

# Peroxisome Proliferator-activated Receptor $\delta$ Activators Induce IL-8 Expression in Nonstimulated Endothelial Cells in a Transcriptional and Posttranscriptional Manner<sup>\*[5]</sup>

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Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that are implicated in the regulation of lipid and glucose homeostasis. PPAR agonists have been shown to control inflammatory processes, in part by inhibiting distinct proinflammatory genes (e.g. IL-1 $\beta$  and IFN- $\gamma$ ). IL-8 is a member of the proinflammatory chemokine family that is important for various functions, such as mediating the adhesion of eosinophilic granulocytes onto endothelial cells. The influence of PPAR $\delta$  activators on the expression of IL-8 in noninduced quiescent endothelial cells is unclear. Therefore, we explored the influence of PPAR $\delta$  activators on the expression of IL-8 in nonstimulated endothelial cells. PPAR $\delta$  agonists induce IL-8 expression in human umbilical vein endothelial cells. This induction is demonstrated at the level of both protein and mRNA expression. Transcriptional activation studies using IL-8 reporter gene constructs and DNA binding assays revealed that PPAR $\delta$  agonists mediated their effects via an NF $\kappa$ B binding site. It is well known that IL-8 is also regulated by mRNA stability. To provide further evidence for this concept, we performed mRNA stability assays and found that PPAR $\delta$  agonists induce the mRNA stability of IL-8. In addition, we showed that PPAR $\delta$  agonists induce the phosphorylation of ERK1/2 and p38, which are known to be involved in the increase of mRNA stability. The inhibition of these MAPK signaling pathways resulted in a significant suppression of the induced IL-8 expression and the reduced mRNA stability. Therefore, our data provide the first evidence that PPAR $\delta$  induces IL-8 expression in nonstimulated endothelial cells via transcriptional as well as posttranscriptional mechanisms.

Peroxisome proliferator-activated receptors (PPARs)<sup>2</sup> are members of the nuclear receptor/ligand-activated transcription factors superfamily and consist of three different subtypes: PPAR $\alpha$ , PPAR $\delta$ , and PPAR $\gamma$ . The role of these receptors was

originally thought to be restricted to lipid and lipoprotein metabolism, glucose homeostasis, and cellular differentiation (1, 2). PPARs are activated by natural ligands, such as eicosanoids and fatty acids. In addition, synthetic antidiabetic thiazolidinediones and lipid-lowering fibrates have been shown to act as activators of PPAR $\gamma$  and PPAR $\alpha$ , respectively (3, 4). To date, PPAR $\delta$ , which is expressed in almost all tissues, is the subtype that still remains an interesting target for new pharmaceutical drugs.

New evidence suggests that PPAR $\delta$  plays a crucial role in the regulation of differentiation, cell growth, and the metabolism of lipids and glucose (5, 6). With respect to the development of novel drugs, the effects and side effects of PPAR $\delta$  activators are therefore of great importance.

In the last few years, knowledge concerning the impact of PPAR $\delta$  in endothelial cell function has increased. During inflammation, proinflammatory stimuli such as LPS, TNF $\alpha$ , or IL-1 $\beta$  lead to a phenotypic change of the quiescent endothelium by induction of proinflammatory factors, such as IL-6, IL-8, MCP-1, or adhesion molecules, such as ICAM-1 or VCAM-1. Recently, Rival *et al.* demonstrated that the PPAR $\delta$  activator L165041 suppresses TNF $\alpha$ -induced VCAM-1 and MCP-1 expression (7). Similar results were demonstrated by Fan *et al.*, revealing that the PPAR $\delta$  agonist GW501516 suppresses IL-1 $\beta$ -induced VCAM-1 and E-selectin expression in human umbilical vein endothelial cells (HUVECs) (8). Piqueras *et al.* demonstrated that PPAR $\delta$  inhibits leukocyte recruitment and cell adhesion molecule expression (9). Recently, Liang *et al.* showed that the PPAR $\delta$  agonist L-165041 suppresses C-reactive protein-induced IL-6 expression (10). The expression profile of both cytokines during treatment with various PPAR $\delta$  agonist concentrations in the endothelial quiescent status has yet to be analyzed. Nonetheless, these studies showed that PPAR $\delta$  agonists could suppress the inflammatory processes in activated endothelial cells. The impact of PPAR agonists on the normally quiescent endothelium, however, remains to be elucidated. This could be of significant importance due to the possible broad range of applications of PPAR $\delta$  agonists in various diseases, such as chronic inflammation, glucose metabolism, dyslipidemia, obesity, and cancer therapy, to mention only a small number of possible medical applications.

During the process of inflammation, proinflammatory cytokines are produced by various cell types. IL-8 is one of these important cytokines. It is secreted at very low levels from non-induced cells, although the secretion is increased rapidly by a

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1–3.

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<sup>2</sup> The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; MCP, monocyte chemoattractant protein; VCAM, vascular cellular adhesion molecule; PPRE, PPAR response element.

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wide range of stimuli, such as TNF and IL-1 or bacterial products, such as LPS (11). There are two main methods of regulation: first, by transcriptional activation of the genes by NF $\kappa$ B, and second, by stabilization of the mRNA by the p38 MAPK pathway (12–16).

Besides the important proinflammatory action, IL-8 plays an important role in the tumor microenvironment. Secretion of IL-8 from cancer cells can aggravate the proliferation and survival of cancer cells, in part by autocrine signaling pathways (17). In addition, tumor-derived IL-8 can activate endothelial cells to promote angiogenesis. IL-8, however, is secreted not only by tumor cells, but also by endothelial cells, thereby enhancing endothelial cell survival, proliferation, and angiogenesis (18).

The present study investigated the influence of PPAR $\delta$  activators on the production, secretion, and regulation of IL-8 in nonstimulated endothelial cells, revealing an induction of IL-8 expression conveyed by transcriptional, NF $\kappa$ B-dependent, and posttranscriptional mechanisms involving mRNA stability.

### EXPERIMENTAL PROCEDURES

**Reagents**—Recombinant human TNF $\alpha$  and IL-1 $\beta$  were purchased from R&D Systems (Minneapolis, MN). L165041, GW501546, PD98059, SB203580, LY294002, and actinomycin D were obtained from Sigma-Aldrich.

