d-PGJ_2. Instead, we demonstrated that stimulation of IL-8 involved induction of oxidative stress, activation of p38 kinases, and increase in stability of IL-8 mRNA. Upregulation of IL-8 promoter, although measurable, seemed to play a less-pronounced role. Additionally, our results indicate the involvement of cAMP elevation and may suggest a role for ATF2 transcription factor. Concomitant induction of heme oxygenase-1 in HMEC-1s did not influence the synthesis of IL-8. In summary, we showed that 15d-PGJ_2, acting through oxidative stress, may exert proinflammatory effects. The upregulation of IL-8 is mostly associated with p38-mediated stabilization of mRNA. *Antioxid. Redox Signal.* 11, 2035–2046.

Introduction

Prostaglandin-D_2 (PGD_2) and prostaglandin-J_2 (PGJ_2) are the major prostaglandins generated by mast cells or macrophages in infected or inflamed tissues. They are also produced at relatively high concentrations in endothelial cells (40).

PGJ_2 is a metabolite of PGD_2, formed spontaneously in biologic fluids in the presence of albumin (52). It is an unstable compound; thus, in almost all experiments, its stable derivative 15-deoxy-$\Delta^{12,14}$-prostaglandin-J_2 (15d-PGJ_2) is used. Direct measurements have confirmed that 15d-PGJ_2 is truly produced in the vessel wall, especially in foam cells within human atherosclerotic plaques, and its synthesis is increased in response to proinflammatory cytokines (44, 45). Increased production of 15d-PGJ_2 in macrophages was suggested as an antiinflammatory mechanism underlying inhibition of IKKβ and, in consequence, NF-κB activities (57).

In human aortic endothelial cells, 15d-PGJ_2 was recognized as essential mediator of laminar flow–induced activation of the Nrf2 pathway, responsible for upregulation of antiatherosclerotic genes, among them the cytoprotective enzyme heme oxygenase-1 (HO-1) (20). Recently, some antiatherogenic activities of statins also were suggested to be mediated by elevation of 15d-PGJ_2 and activation of PPARγ (58). We demonstrated, however, that in endothelial cells, 15d-PGJ_2 acts mostly in a PPARγ-independent manner (23, 25).

Endothelium lines the inner surface of blood vessels and is a primary target for inflammatory agents. Its exposure to cytokines or to bacterial LPS induces the secretion of proinflammatory mediators, among them interleukin-8 (IL-8) (29). This chemokine stimulates angiogenesis and plays a crucial role in recruiting leukocytes to the sites of acute inflammation. It has been commonly believed that the locally produced 15d-PGJ_2 may function as a negative-feedback regulator of inflammation by the inhibition of proinflammatory genes (4). However, our previous study indicated that 15d-PGJ_2 stimulates microvascular endothelial cells to produce IL-8, and thereby plays a proinflammatory role as well. This effect was independent of PPARγ, but its mechanism was not known (23).

We demonstrate that induction of IL-8 in endothelial cells involves 15d-PGJ_2–induced oxidative stress, activation of p38 kinases, and stabilization of IL-8 mRNA.

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Materials and Methods

Reagents

The 15d-PGJ_2, troglitazone, and ciglitazone were obtained from Biomol (Warszawa, Poland). IL-1β, NAC, BW245C, AH6809, Act-D, L-glutamine, EGF, hydrocortisone, PEG-catalase, and ODQ were purchased from Sigma (Poznan, Poland). Hemin was obtained from Fluka, SnPPIX was from Porphyrin Products (Carnforth, Lancashire, England), and hemoglobin from Calzyme Laboratories (Croydon, Surrey, England). Fetal calf serum (FCS) was procured from PromoCell (Heidelberg, Germany). Total RNA Extraction Kit, Reverse Transcription System, PCR Core System, Luciferase Assay Reagents, and pGL-2 and pSVβgal plasmids were obtained from Promega (Gdansk, Poland), whereas pSG5 and pBK-CMV plasmids were from Stratagene (Gdansk, Poland). Maxiprep QIAfilter EndoFree Plasmid Isolation Kit and SuperFect Transfection Reagent were purchased from Qiagen (Wroclaw, Poland). ELISA kit for human IL-8 protein was obtained from R&D Systems (Warszawa, Poland). Total Protein Test Kit was procured from Biorad (Warszawa, Poland). Immunoenzymatic assay for cAMP measurements was from Amersham Pharmacia Biotech (Warszawa, Poland). TransAM ELISA for ATF2 and FACE ELISA for p38 were purchased from Active Motif (Rixensart, Belgium). All others reagents were procured from Gibco BRL (Warszawa, Poland).

Cell culture

Human microvascular endothelial cells (HMEC-1s) were purchased from The Centers for Disease Control and Prevention (Atlanta) and cultured in DMEM F-12 medium containing 10% FCS, L-glutamine (2 m*M*), EGF (10 ng/ml), hydrocortisone (1 μg/ml), penicillin (100 U/ml), and streptomycin (10 μg/ml).

Transient transfection

Human SOD1 cDNA (495 bp) was cloned into pSG5 expression vector, whereas human catalase cDNA (1.68 kb) was ligated to pBK-CMV expression plasmid, under control of SV40 and CMV promoters, respectively. pSVβgal construct (bacterial β-galactosidase gene driven by SV40 promoter) served as a control for transfection efficacy. Activity of the IL-8 promoter was measured by using the pGL2-IL8 plasmid, containing the firefly luciferase gene regulated by a full-length promoter of human IL-8 (construct was kindly supplied by Dr. Rainer De Martin, Austria), whereas an empty pGL2 plasmid was used as a control. Cells were grown in 24-well plates to 80% confluence and were then transfected with 0.5 μg of plasmid DNA and 2.5 μl of SuperFect Reagent per well, according to vendor's protocol. Experiments were performed 24 h after transfection.

Reporter gene assay

After transfection, cells were exposed to the compounds studied for 24 h. Luciferase activity was measured in the cell lysates according to the manufacturer's instructions. Results were normalized to total protein concentrations.

Measurement of p38 kinase activity

Cells were incubated in 96-well plates and stimulated with 15d-PGJ_2 for 20 min or 1 h. Then total and phosphorylated p38 kinase proteins were measured by using FACE ELISA, according to vendor's protocol.

TransAM ELISA

Cells were cultured in six-well plates and stimulated with 15d-PGJ_2 for 1.5 h. Bindings of nuclear cell extracts to oligonucleotides containing consensus sequences for ATF2 were assayed by using TransAM ELISA, according to vendor's protocol.

RT-PCR

Total RNA was isolated from the cells grown in 24-well plates by acid guanidinium thiocyanate-phenol-chloroform (GTC) extraction by using 300 μl of GTC per well. The procedure was performed according to the vendor's protocol. After isolation, RNA was dissolved in 30 μl of RNase-free H_2O. Reverse transcription was carried out on 1 μg of total RNA for 1 h at 42°C by using random primers or oligo-(dT) primers and AMV reverse transcriptase, according to the vendor's protocol. Then PCR was performed according to the manufacturer's instructions by using Taq DNA polymerase and primers recognizing IL-8 (F: 5′-CTC TCT TGG CAG CCT TCC TGA; R: 5′-CCC TCT GCA CCC AGT TTT CCT T; length of the product: 240 bp). Reaction was performed for 35 cycles by using the following protocol: 95°C, 40 s; 58°C, 40 s; and 72°C, 50 s. PCR products were analyzed by electrophoresis in 2% agarose gels.