**Cell Culture**—HUVECs were purchased from PromoCell (Heidelberg, Germany) and were cultured until the fifth passage at 37 °C and 5% CO<sub>2</sub> in endothelial cell growth medium (Cambrex, East Rutherford, NJ).

**Enzyme-linked Immunosorbent Assay (ELISA)**—The concentrations of IL-8 and IL-6 in cell culture supernatants were determined by ELISA. Commercially available ELISA kits (R&D Systems) for the quantification of IL-8 and IL-6 were used as described in the manufacturer's instructions.

**Western Blot Analysis**—Whole cell protein and cytoplasmic and nuclear protein extracts were prepared as described previously (19). Membranes were incubated with the indicated primary antibodies. Antibodies were as follows: anti-p-p65, anti-I $\kappa$ B- $\alpha$ , anti-phospho-I $\kappa$ B- $\alpha$ , anti-p65, anti-ERK1/2, anti-phospho-ERK1/2, anti-phospho-p38, anti-p38, anti-phospho-Akt (Ser<sup>473</sup>), anti-phospho-Akt (Thr<sup>308</sup>), anti-Akt, and anti-SP1 from Santa Cruz Biotechnology (Heidelberg, Germany) and anti-tubulin from LabVision (Fremont, CA). Primary antibody application was followed by incubation with horseradish peroxidase-conjugated secondary antibodies (anti-mouse and anti-rabbit IgG, Amersham Biosciences; anti-goat, Dako, Glostrup, Denmark). Blots were developed using an enhanced chemiluminescence detection system (ECL) (Amersham Biosciences), according to the manufacturer's instructions.

**Cytotoxicity Assay**—The cytotoxic potential of L165041 was determined using the Cytotoxicity Detection kit (lactate dehydrogenase) from Roche, according to the manufacturer's instructions. Twenty-four hours after seeding, cells were exposed to L165041 for 24 h at the indicated concentrations.

**RNA Extraction and RT-PCR**—RT-PCR analyses were performed using total RNA (150 ng) extracted from subconfluent cell cultures. Total cellular mRNA was isolated by the RNeasy Mini Procedure (Qiagen, Hilden, Germany) after DNase diges-

tion. RT-PCR analyses for IL-6, IL-8, and GAPDH were performed using the One-Step RT-PCR kit (Qiagen). PCR products were resolved by 1–2% agarose gel electrophoresis, and ethidium bromide-stained bands were visualized with an ultraviolet transilluminator. A densitometric analysis was used to quantify band intensities using the public domain Java image-processing program ImageJ (v1.29s). Optical densities (ODs) of the IL-8 bands were corrected for loading differences based on the corresponding GAPDH bands. The primer sets for IL-8, IL-6, and GAPDH have been published previously (20).

**Transient Transfection and Analysis of Reporter Gene Expression**—HUVECs ( $1.0 \times 10^5$  cells/well in 12-well plates) were transfected with 0.5  $\mu$ g of the appropriate firefly luciferase construct and 0.1  $\mu$ g of pHRG-TK vector (Promega, Madison, WI) using the SuperFect transfection reagent (Qiagen). Human IL-8 reporter gene constructs were generously provided by Dr. Naofumi Mukaida (Division of Molecular Bioregulation, Cancer Research Institute, Kanazawa University, Japan). Twenty-four hours after transfection, cells were treated with vehicle (0.3% dimethyl sulfoxide) or with L165041 for 24 h. Luciferase activities were measured with the Dual-Luciferase Reporter Assay system (Promega).

**Preparation of Nuclear Extracts and Gel Mobility Shift Analysis**—HUVECs were treated with vehicle or incubated with L165041 for 60 min. Nuclear proteins were extracted as described previously (21). DNA binding reactions were performed with or without excess unlabeled NF $\kappa$ B consensus oligonucleotide (Promega) and p65 antibody (Santa Cruz Biotechnology). The electromobility shift analysis (EMSA) was performed as described previously (21).

**Immunolocalization of p65 NF $\kappa$ B**—For immunolocalization studies, HUVECs were plated onto 8-well chamber slides (Lab-Tek, Christchurch, New Zealand) at a density of 80%/well. The cells were grown in endothelial cell basal medium with 5% FCS for 24 h, fixed in 4% paraformaldehyde at room temperature for 10 min, and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. Permeabilized cells were rinsed three times with PBS and incubated in blocking solution (1% bovine serum albumin/PBS) for 30 min at room temperature to remove non-specific binding of the antibody. All subsequent steps were carried out at room temperature, and cells were rinsed three times in 1% bovine serum albumin/PBS between each of the steps. RelA/p65 was detected using a rabbit polyclonal anti-RelA/p65 antibody (C-20; Santa Cruz Biotechnology) and an Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody. The slides were mounted in Vectashield immunofluorescence mounting medium (Vector Laboratories) and viewed with fluorescence microscopy. Cells were counterstained for nuclei with Hoechst staining.

**Statistical Analyses**—The data are expressed as means  $\pm$  S.D. from at least three independent experiments. Statistical analyses were performed using Student's *t* test.

### RESULTS

**PPAR $\delta$  Agonists Induce IL-8 Expression in Nonstimulated Endothelial Cells**—To evaluate the influence of the PPAR $\delta$  agonists L165041 and GW501516 on quiescent nonstimulated endothelial cells, ELISA analyses were performed. Interest-

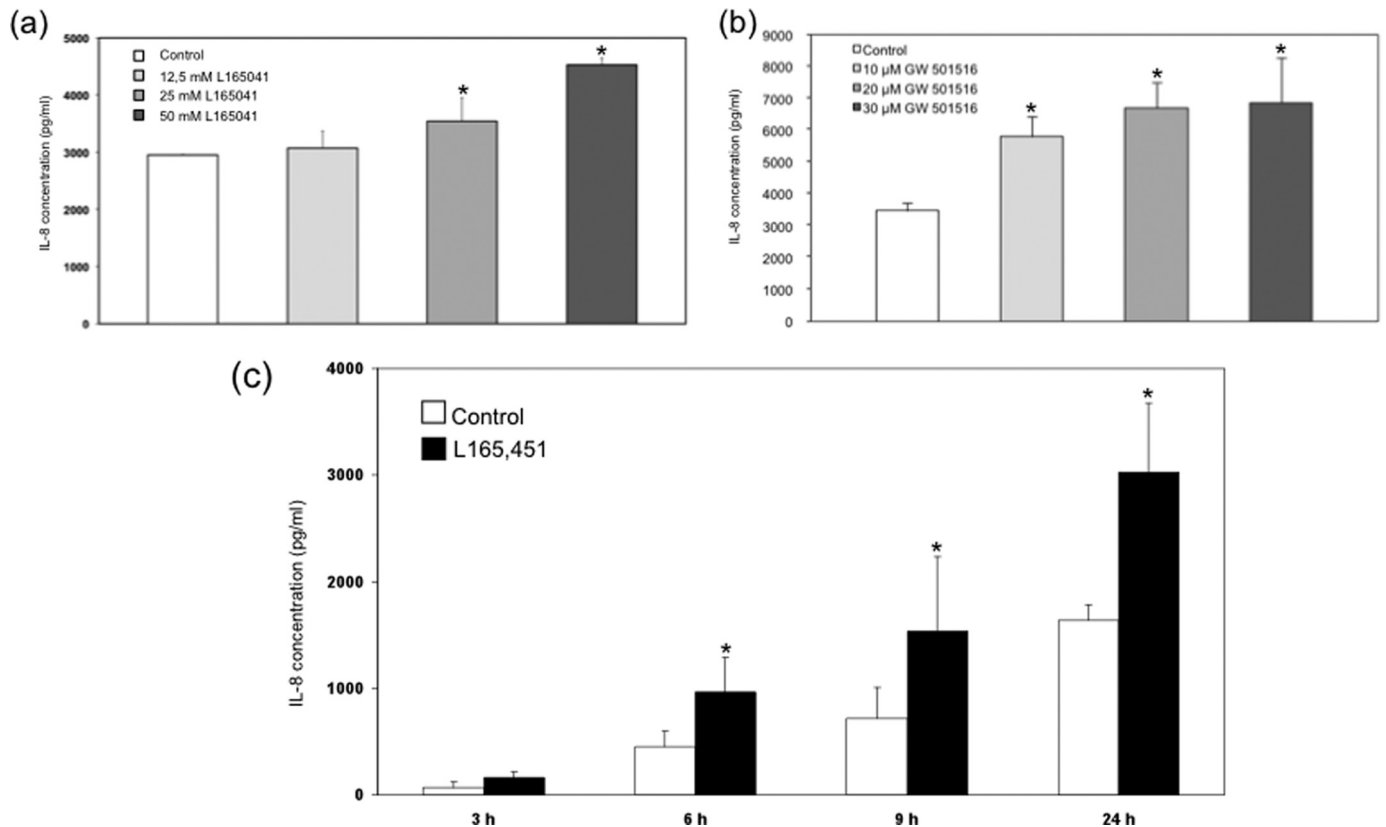


FIGURE 1. **Effects of GW501516 and L165041 on the IL-8 expression in supernatants of HUVEC.** IL-8 protein content was assayed in culture supernatants by IL-8 ELISA (R&D Systems) according to the manufacturer's instructions. *a* and *b*, HUVECs were left untreated (solvent only, 0.1% dimethyl sulfoxide) or were treated with L165041 (*a*) and GW501516 (*b*) for 24 h at the indicated concentrations. *c*, HUVECs were left untreated (solvent only, 0.1% dimethyl sulfoxide) or were treated with L165041 for the indicated times. Mean values from triplicate experiments are depicted  $\pm$  S.E. (error bars). \*,  $p < 0.05$  was considered significant.

ingly, these experiments demonstrated a time- and concentration-dependent induction of IL-8 expression in nonstimulated HUVECs treated with L165041 and GW501516, respectively (Fig. 1). The results were comparable for both PPAR $\delta$  agonists, indicating that the effects are specific to PPAR $\delta$ . To analyze whether the concentrations used have any cytotoxic effects, we performed cytotoxicity lactate dehydrogenase assays with our model compound, L165041, and found no relevant cytotoxic effects (supplemental Fig. 1). In further experiments we could demonstrate comparable results analyzing the expression of IL-6 in HUVEC treated with L165041 and GW501516, respectively (supplemental Fig. 2).

**PPAR $\delta$  Agonists Up-regulate the Steady State of IL-8 mRNA**—To determine whether activation of PPAR $\delta$  plays a role in the steady state of IL-8 mRNA expression, we performed RT-PCR analysis after treatment with the PPAR $\delta$  agonist L165041. Consistent with our protein expression data, application of the agonist induced IL-8 mRNA expression in a concentration- and time-dependent manner (Fig. 2). Comparable results could be demonstrated for IL-6 (supplemental Fig. 3). The expression of IL-8 can be regulated in a transcriptional and a posttranscriptional manner. We therefore first examined a possible transcriptional mechanism of control.

**PPAR $\delta$  Agonists Up-regulate IL-8 Promoter Activity in an NF $\kappa$ B-dependent Manner**—To analyze the underlying molecular mechanisms that mediate PPAR $\delta$ -conveyed induction of IL-8 mRNA expression, luciferase reporter assays were