Estimation of mRNA stability

To investigate IL-8 mRNA stability, cells were treated with LPS (100 ng/ml) for 4 h to induce expression of the studied genes, and then transcription was terminated by addition of actinomycin-D (1 μg/ml) in the presence or absence of 15d-PGJ_2 (10 μ*M*). After 0.5- to 48-h incubation, total RNA isolation was performed, and expression of gene of interest was determined by RT-PCR. Results were assessed after electrophoresis of RT-PCR products in 2% agarose gel. Densitometric measurements were performed by using the ImageJ program (http://rsb.info.nih.gov/ij/). The mean value was recorded for each band. Background values for each band also were measured and subtracted to obtain final values of band intensities. The area of analysis was identical for each band in the same gel.

Measurements of IL-8 protein concentration

Cells were seeded into 24-well plates and grown to confluence. Then the medium was replaced and supplemented with compounds tested. Culture media were collected after 24 h, and concentrations of IL-8 protein in the culture media were quantified by using ELISA, following the manufacturer's instructions.

Measurements of cyclic AMP

Total production of cAMP in confluent HMEC-1s cultured in 96-well plates was quantified by immunoenzymatic assay in cell lysates, according to the vendor's protocol. Samples were acetylated before measurements.

Determination of ROS production

The level of intracellular ROS was assessed by measuring the oxidation of DCFH-DA (2′,7′-dichlorodihydrofluorescin diacetate). Cells were seeded into 12-well plates and grown until reaching confluence. Then the medium was removed, the cells were washed with PBS, and fresh serum-free medium was added. The cells were loaded with 10 μM DCFH-DA for 30 min in the incubator, and then 15d-PGJ$_2$ (1–10 μM) was applied. After 20 min or 1 h, the plates were placed on ice, and the cells were scraped in the dark in 100 μl of PBS containing 1% Triton X-100. Lysates were centrifuged at 14,000 g for 10 min at 4°C. The samples were put into a black 96-well plate, and fluorescence (excitation, 485 nm; emission, 535 nm) was measured. Values were normalized to the protein content.

(^{35}S)GTPγS binding assay

HMEC-1 cells were harvested nonenzymatically, washed with PBS, and resuspended in assay buffer (20 mM Hepes, pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl), supplemented with complete protease inhibitors. Cells were homogenized in a nitrogen cavitation chamber, and unbroken cells and nuclei were pelleted by centrifugation (500 g, 10 min, 4°C). Then the supernatant fraction was centrifuged at 45,000 g for 45 min at 4°C. Membrane pellets were resuspended in assay buffer, titrated through a fine-gauge needle, and stored at −80°C until required.

For (^{35}S)GTPγS binding experiments, membranes were incubated with or without 15d-PGJ$_2$ (10 μM) or IL-1β (10 ng/ml) for 5 or 20 min at 25°C in assay buffer containing (^{35}S)GTPγS (100 nCi/point), saponin (20 μg/point), and 0.1 μM GDP. After incubation, membrane proteins were solubilized with 1.25% NP-40 and 0.4% SDS, and after preclearance by using nonimmune serum, Gα subunits were immunoprecipitated with antiserum, used at a dilution of 1 in 200. Nonspecific binding was determined by the addition of 100 μM unlabeled GTPγS. Bound radioactivity was measured in a liquid scintillation counter (Becton-Dickinson).

Statistical analysis

All experiments were performed in duplicate and were repeated 2 to 7 times. Data are presented as mean ± 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

Effects of 15d-PGJ$_2$ on generation of IL-8 protein

Resting HMEC-1s released to the culture medium 143 ± 13 pg/ml of IL-8 protein after a 24-h incubation period, as measured by using ELISA. According to earlier observations, described in our previous article (23), 15d-PGJ$_2$ (1–10 μM) strongly and dose-dependently augmented IL-8 synthesis. Such a treatment did not influence the viability of HMEC-1, as measured by using MTT reduction or lactate dehydrogenase (LDH) activity assays (23, and data not presented).

The mechanism responsible for upregulation of IL-8 is not known. In many cell types, including endothelium, 15d-PGJ$_2$ may activate the PPARγ nuclear receptor. This pathway, however, seemed to be not involved here, as ciglitazone, a PPARγ-specific agonist, did not influence IL-8 production in HMEC-1s (Fig. 1) (23).

The second potential mediator of prostaglandin-J$_2$ activities could be DP-1, a surface receptor recognizing cyclopentenone prostaglandins, which is coupled to G_i protein (10). To test this possibility, we incubated HMEC-1 cells in the presence of BW245C (DP-1 agonist) and AH6809 (DP-1 antagonist). Results of ELISA demonstrated that BW245C, at the concentration of 10 μM, increased by ~60% the release of IL-8 protein, which would suggest the contribution of the DP-1 receptor (Fig. 2A). However, this effect could not be reversed by preincubation of cells with AH6809. Similarly, the DP-1 antagonist did not modulate effect of 15d-PGJ$_2$ (Fig. 2A). Thus, we conclude that DP-1 receptor is not involved in 15d-PGJ$_2$–stimulated induction of IL-8 synthesis.

To verify definitely the involvement of a surface receptor in the effects of 15d-PGJ$_2$ we used the (^{35}S)GTPγS binding assay to investigate the activation of G proteins. As shown in Fig. 2B, stimulation of HMEC-1 with IL-1β (a positive control) for 5 min led to a 2.5-fold increase in the activity of G-proteins. In contrast, 15d-PGJ$_2$ did not show any effect. The same results were obtained after a 20-min incubation (data not shown). Thus, we conclude that surface receptors coupled to G proteins are not activated by 15d-PGJ$_2$ in HMEC-1s.

Effect of oxidative stress on 15d-PGJ$_2$-induced IL-8 expression

Although some reports show that preincubation of cells with 15d-PGJ$_2$ reduces apoptosis caused by H_2O_2 and protects against oxidative injury (16), many experiments have demonstrated that 15d-PGJ$_2$ itself induces generation of reactive oxygen species (ROS) and leads to oxidative stress (31). Thus, we decided to check the effect of 15d-PGJ$_2$ on ROS formation and to test the role of oxidative stress in induction of IL-8.

Production of ROS was detected by measuring the fluorescence of dichlorofluorescein (DCF). As shown in Fig. 3A, the exposure of HMEC-1s to 15d-PGJ$_2$ for 20 min signifi-

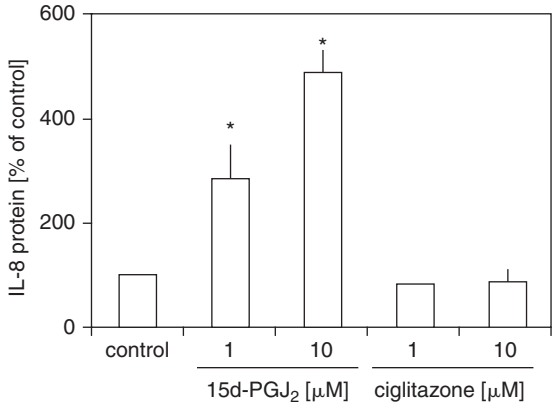

FIG. 1. Effect of 15d-PGJ$_2$ and ciglitazone (1 μM and 10 mM) on the release of IL-8 protein from HMEC-1 after 24-h incubation. Data are shown as percentage of control value (control: unstimulated cells). *$p < 0.05$ compared with control. ELISA.

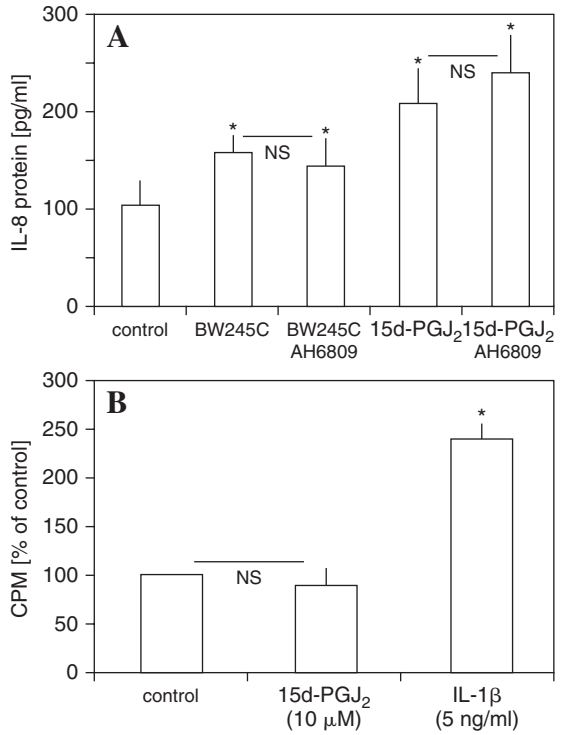

FIG. 2. (A) Effect of DP-1 ligands on the basal and 15d-PGJ_2–induced release of IL-8 protein from HMEC-1: BW245C (10 μM, DP-1 agonist), AH6809 (10 μM, DP-1 antagonist). Data are shown as percentage of control value (control: unstimulated cells). Cells were incubated for 24 h. Some samples, before stimulation, were preincubated with AH6809 for 30 min. *$p < 0.05$ compared with control; NS, not significant. ELISA. (**B**) No effect of 15d-PGJ_2 (10 μM, 5 min) on the activation of G proteins in membranes prepared from HMEC-1s. Exposure of membranes to IL-1β (5 ng/ml) served as a positive control. Data are shown as percentage of control value (control: unstimulated cells). *$p < 0.05$ compared with control; NS, not significant. (^{35}S)GTPγS binding assay.

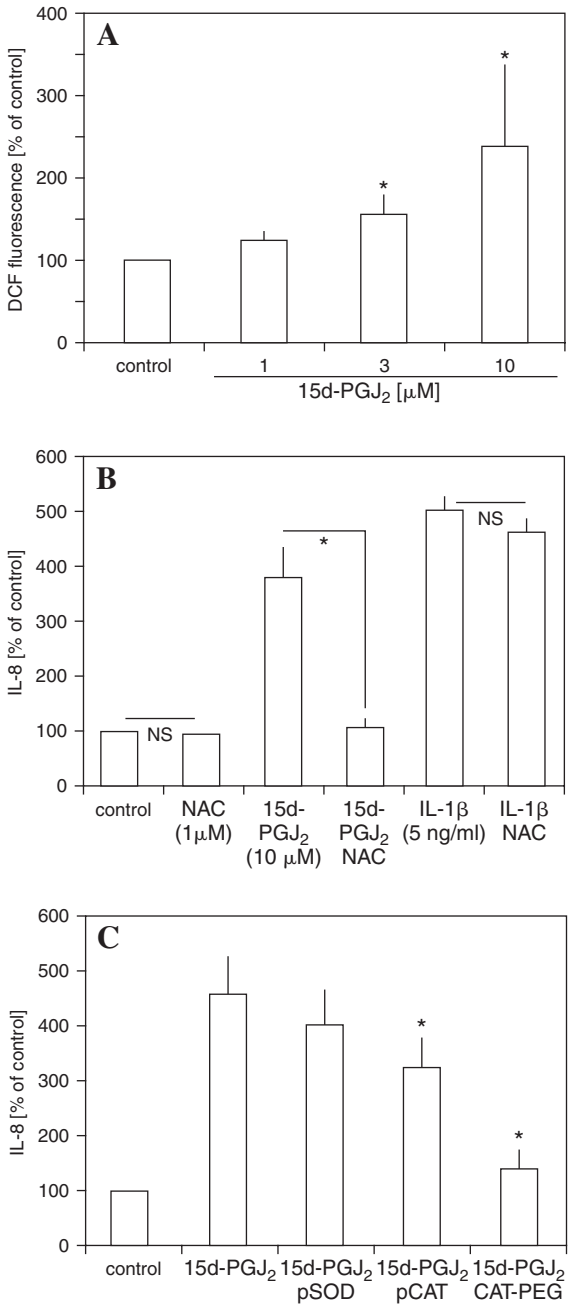

FIG. 3. (A) Effect of 15d-PGJ_2 (1–10 μM, 20 min) on the formation of ROS in HMEC-1s, shown as percentage of control value (control: unstimulated cells). *$p < 0.05$ compared with control. DCF fluorescence assay. (**B**) Effect of NAC (1 mM) on the basal, 15d-PGJ_2–induced, and IL-1β–induced release of IL-8 protein from HMEC-1s after 24-h incubation. Before stimulation, cells were pretreated with NAC for 30 min. Data are shown as percentage of control value (control: unstimulated cells). *$p < 0.05$ compared with cells without NAC; NS, not significant. (**C**) Effect of transfection of HMEC-1s with expression plasmids containing SOD1 (pSOD) or catalase (pCAT) and influence of supplementation with catalase-PEG enzyme (CAT-PEG, 100 U/ml) on 15d-PGJ_2–induced release of IL-8 after 24-h incubation. CAT-PEG was added to the cells 1 h before stimulation with 15d-PGJ_2. Data are shown as percentage of control values (control: intact cells or pSVβgal-transfected cells). *$p < 0.05$ compared with cells treated with 15d-PGJ_2 only. ELISA.

cantly and dose-dependently increased the generation of ROS. The same effects were observed after a 1-h incubation period (data not shown). Thus, 15d-PGJ_2 induces oxidative stress in HMEC-1s.

To test the possible contribution of oxidative stress in upregulation of IL-8, we preincubated the cells with *N*-acetylcysteine (NAC, 1 mM) before the exposure to 15d-PGJ_2. Such a treatment abrogated completely the stimulatory effect of 15d-PGJ_2 on the release of IL-8 protein (Fig. 3B). Importantly, this inhibition was specific for 15d-PGJ_2, as NAC did not influence the upregulation of IL-8 in HMEC-1s treated with IL-1β. Similarly, supplementation of control cells with NAC did not affect IL-8 production (Fig. 3B).