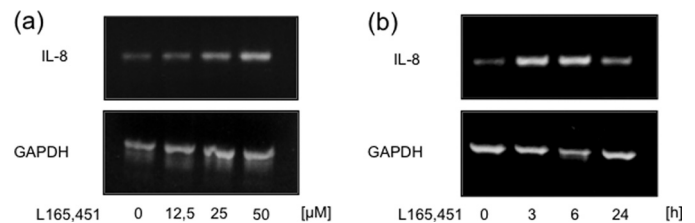
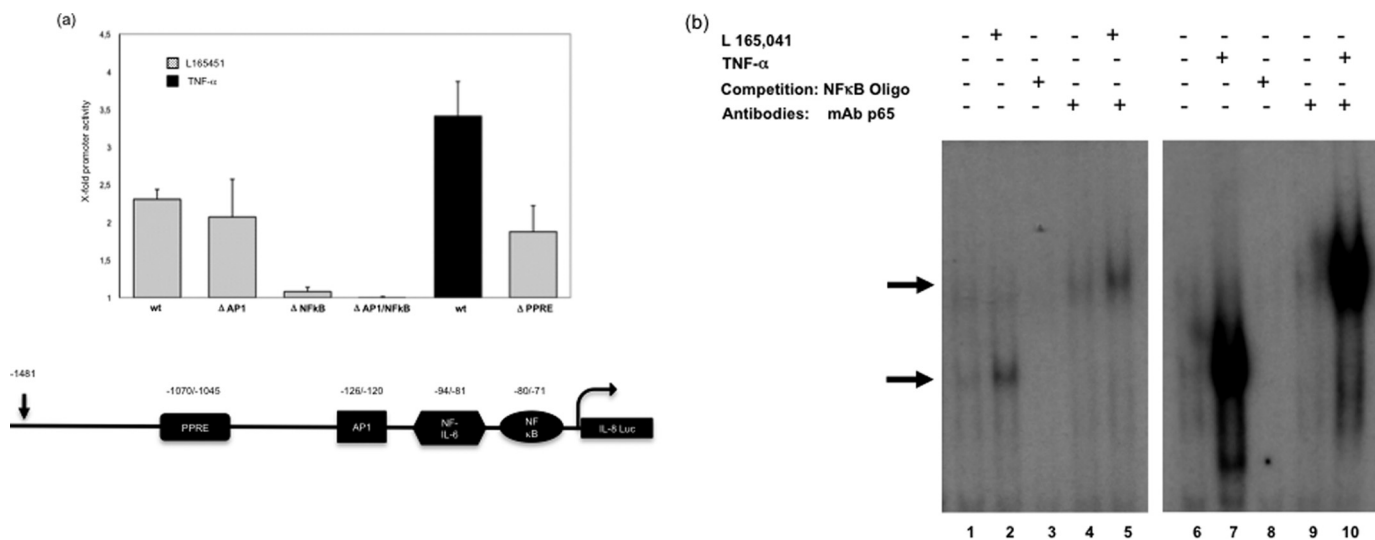


FIGURE 2. **IL-8 mRNA expression is induced by PPAR $\delta$  agonists.** RT-PCR analyses of total mRNA extracted from HUVECs that were treated with vehicle or L165041 for varying times (*a*) or varying concentrations (*b*) are shown. Results were confirmed in three independent sets of experiments.

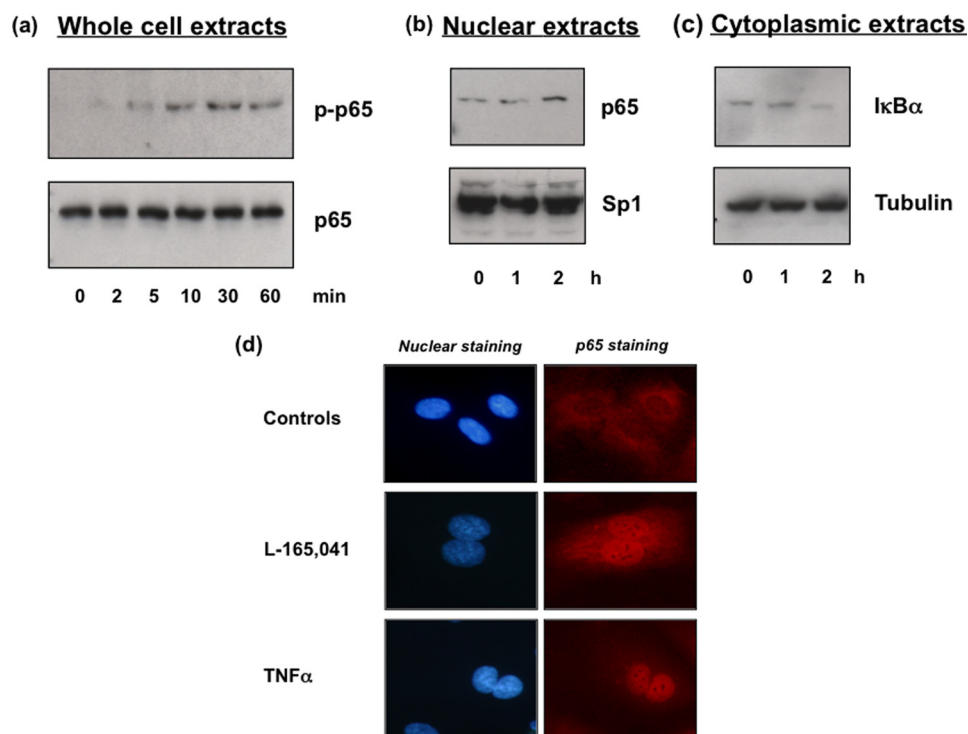
employed. Luciferase reporter constructs containing the 5'-region of the IL-8 promoter and a series of deletion constructs were transiently transfected into vehicle- and PPAR $\delta$  agonist-treated HUVECs.

Analysis of the expression of luciferase in vehicle and PPAR $\delta$  agonist-treated cells revealed an approximate 2.3-fold induction of basal luciferase activity with the wild type IL-8 construct (Fig. 3*a*). Deletion of the AP1 site did not significantly influence IL-8 promoter activity, whereas the deletion of NF $\kappa$ B led to a complete loss (Fig. 3*a*). Recently, the IL-8 promoter was shown to contain a PPRE site. To exclude the possibility that this site is also an important point of action of PPAR $\delta$  agonists, we analyzed an IL-8 promoter construct with a deletion of the PPRE site. Interestingly, the deletion did not significantly influence luciferase promoter activity during treatment with PPAR $\delta$  ago-

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**FIGURE 3. PPAR $\delta$  agonists induce IL-8 transcription in an NF $\kappa$ B-dependent manner.** *a*, analyses of deletional IL-8 luciferase (*Luc*) reporter constructs in HUVECs. The *Luc* activities are expressed as x-fold activity compared with untreated controls (mean  $\pm$  S.D. (error bars) of five independent triplicate assays). *b*, representative EMSAs using nuclear extracts of vehicle- and L165041-treated (50  $\mu$ M/liter, 1 h) HUVECs. Competition with unlabeled excess double-stranded NF $\kappa$ B consensus oligonucleotides is shown in lanes 3 and 8 (0.35  $\mu$ M/liter). A representative autoradiography from three independent experiments is presented. Supershift analyses were performed by addition of a p65-specific antibody (lanes 4 and 5 and lanes 9 and 10; 100 ng/ $\mu$ l). Arrows on the left indicate NF $\kappa$ B-containing transcription factor-binding complexes. Comparable results were obtained from three independent experiments.