However, NAC is a thiol-containing antioxidant, which can bind 15d-PGJ_2, reducing its effects by direct conjugation. Therefore, we checked the 15d-PGJ_2–induced IL-8 synthesis in HMEC-1s transfected with plasmids harboring cDNA of SOD1 or catalase. As shown in Fig. 3C, overexpression of SOD1 did not influence the IL-8 production. Conversely, delivery of catalase cDNA resulted in moderate, but statistically significant inhibition. Because the transfection efficacy of HMEC-1s did not exceed 20%, we preincubated cells for

1 h with pegylated catalase enzyme (100 U/ml). Such a treatment very potently reduced the effect of 15d-PGJ_2, suggesting that elevation of H_2O_2 is involved in upregulation of IL-8 synthesis.

Additionally, we demonstrated that depletion of GSH cellular stores by administration of a GSH scavenger, diethylmaleimide (DEM, 100 μM) (17), significantly increased both basal and 15d-PGJ_2–induced IL-8 production (Fig. 4A). Similar results were obtained after pretreatment of HMEC-1s with ethacrynic acid (EA, 20 μM) (Fig. 4B), which decreases both cytosolic and mitochodrial GSH levels and inhibits glutathione S-transferase (GST), which catalyzes GSH-substrate conjugation (43). Taken together, our results may indicate that the influence of 15d-PGJ_2 on IL-8 synthesis is mediated by induction of oxidative stress and that cellular GSH modulates the effects of 15d-PGJ_2. They might also suggest that 15d-PGJ_2 likely can conjugate in some form with GSH and that this conjugation attenuates 15d-PGJ_2 activity.

Oxidative stress generated by 15d-PGJ_2 in HMEC-1s leads to strong upregulation of heme oxygenase-1 (HO-1) (24), an enzyme degrading heme to biliverdin, iron ions, and carbon monoxide (CO) (39). Because HO-1 has been suggested to increase production of IL-8 in endothelial cells (41), we decided to check its potential involvement in IL-8 induction in our experimental setting. We tested the effects of activation or inhibition of the HO-1 pathway on the generation of IL-8. As shown in Fig. 5, IL-8 synthesis was not influenced by tin protoporphyrin (SnPPIX, the inhibitor of HO-1 enzymatic activity), hemoglobin (the scavenger of CO), or ODQ (the inhibitor of cGMP generation, one of mediators of CO action). Accordingly, activation of HO-1 by hemin did not exert any effect on IL-8 release from HMEC-1s, indicating that the induction of IL-8 by 15d-PGJ_2 is independent of HO-1.

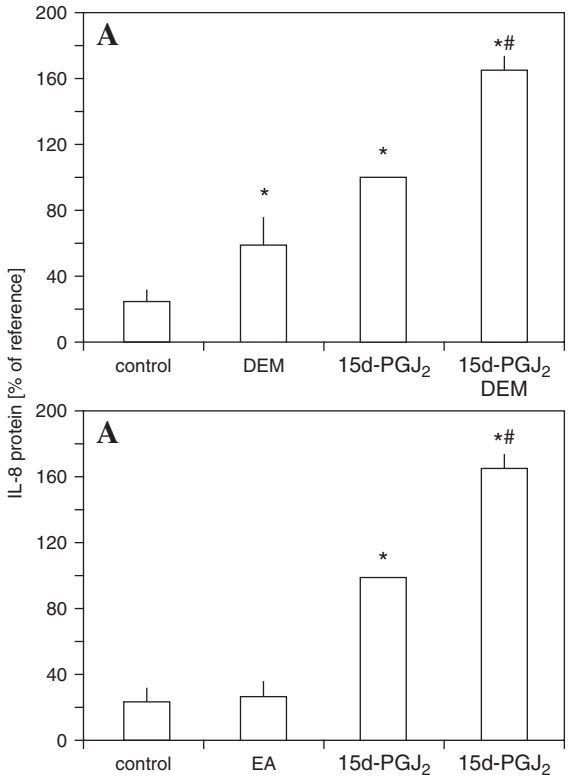

FIG. 4. Effect of modulation of glutathione pathway on the release of IL-8 protein from HMEC-1 after 24-h incubation. (**A**) Effect of DEM (100 μM, glutathione scavenger). (**B**) Effect of EA (20 μM, inhibitor of glutathione S-transferase). Before stimulation with 15d-PGJ_2, cells were incubated with DEM for 2 h, or with EA for 30 min. Data are shown as percentage of reference value (reference: cells treated with 15d-PGJ_2 alone).*$p < 0.05$ compared with control; #$p < 0.05$ compared with 15d-PGJ_2–treated cells. ELISA.

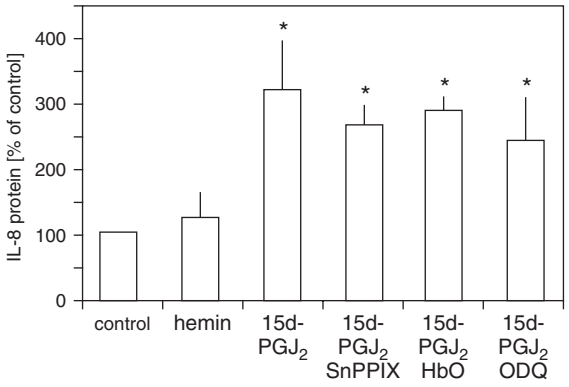

FIG. 5. Effect of hemin (10 μM, HO-1 activator) and inhibitors of HO-1 pathway on the 15d-PGJ_2–induced release of IL-8 protein from HMEC-1s after 24-h incubation: SnPPIX (10 μM, HO-1 inhibitor), HbO (5 μM, CO scavenger), and ODQ (5 μg/ml, inhibitor of cGMP synthesis). Data are shown as percentage of control value (control: unstimulated cells). Inhibitors were added to the cells 30 min before stimulation with 15d-PGJ_2. *$p < 0.05$ compared with control. ELISA.

Effect of cAMP elevation on 15d-PGJ_2–induced IL-8 synthesis

The 15d-PGJ_2 increased the generation of cAMP in HMEC-1s (Fig. 6A). Activation of the cAMP-dependent pathway was additionally confirmed by measuring the activity of ATF2 transcription factor. As demonstrated by TransAM ELISA, 15d-PGJ_2 significantly and dose dependently induced binding of ATF2 to the target DNA sequences (Fig. 6B).

We found that elevation of cAMP by forskolin mimicked the effect of 15d-PGJ_2 and strongly augmented the release of IL-8 (Fig. 6C). It suggests that induction of cAMP by 15d-PGJ_2 contributes to the upregulation of IL-8 expression. This supposition was confirmed by the further experiments, in which we preincubated HMEC-1s with MDL12330A, an adenylate cyclase inhibitor, and then stimulated the cells with 15d-PGJ_2 (10 μM). Such a treatment strongly reduced the augmentation of IL-8 synthesis. Finally, the stimulatory effect of the lower concentration of 15d-PGJ_2 (1 μM) was potentiated by IBMX, an inhibitor of phosphodiestereases (Fig. 6C). Taken together, these results strongly imply that elevation of cAMP contributes to 15d-PGJ_2–mediated upregulation of IL-8 production.

Effect of inhibition of protein kinases on 15d-PGJ_2-induced IL-8 synthesis

In the next experiments, we tested the effects of preincubation of HMEC-1s with inhibitors of several protein kinases

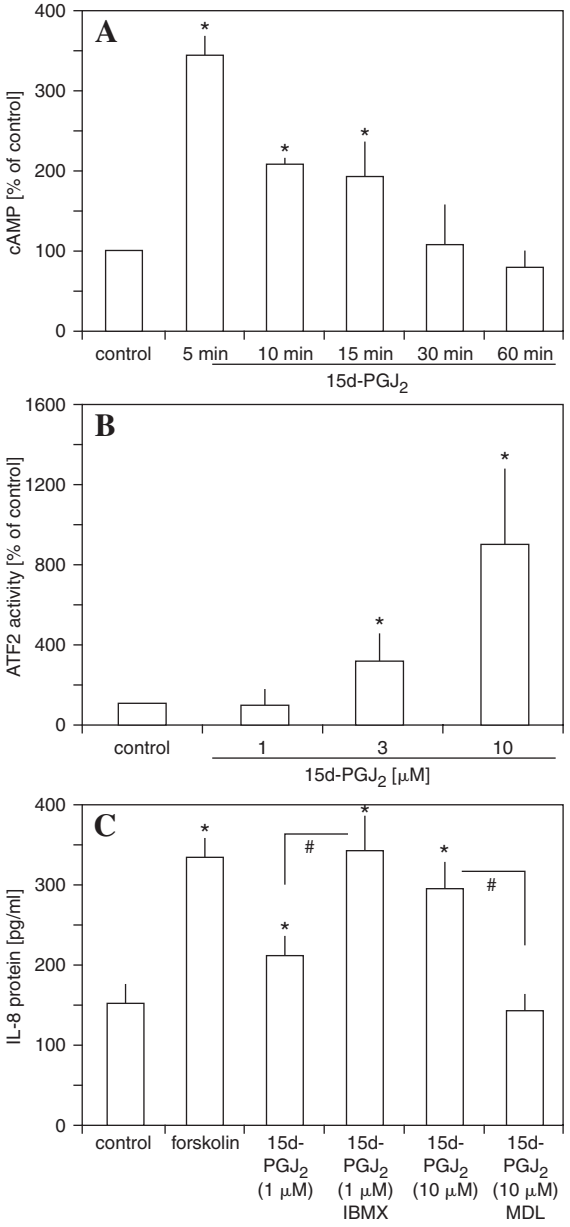

FIG. 6. (A) Effect of 15d-PGJ_2 (10 μM) on the generation of cAMP in HMEC-1s at different times after stimulation. Data are shown as percentage of control value (control: unstimulated cells). Immunoenzymatic assay. (**B**) Effect of 15d-PGJ_2 (1–10 μM, 1.5 h) on DNA-binding activity of ATF2 transcription factor. TransAM ELISA assay. (**C**) Effect of modulation of cAMP turnover on the basal and 15d-PGJ_2–induced release of IL-8 protein from HMEC-1s: IBMX (20 μM, inhibitor of phosphodiesterases), MDL12330A (1 μM, inhibitor of adenylate cyclase), and forskolin (1 μM, activator of cAMP synthesis). Cells were incubated for 24 h; inhibitors were added 30 min before stimulation with 15d-PGJ_2. $^*p < 0.05$ compared with control; $\#p < 0.05$ compared with 15d-PGJ_2–treated cells. ELISA.

on activity of 15d-PGJ_2. The results show that induction of IL-8 was not changed in the presence of wortmannin (100 nM), myristolated PKI 14-22 amide (5 μM), EGF-R651-658 fragment (5 μM), and PD 98059 (20 μM) (Fig. 7A). It suggests that PI3K, PKA, PKC, and ERK1/2 kinases do not play

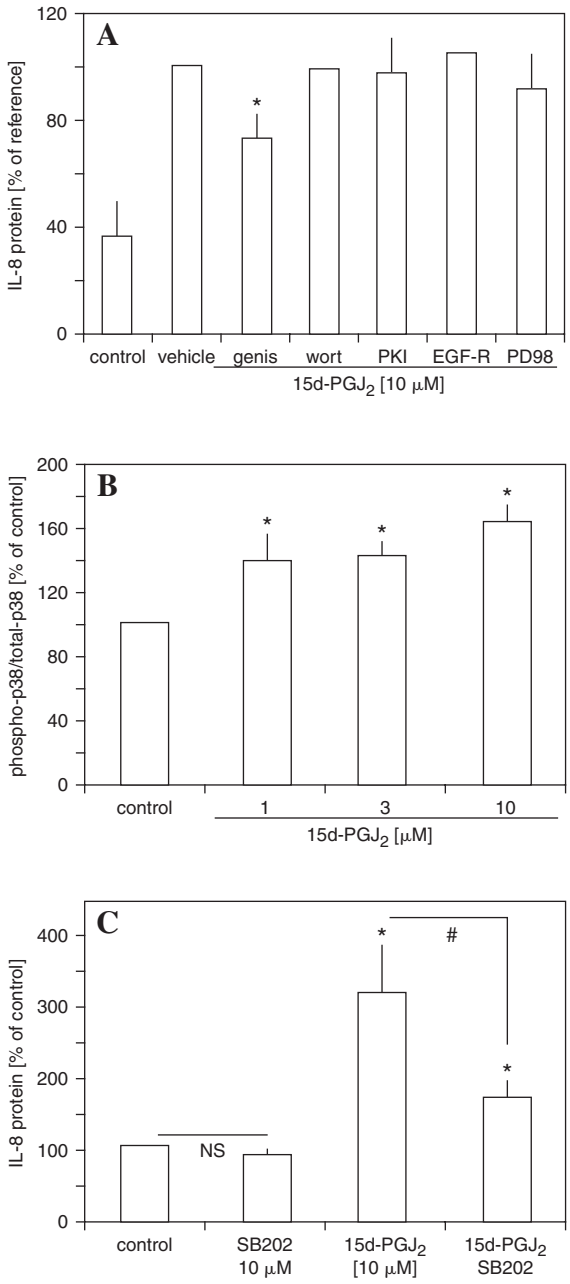

FIG. 7. (A) Effect of kinase inhibitors on the 15d-PGJ_2–induced release of IL-8 protein from HMEC-1s: genistein (20 μM, tyrosine kinase inhibitor), wortmannin (0.1 μM, PI3K/Akt inhibitor), myristolated PKI14-22 amide (5 μM, PKA inhibitor), myristolated EGF-R651-658 fragment (5 μM, PKC inhibitor), PD98059 (20 μM, ERK1/2 inhibitor). Cells were incubated for 24 h; inhibitors were added 30 min before stimulation with 15d-PGJ_2. Data are shown as percentage of reference value (reference: cells stimulated with 15d-PGJ_2). $^*p < 0.05$ compared with the cells treated with 15d-PGJ_2 alone. ELISA. (**B**) Effect of 15d-PGJ_2 on the activation of p38 kinases in HMEC-1s. Results were counted as a ratio of phosphorylated p38 to total p38 protein. FACE-ELISA. (**C**) Effect of SB 202190 (10 μM, p38 inhibitor) on the basal and 15d-PGJ_2–induced release of IL-8 protein from HMEC-1s. Data are shown as percentage of control value (control: unstimulated cells). $^*p < 0.05$ compared with control; $\#p < 0.05$ compared with the cells treated with 15d-PGJ_2 alone.

an important role in the signal transduction. The only inhibitor of this set able to inhibit 15d-PGJ$_2$ activity was genistein (20 μM), suggesting the involvement of tyrosine kinases.