**FIGURE 4. PPAR $\delta$  agonists induce p65 phosphorylation and nuclear translocation.** Representative Western blot analysis of HUVECs that were treated with vehicle or L165041 (50  $\mu$ M) for the indicated times is shown. Protein was separated by 8% SDS-PAGE, and proteins were detected by enhanced chemiluminescence. All comparable results were obtained from three independent experiments. *a*, total cellular protein showing the amount of phospho-p65 and p65. *b*, nuclear protein showing the amount of p65. Sp1 protein served as a control. *c*, cytoplasmic protein showing the amount of I $\kappa$ B $\alpha$ . Tubulin protein served as a control. *d*, representative immunofluorescent analysis of p65 in HUVECs that were treated with vehicle, L165041 (50  $\mu$ M), or TNF $\alpha$  (20 ng/ml). Comparable results were obtained from three independent experiments.

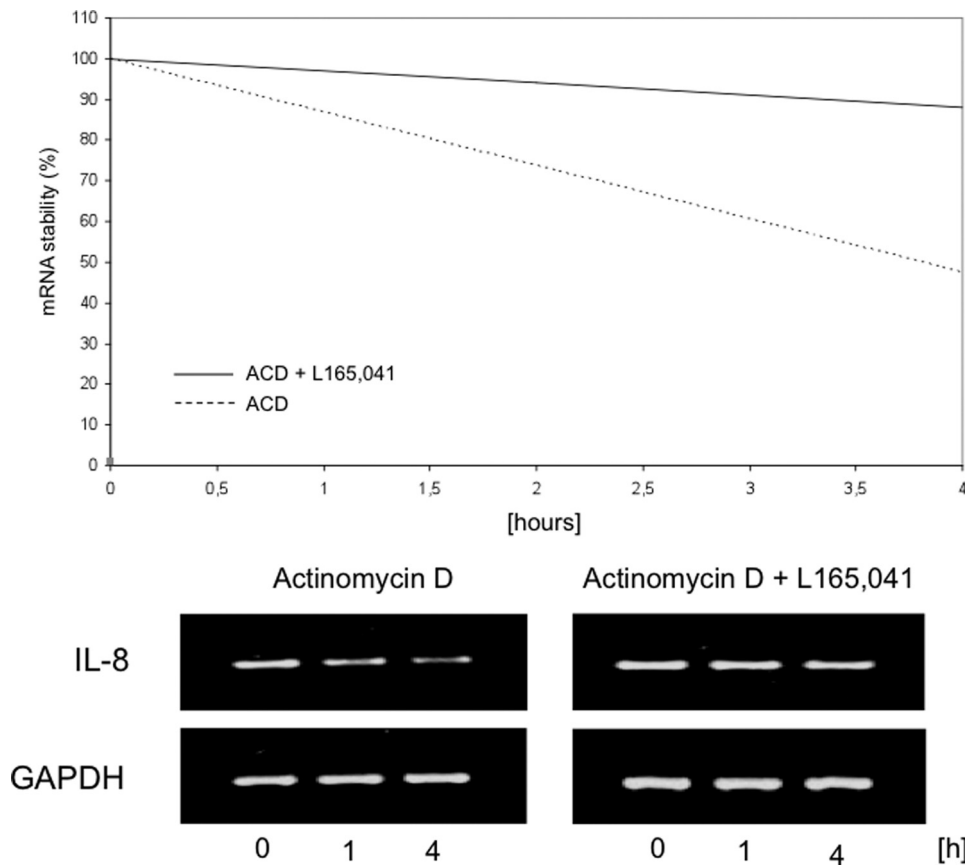
nists, demonstrating that the inducing effect of IL-8 is conveyed only by the NF $\kappa$ B site (Fig. 3a).

**NF $\kappa$ B-dependent Binding to the IL-8 Promoter Is Induced by PPAR $\delta$  Agonist Treatment**—To determine the nuclear factors that bind to the NF $\kappa$ B binding site, we performed EMSAs using

nuclear extracts from HUVECs and a  $^{32}$ P-labeled NF $\kappa$ B oligonucleotide probe. In untreated HUVECs, a distinct complex was observed to bind and shift the migration pattern of the oligonucleotide (Fig. 3b, lane 1). A significant increase in DNA binding activity, however, was observed in the lysates of cells treated with L165041 (Fig. 3b, lane 2) and in cells treated with TNF $\alpha$  as a control (Fig. 3b, lane 6). A supershift was observed upon addition of the p65 antibody (Fig. 3b, lanes 4 and 5 and lanes 9 and 10), confirming that p65 binds to the IL-8 promoter. Competition assays using excess unlabeled oligonucleotide supported the assumption that nuclear proteins bind to the IL-8 promoter sequence in an NF $\kappa$ B-specific manner (Fig. 3b, lanes 3 and 8).

**PPAR $\delta$  Agonists Lead to the Phosphorylation and Nuclear Translocation of NF $\kappa$ B**—After obtaining data suggesting that PPAR $\delta$  agonists convey the IL-8 induction in an NF $\kappa$ B-dependent manner, we analyzed the phosphorylation status of p65 in our PPAR $\delta$  agonist-treated

HUVECs. Interestingly, an increase in p65 phosphorylation and p65 nuclear translocation was detected by Western blot analysis (Fig. 4, *a* and *b*). Consistent with these results, we could demonstrate an increase in I $\kappa$ B $\alpha$  degradation (Fig. 4c). In addition, we found a significant nuclear translocation of p65 by



**FIGURE 5. IL-8 mRNA half-life is induced by PPAR $\delta$  agonists.** HUVEC were incubated with vehicle or L165041 (50 nM) for 1 h, followed by incubation with fresh medium containing actinomycin D (10  $\mu$ g/ml) for 0, 1, and 4 h. RT-PCR analyses for IL-8/GAPDH of total RNA extracted from subconfluent cell cultures were performed. The PCR products were separated by 2% agarose gel electrophoresis, and ethidium bromide-stained bands were visualized using an ultraviolet transilluminator. IL-8 bands were quantified by densitometric scanning, the results of which were normalized to amounts of GAPDH mRNA. Comparable results were obtained from three independent experiments.

immunofluorescence staining of NF $\kappa$ B p65 in HUVECs treated with PPAR $\delta$  agonists (Fig. 4*d*). The increase in p65 staining was comparable with the influence of TNF $\alpha$ .