Finally, we tested more thoroughly the involvement of p38 kinases, the essential mediators of signal transduction under oxidative stress (54). First, we measured the activity of p38 kinases in HMEC-1s treated with 15d-PGJ$_2$. By using ELISA-recognizing nonphosphorylated and phosphorylated forms of p38, we demonstrated that a 20-min exposure of endothelial cells to 15d-PGJ$_2$ (1–10 μM) increased the amount of phospho-p38, whereas the total p38 level remained unchanged (Fig. 7B). Thus, treatment of HMEC-1s with 15d-PGJ$_2$ leads to phosphorylation and activation of p38 kinases.

Furthermore, inhibition of p38 kinases with SB 202190 (10 μM) did not alter significantly the basal IL-8 synthesis, but potently abrogated the stimulatory effect of 15d-PGJ$_2$, reducing the expression of IL-8 by ~50% (Fig. 7C). This observation confirms that p38 kinases play an important role in 15d-PGJ$_2$–induced upregulation of IL-8.

Effect of 15d-PGJ$_2$ on IL-8 promoter activity and mRNA stability

As ATF2 is one of the regulators of IL-8 transcription (13), we decided to check whether the synthesis of IL-8 by 15d-PGJ$_2$ is upregulated at the transcriptional or also at the post-transcriptional level. To investigate the rate of IL-8 transcription, we transfected HMEC-1s with a reporter plasmid, pGL2-IL8, containing a luciferase cDNA under control of the full-length human IL-8 promoter. After transfection, some cells were supplemented with 15d-PGJ$_2$ (10 μM), cultured for 24 h, and then subjected to luciferase assay. We found that 15d-PGJ$_2$ increased the transcription rate from IL-8 promoter by ~50% ($p < 0.038$; Fig. 8A). Interestingly, this upregulation was not reduced in the presence of SB 202190 ($p < 0.018$ when compared with control, unstimulated cells), suggesting that the increase in transcription is independent of p38 kinases (Fig. 8A).

Activation of the IL-8 promoter, although statistically significant, was not very prominent. Therefore, we investigated the effect of 15d-PGJ$_2$ on mRNA stability. The HMEC-1s were incubated with Act-D (1 μg/ml) in the presence or absence of 10 μM 15d-PGJ$_2$. After the 4-, 8-, 12-, and 16-h incubation periods, RNA was isolated from the cells, and RT-PCR with primers specific for IL-8 was performed. We found that signal for IL-8 was detectable in control cells (not stimulated with 15d-PGJ$_2$) until 4 h after addition of Act-D, but almost disappeared after 8 h (Fig. 8B and D). Conversely, in cells supplemented with Act-D in the presence of 15d-PGJ$_2$, IL-8 mRNA was present even 12 h after stimulation. This suggests that an increase in mRNA stability is a mechanism involved in the upregulation of IL-8 synthesis in response to 15d-PGJ$_2$.

Interestingly, in contrast to the promoter activity, inhibition of p38 strongly influenced the posttranscriptional regulation of IL-8 expression. In the presence of SB 202190, 15d-PGJ$_2$ was unable to increase the stability of mRNA (Fig. 8C and D), whereas in the absence of the p38 inhibitor, the stability was strongly augmented (Fig. 8B and D). Therefore, we conclude that the stimulatory effect of 15d-PGJ$_2$ on IL-8 generation is mediated in part by p38-dependent stabilization of IL-8 mRNA. We observed the same effects in LPS-stimulated and -unstimulated cells (data not shown).

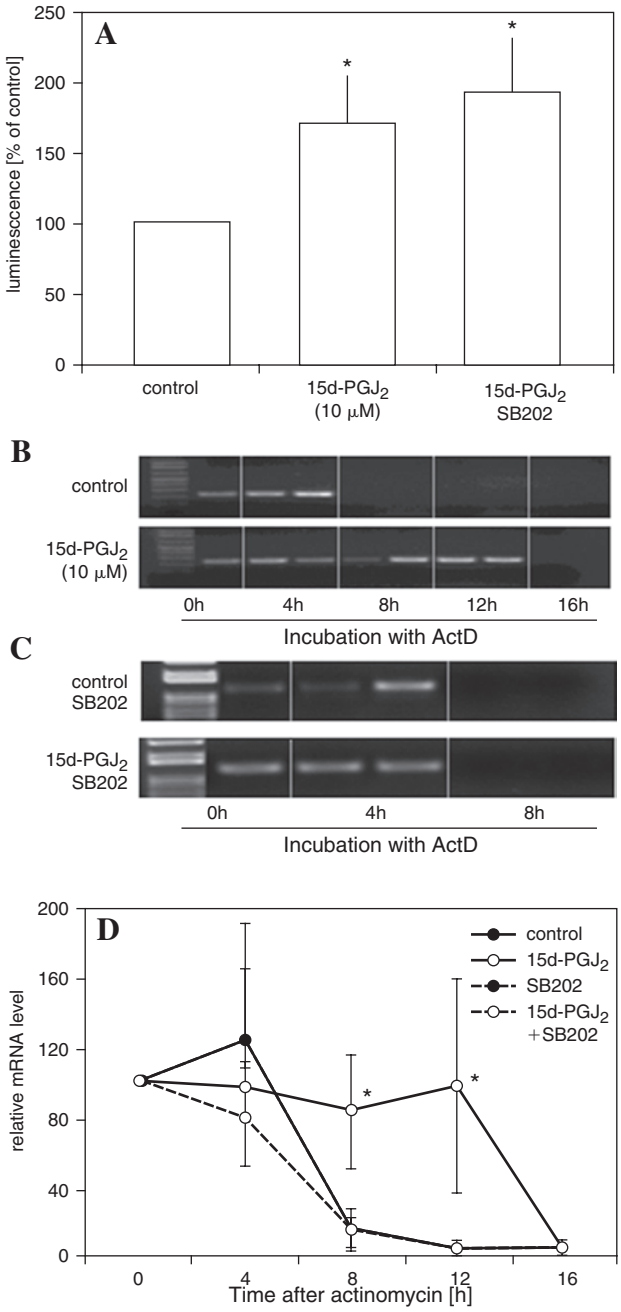

FIG. 8. (A) Effect of SB 202190 (10 μM) on the influence of 15d-PGJ$_2$ (10 μM, 24 h) on activity of IL-8 promoter in HMEC-1s transiently transfected with pGL2-IL8 plasmid. Data are shown as percentage of control value (control: unstimulated cells). *$p < 0.05$ compared with control. Luciferase assay. (**B**) Effect of 15d-PGJ$_2$ on IL-8 mRNA stability. HMEC-1s were exposed to Act-D (1 μg/ml) with or without 15d-PGJ$_2$ (one of five experiments). (**C**) Effect of SB 202190 (10 μM) on the influence of 15d-PGJ$_2$ on IL-8 mRNA stability. HMEC-1s were supplemented with SB 202190, and then exposed to Act-D with or without 15d-PGJ$_2$. After different time points, HMEC-1s were subjected to total RNA isolation followed by RT-PCR with electrophoresis of RT-PCR products. SB 202190 was added to the cells 30 min before stimulation with 15d-PGJ$_2$ (one of four experiments). (**D**) Densitometric analysis of the gels. Each point represents mean ± SD of four to five experiments, calculated as percentage of reference (0-h) values. *$p < 0.05$ in comparison with control cells, cultured without 15d-PGJ$_2$, or SB 202190.