**PPAR $\delta$  Agonists Induce IL-8 mRNA Stability**—IL-8 expression is not only controlled on the transcriptional level, but can be also influenced by the stability of IL-8 mRNA. Therefore, we next analyzed IL-8 mRNA stability in solvent- and PPAR $\delta$  agonist-treated endothelial cells. Surprisingly, a significant increase in IL-8 mRNA stability was found (Fig. 5), providing an additional mechanism for the induction of IL-8 expression.

**PPAR $\delta$  Agonists Induce ERK1/2, p38, and Akt Phosphorylation**—It is known that the p38 and ERK1/2 MAPK signaling pathways are involved in the regulation of the mRNA stability of IL-8. We therefore analyzed the phosphorylation status of MAPK ERK1/2 and p38 as well as Akt by Western blot analysis. Interestingly, the MAPK ERK1/2 and p38 were quickly phosphorylated after treatment with PPAR $\delta$  agonists, comparable results could be demonstrated for Akt (Fig. 6*a*). To analyze whether these signaling pathways influence PPAR $\delta$ -induced IL-8 protein expression, we blocked the ERK1/2, p38, and Akt signaling pathways using the specific inhibitors PD98059, SB203580, and LY294002, respectively. Except for Akt signaling, these treatments led to par-

tial and significant neutralization of PPAR $\delta$  agonist-induced IL-8 expression, demonstrating the importance of the p38 and ERK1/2 MAPK signaling pathways in PPAR $\delta$ -mediated IL-8 expression (Fig. 6*b*). The phosphorylation of Akt seems to have no significant influence on IL-8 expression.

These results could also be bolstered by mRNA analysis. To analyze whether p38 and ERK1/2 influence PPAR $\delta$ -induced IL-8 mRNA expression, we blocked the ERK1/2 and p38 signaling pathways using the specific inhibitors PD98059 and SB203580, respectively. These treatments led to partial neutralization of PPAR $\delta$  agonist-induced IL-8 mRNA expression (Fig. 6*c*).

**Inhibition of MAPK Signaling Pathways p38 and ERK1/2 Results in Decreased IL-8 mRNA Stability**—To provide evidence that the MAPK signaling pathways influence PPAR $\delta$ -induced IL-8 mRNA expression and stability, we performed mRNA stability assays while inhibiting the MAPK signaling pathways. We demonstrated that blockade of the ERK1/2 and p38 signaling pathways using PD98059 and SB203580, respectively, resulted in a reduced mRNA stability compared with the

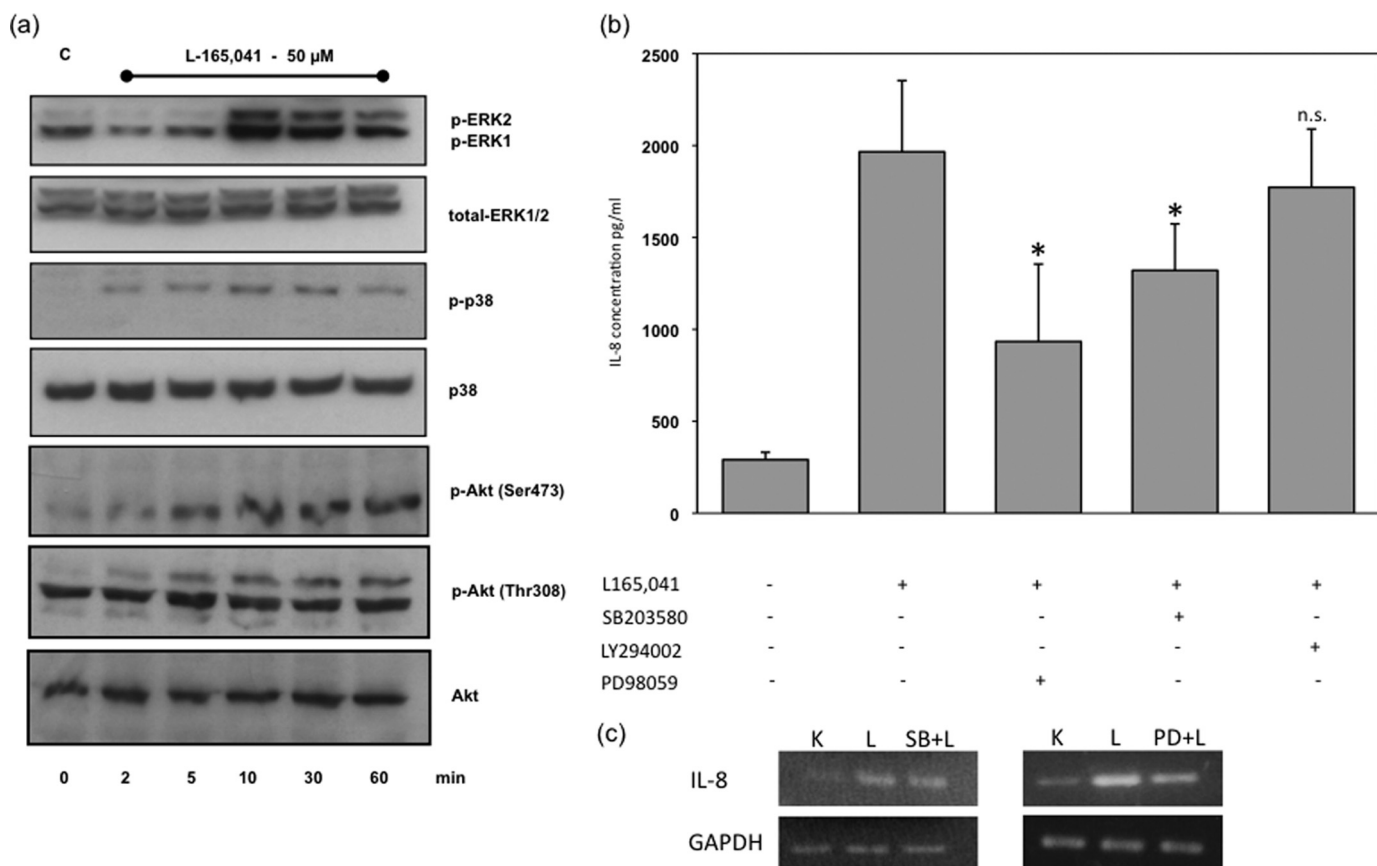
PPAR $\delta$  agonist only (Fig. 7).

## DISCUSSION

In the last decade, knowledge concerning PPAR activators has increased rapidly. The research community has primarily focused on PPAR $\alpha$  and PPAR $\gamma$  agonists, which are already in clinical use. In the last 2 years, however, PPAR $\delta$  agonists have raised significant interest, even though the research concerning this nuclear receptor and its ligands has been limited. Emerging evidence has demonstrated that PPAR $\delta$  plays an important role in the regulation of differentiation, cell growth, and lipid and glucose metabolism (5, 22). Recently, the impact of PPAR $\delta$  agonists in endothelial cell function and angiogenesis has been addressed (24). These results highlight the growing importance and the possible expanding area of employing PPAR $\delta$  agonists for clinical use.

In the current study, we demonstrated that PPAR $\delta$  agonists effectively induced IL-8 expression in nonstimulated quiescent endothelial cells. We identified two responsible mechanisms underlying this effect: a transcriptional mechanism conveyed by NF $\kappa$ B and a posttranscriptional mechanism mediated by a p38- and ERK1/2-dependent increase in mRNA stability. The influence of the major transcription factor NF $\kappa$ B on IL-8 expression is an established method of regulation (11, 25).

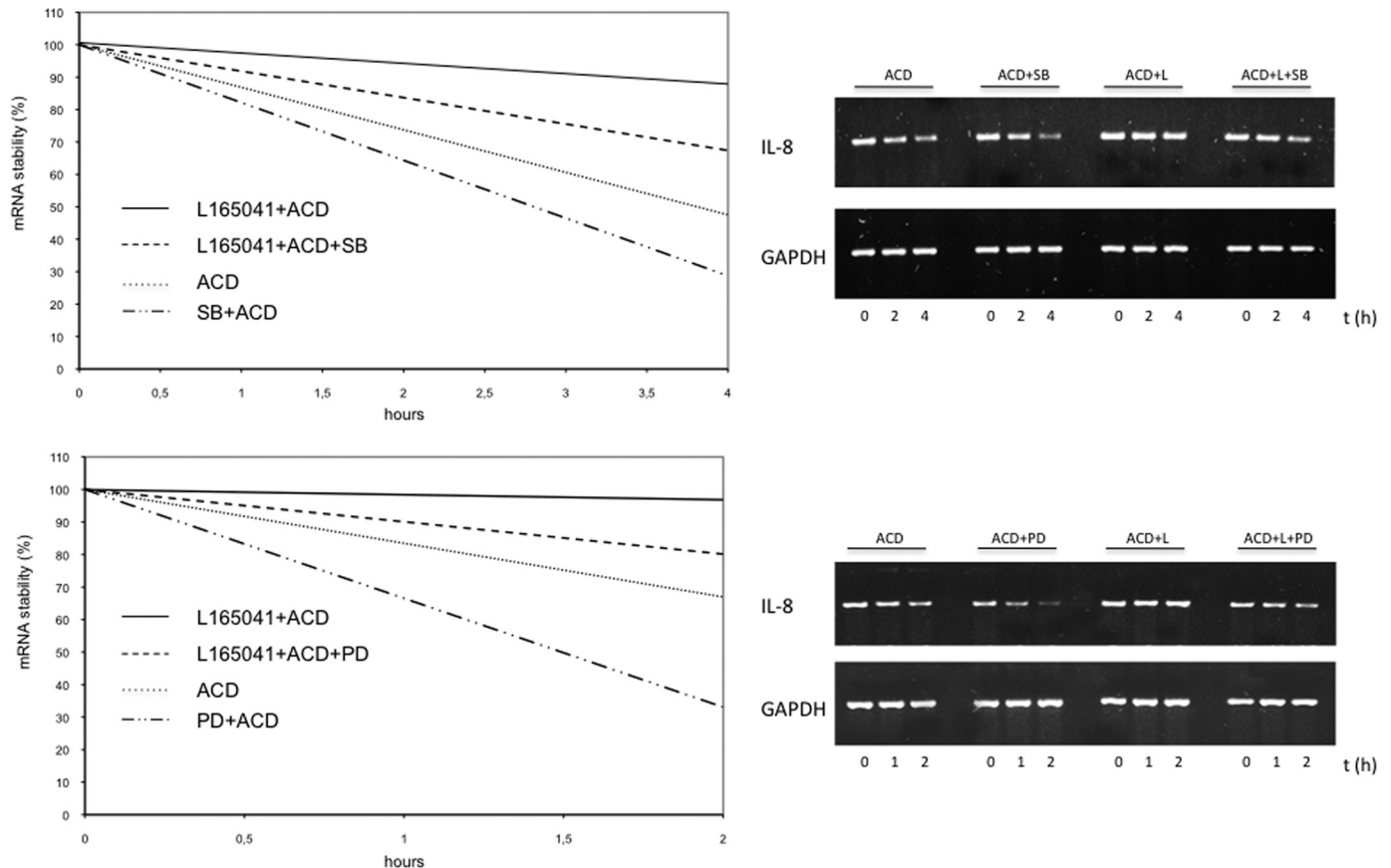
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**FIGURE 6. PPAR $\delta$  agonists activate MAPK signaling pathways.** *a*, representative Western blot analysis of HUVECs that were treated with vehicle or L165041 (50  $\mu$ M) for the indicated times up to 60 min. Total cellular protein was separated by 8% SDS-PAGE. ERK1/2, phospho-ERK1/2, p38, phospho-p38, Akt and phospho-Akt (Thr<sup>308</sup> and Ser<sup>473</sup>) protein were detected by enhanced chemiluminescence. Comparable results were obtained from three independent experiments. *b*, effects of MAPK signaling pathway inhibition on L165041-induced IL-8 expression in supernatants of HUVEC. HUVECs were pretreated for 30 min with 30  $\mu$ M PD98059, 1  $\mu$ M SB203580, and 10  $\mu$ M LY294002 and then exposed to 50  $\mu$ M L165041 for 6 h. The amount of IL-8 released by HUVECs into the culture medium was assessed by ELISA. Values are expressed as mean  $\pm$  S.E. (error bars) ( $n = 4$ ). \*,  $p < 0.05$  was considered significant. *c*, effects of MAPK signaling pathway inhibition on L165041-induced IL-8 mRNA expression in HUVECs. HUVECs were pretreated 30 min with 30  $\mu$ M PD98059 and 1  $\mu$ M SB203580 and then exposed to 50  $\mu$ M L165,041 for 6 h. The amount of IL-8 mRNA expression by HUVEC was assessed by RT-PCR. Results were confirmed in three independent sets of experiments.