Discussion

The meaning of endogenous 15d-PGJ_2 is not fully elucidated. It was found that endothelial expression of lipocalin-type PGD synthase (L-PGDS), which catalyzes the conversion of PGH_2 to PGD_2, is stimulated by laminar fluid shear stress and is upregulated in diseases associated with vascular injuries, such as hypertension and diabetes (45). Some data indicate higher production of 15d-PGJ_2 in patients with diabetes, hypertension, atherothrombotic stroke (6), or rheumatoid arthritis (47). In patients with acute stroke, increased plasma 15d-PGJ_2 levels (remaining, however, within low-nanomolar range) were associated with good neurologic outcome and smaller infarct size (6). Early articles indicate that cyclopentenone prostaglandins in some tissues can reach low-micromolar concentrations (55). In more recent studies, 15d-PGJ_2 was found to be increased to ~0.1 μM in the murine ischemic cortex (36) or ~0.2 μM in the blood of endotoxemic, selenium-supplemented mice (57). The vast majority of articles describe, however, effects of *in vitro* application of 15d-PGJ_2 at the low-micromolar doses. Because our article aimed to shed light on mechanisms responsible for the effect observed earlier, we decided to use similar concentrations.

The most important finding of our study is the demonstration that 15d-PGJ_2 strongly increases the synthesis of IL-8 protein in human microvascular endothelial cells (HMEC-1s) through induction of oxidative stress, activation of p38 kinases, and increase in IL-8 mRNA stability. Upregulation of the IL-8 promoter, although measurable, seems to play a less-pronounced role. Additionally, our data indicate the involvement of cAMP elevation and may suggest a role for the ATF2 transcription factor. We also excluded the contribution of the surface prostaglandin receptors and confirmed that the effect of 15d-PGJ_2 is independent of PPARγ.

Lack of involvement of PPARγ in regulation of basal and LPS-stimulated expression of IL-8 was already demonstrated in our earlier experiments, in which the effects of 15d-PGJ_2 were not mimicked by thiazolidinediones, the specific PPARγ ligands, and were not changed by the overexpression of PPARγ (23). These results are in accordance with studies performed on macrophages, in which the specific activators of PPARγ failed to modulate the synthesis of IL-8 (50). In contrast, in intestinal cell lines or in human aortic endothelial cells, PPARγ agonists were strong inhibitors of IL-8 (32), showing that the role of PPARγ can be tissue specific.

Apart from the binding to PPARγ, prostaglandins of the D_2 and J_2 series can activate DP-1/DP and DP-2/CRTH2 transmembrane receptors, associated with $G_{\alpha s}$ and $G_{\alpha i}$ proteins, respectively (46). Whereas the DP-2 receptor is expressed only on Th-2 lymphocytes, eosinophils, and basophils, low levels of DP-1 mRNA have been detected in most tissues tested (8).

Most actions of the cyclopentenone prostaglandins do not appear to be mediated by binding to G protein–coupled receptors. However, DP-1 is responsible for the PGD_2-induced stimulation of endothelial nitric oxide synthase (eNOS) expression in the choroids (12). It was also shown that activation of DP-1 leads to elevated generation of cAMP (8), a feature observed in our cells in response to 15d-PGJ_2. Therefore, it seemed possible that induction of IL-8 in HMEC-1s might be mediated by activation of DP-1.

The data obtained after incubation of cells with the DP-1 agonist and antagonist clearly showed, however, that the DP-1 receptor is not involved. Additionally, lack of effect of 15d-PGJ_2 on activity of G proteins in HMEC-1s excluded the potential role of any other G protein–coupled transmembrane receptor. Instead, stimulation of the IL-8 observed in the cells treated with BW245C, a DP-1 agonist, can result from its nonspecific action. It has been shown that this compound may bind to PF-R, a receptor for $PGF_{2\alpha}$, associated with a G_s protein (13). Such an interaction could be suggested also in our cells, as we have found the upregulation of IL-8 in response to $PGF_{2\alpha}$ (23).

Because neither nuclear nor surface receptors were involved in upregulation of IL-8, we supposed that the observed effects might result from an interaction of 15d-PGJ_2 with cellular proteins by the reactive α,β-unsaturated carbonyl group located in the cyclopentenone ring and induction of oxidative stress (46). Measurement of DCF fluorescence showed that treatment of HMEC-1s with 15d-PGJ_2 led to the rapid generation of reactive oxygen species. Inhibition of 15d-PGJ_2–induced synthesis of IL-8 in cells overexpressing catalase or supplemented with catalase-PEG enzyme indicates a role for ROS, especially H_2O_2. Complete abrogation of IL-8 synthesis by pretreatment of cells with NAC further confirms dependence of 15d-PGJ_2 activity on alternations in the cellular redox state.

It should be stressed, however, that NAC may act not only as an antioxidant, but also by direct binding of 15d-PGJ_2 to the thiol groups. Thus, one can expect that the inhibitory effect of NAC results from decreased availability of 15d-PGJ_2. This possibility cannot be excluded. It seems, however, that direct binding to NAC is not enough to block the activity of 15d-PGJ_2 in our experimental setting, as in earlier experiments with the same concentrations of 15d-PGJ_2 (1–10 μM) and NAC (1 mM), we did not observe any effect of NAC on 15d-PGJ_2–exerted inhibition of eNOS in HUVECs and uPA in HMEC-1s (26, 27).

Involvement of ROS and changes of oxidative status in 15d-PGJ_2–elicited regulation of gene expression was described in many earlier articles (*e.g.*, 31). However, 15d-PGJ_2, may produce oxidative stress by several mechanisms. Apart from the induction of ROS generation, it can bind to the thiol groups of different proteins, directly modulating their properties. Formation of conjugates with cytoprotective enzymes, such as biliverdin reductase (2), glutathione peroxidase (31), glutathione-S-transferase (GSTP1) (44), or thioredoxin (49), leads to decreased activities of these enzymes and thereby lowers the cellular resistance against free radicals. Binding to GSH, catalyzed by glutathione-S-transferases, attenuates the effects of 15d-PGJ_2 (*e.g.*, leading to inhibition of its antiproliferative effects or antagonizing expression of genes transactivated by PPARγ and Nrf2) (19).

In our experiments, the increased activity of 15d-PGJ_2 in cells pretreated with ethacrynic acid (EA), a GST inhibitor, suggests the involvement of this pathway in regulation of IL-8 expression in HMEC-1s. The role of an appropriate GSH level in IL-8 control is also supported by observation of the stronger effects of 15d-PGJ_2 in the HMEC-1s depleted of GSH by preincubation of cells with DEM, a GSH scavenger.

Depletion of GSH, induction of mitochondrial generation of ROS, and direct binding of 15d-PGJ_2 to the cysteines of Keap1 potently activate the Nrf2 transcription factor and up-

regulate several antioxidative genes, including HO-1 (24, 20, 35). This stimulation is independent of PPARγ but can be reduced by NAC and by inhibition of p38 kinase (35). 15d-PGJ$_2$ is one of the strongest inducers of HO-1, and some of its cellular effects result just from activation of HO-1 pathway (24, 30). We have shown that 15d-PGJ$_2$–mediated induction of HO-1, followed by increased production of CO and elevation of cGMP, is responsible for stimulation of vascular endothelial growth factor (VEGF) expression in HMEC-1s (24). The current study evidences, however, that production of IL-8 was not modulated by SnPPIX (HO-1 inhibitor), hemoglobin (CO scavenger), or ODQ (guanyl cyclase inhibitor, decreasing cGMP production). This indicates that differently from that in the case of VEGF, HO-1 pathway is not involved in regulation of IL-8 synthesis in HMEC-1s treated with 15d-PGJ$_2$.

Interestingly, we observed that 15d-PGJ$_2$ rapidly increased generation of cAMP, whereas induction of cAMP by forskolin potently upregulated the production of IL-8. This pathway has not been regarded as an important mediator of 15d-PGJ$_2$ activities. In contrast, cAMP was shown to be not involved in increased cytotoxicity in hepatocytes or in reduced expression of iNOS in microglia treated with 15d-PGJ$_2$ (5, 38). However, our experiments showed that inhibition of adenylyl cyclase activity by MDL 12330A reduced the production of IL-8. Accordingly, inhibition of phosphodiesterases by IBMX, resulting in an increased cAMP level, augmented release of IL-8. This observation is consistent with the earlier reports on the stimulatory effects of cAMP on IL-8 synthesis (22, 59). We demonstrated for the first time that cAMP elevation may contribute to 15d-PGJ$_2$–exerted signal transduction and upregulation of IL-8.

Our results do not clarify which mechanism can be responsible for the increased transcription from IL-8 promoter. One of the most important transcription factors regulating the expression of IL-8 is NF-κB (7). It is well known, however, that NF-κB and its activating kinase are key targets for the antiinflammatory activity of 15d-PGJ$_2$, which inhibits NF-κB–mediated transcriptional activation by PPARγ-dependent and independent mechanisms (46). Interestingly, NF-κB was not involved in the induction of IL-8 by 15d-PGJ$_2$ in HMEC-1s, as 15d-PGJ$_2$ did not influence NF-κB nuclear translocation and DNA binding (23).

Here we demonstrate that 15d-PGJ$_2$ increased the activity of ATF2, a cAMP-dependent transcription factor. ATF2 is the important regulator of IL-8 transcription (13). One can suggest that ATF2 may play a role also in the 15d-PGJ$_2$– and cAMP-mediated induction of IL-8 in HMEC-1s. This supposition, however, requires further experiments.

15d-PGJ$_2$ is usually regarded as a compound inhibiting inflammatory reactions in endothelial cells. For example, in HUVECs, 15d-PGJ$_2$ decreased TNF-induced expression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). Interestingly, expression of E-selectin and platelet–endothelial cell adhesion molecule-1 (PECAM-1) was not altered, indicating that the antiinflammatory potential of 15d-PGJ$_2$ may depend on the target protein analyzed (42). The proposed mechanism of downregulation of inflammatory mediators included activation of PPARγ (42), direct inhibition of NF-κB (28), or attenuation of the diacylglycerol–PKC pathway (56).

Induction of IL-8 by 15d-PGJ$_2$ has been already reported in monocytes (15, 60) or in endothelial cells (21, 23). This regulation seems to be tissue specific, as in epithelial cells, 15d-PGJ$_2$ inhibited the IL-8 synthesis (3, 14), and even in the same cell line, the effects of 15d-PGJ$_2$ may vary depending on the additional stimuli used for regulation of IL-8 (60).

Inhibition of IL-8 is usually regarded a result of reduced NF-κB or AP-1 activities (3, 14). The mechanism underlying the induction of IL-8 is less understood. Experiments performed in macrophages suggested that 15d-PGJ$_2$ upregulates the expression of the IL-8 gene through the Erk1/2 pathway (15). Our experiments indicate that ERK1/2 MAPK kinases are not involved. Preincubation of HMEC-1s with inhibitors of different protein kinases showed that the upregulation of IL-8 is reduced by genistein and SB 202190, whereas blockers of ERK1/2, PKA, PKC, and PI3K/Akt did not display a significant influence.

The effect of genistein may suggest the involvement of tyrosine kinases, as already observed in human endothelial cells (53). It should be stressed, however, that genistein displays many nonspecific activities. Thus, further studies are required to confirm conclusively the role of genistein and involvement of tyrosine kinases in 15d-PGJ$_2$–mediated IL-8 upregulation.

It seems possible that 15d-PGJ$_2$, through the induction of oxidative stress, may lead to activation of the p38 MAPK cascade. Phosphorylation of p38 in response to 15d-PGJ$_2$ has been already described in chondrocytes or cancer cell lines, where it initiated apoptosis (11, 18, 47). Also in hepatic myofibroblasts or macrophages, activation of p38 was necessary for induction of HO-1 (33, 34). We demonstrated that 15d-PGJ$_2$ leads to the increase in phosphorylation of p38 kinases in HMEC-1s. Additionally, the importance of the p38 in the regulation of IL-8 was illustrated by the prominent effects of SB 202190, a p38 inhibitor. Pretreatment of HMEC-1s with SB 202190 prevented completely the induction of IL-8 synthesis in response to 15d-PGJ$_2$. Similar effects were observed in human aortic endothelial cells, in which stimulation of IL-8 was caused by a high concentration of glucose (51). Because p38 can phosphorylate and activate the ATF2 transcription factor (1), one can hypothesize that such activation contributes to the increased synthesis of IL-8. However, the effect of 15d-PGJ$_2$ on the IL-8 promoter is relatively weak, and regulation at the transcriptional level cannot fully explain the observed IL-8 induction.

It is well established that activation of p38 may increase the stabilities of mRNA. For example, in human monocytes, nitric oxide (NO) increased IL-8 expression acting posttranscriptionally, through p38-dependent mRNA stabilization (37). Moreover, it has been demonstrated that in hepatic myofibroblasts, 15d-PGJ$_2$ induced oxidative stress and activated p38 MAPK, resulting in increased HO-1 mRNA stability (34). We found that a similar mechanism is responsible, at least in part, for the upregulation of IL-8 synthesis in HMEC-1s treated with 15d-PGJ$_2$. The increase in stability of IL-8 mRNA observed in the cells exposed to 15d-PGJ$_2$ was abrogated in the presence of a p38 blocker, whereas transcriptional activity was not altered.

In summary, we demonstrated that 15d-PGJ$_2$ potently increases the expression of IL-8 in human endothelial cells, acting independent of PPARγ and G protein–coupled surface receptors. Instead, it induces oxidative stress, activates p38

kinases, and thereby increases the stability of IL-8 mRNA. Thus, this pathway may contribute to the proinflammatory effects of 15d-PGJ_2.

Acknowledgments

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Abbreviations

Act-D, actinomycin D; DCF, dichlorofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescin diacetate; DEM, diethylmaleimide; EA, ethacrynic acid; HMEC-1, human microvascular endothelial cell line; HUVECs, human umbilical vein endothelial cells; IBMX, 3-isobutyl-1-methylxanthine; MDL-12330A, cis-*N*-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine; NAC, *N*-acetylcysteine; ODQ, 1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one); PGD_2, prostaglandin D_2; SnPPIX, tin protoporphyrin-IX;15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin-J_2.

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