Important proinflammatory cytokines, such as TNF $\alpha$  or IL-1 $\beta$ , as well as LPS are known to induce IL-8 expression via NF $\kappa$ B-containing transcription factor complexes targeting the IL-8 promoter (25–27). To our knowledge, this is the first study to demonstrate that PPAR $\delta$  agonists lead to a concentration-dependent phosphorylation and nuclear translocation of p65 in nonstimulated endothelial cells, thereby increasing NF $\kappa$ B DNA binding. In addition, we found that the PPRE element at the IL-8 promoter did not significantly influence IL-8 promoter activity. These data are contrary to those obtained for stimulated endothelial cells. Liang *et al.* convincingly demonstrated that C-reactive protein in stimulated endothelial cells suppresses NF $\kappa$ B phosphorylation when the cells are treated with the PPAR agonist GW501516, thereby resulting in reduced IL-6 and IL-8 expression (10). A similar result was demonstrated by Rodriguez-Calvo *et al.* for LPS-stimulated adipocytes, in which reduced IL-6 expression and NF $\kappa$ B DNA binding activity were detected after the previously LPS-stimulated adipocytes were treated with the PPAR $\delta$  activator GW501516 (28). The same group also showed an increase of IL-6 mRNA and protein in the control group treated only with GW501516 at very low PPAR $\delta$  concentrations (28). These results were bolstered by

our analysis concerning the expression of IL-6 in endothelial cells. In addition, Rival *et al.* demonstrated that the PPAR $\delta$  activator L165041 inhibits TNF $\alpha$ -induced VCAM-1 and MCP1 expression at concentrations up to 100  $\mu$ M (7). The important difference between those studies and the current work is that nonstimulated quiescent endothelial cells were used in the present study, revealing the possibility of a dual function of the PPAR $\delta$  agonist in endothelial cells that is dependent on the activation status of the cell. This novel information may be important, because this would implicate a differentiated use of these compounds regarding their application in the treatment of various diseases. It is possible that this dual action also explains the different results concerning the influence of PPAR $\delta$  activators in the treatment and development of various tumor entities. Recently, Bility *et al.* demonstrated that PPAR $\delta$  agonists inhibit chemically induced skin carcinogenesis, whereas Kim *et al.* demonstrated that chemically induced skin carcinogenesis is not influenced by PPAR $\delta$  (29, 30). Han *et al.* demonstrated that PPAR $\delta$  agonists induce human lung cancer cell proliferation, whereas He *et al.* found that they do not influence lung cancer cell proliferation (31, 32). It is quite possible that the activation status of the cancer cells or the cancer micro-



**FIGURE 7. Inhibition of MAPK signaling pathways reduces IL-8 mRNA stability.** *Top*, HUVECs were incubated with vehicle, L165041 (50  $\mu$ M), SB203580 (30 mM), and L165041 + SB203580 for 30 min, followed by incubation with fresh medium containing actinomycin D (10  $\mu$ g/ml) for 0, 1, and 4 h. RT-PCR analyses for IL-8/GAPDH of total RNA extracted from subconfluent cell cultures were performed. The PCR products were separated by 2% agarose gel electrophoresis, and ethidium bromide-stained bands were visualized using an ultraviolet transilluminator. IL-8 bands were quantified by densitometric scanning, the results of which were normalized to amounts of GAPDH mRNA. Comparable results were obtained from three independent experiments. *Bottom*, HUVECs were incubated with vehicle, L165041 (50  $\mu$ M), PD98059 (30 mM), and L165041 + PD98059 for 30 min, followed by incubation with fresh medium containing actinomycin D (10  $\mu$ g/ml) for 0, 1, and 2 h. RT-PCR analyses for IL-8/GAPDH of total RNA extracted from subconfluent cell cultures were performed. The PCR products were separated by 2% agarose gel electrophoresis, and ethidium bromide-stained bands were visualized using an ultraviolet transilluminator. IL-8 bands were quantified by densitometric scanning, the results of which were normalized to amounts of GAPDH mRNA. Comparable results were obtained from three independent experiments.

environment, including macrophages, fibroblasts, endothelial cells, and others, influences the way PPAR $\delta$  exerts its effects.

It was recently demonstrated that PPAR $\delta$  activation induces endothelial cell proliferation, VEGF production, and angiogenesis (33). These results are consistent with those of our study; however, we employed quiescent cells instead of cytokine-induced endothelial cells. We showed that PPAR $\delta$  activation led to a proangiogenic and therefore a proinflammatory effect and that PPAR $\delta$  activation led to the induction of VEGF expression. The induction of the proinflammatory and proangiogenic cytokine IL-8 by PPAR $\delta$  agonists supports these results, as Li *et al.* demonstrated that IL-8 enhances endothelial cell survival and proliferation, regulates the production of matrix metalloproteinases, and can induce angiogenesis (18).

Aside from the mechanism involving the conveying of NF $\kappa$ B, we demonstrated an influence of PPAR $\delta$  on the mRNA stability of IL-8. The regulation of IL-8 mRNA stability is a well accepted and important mechanism of IL-8 mRNA expression in various cell types and conditions (11, 34, 35). Many authors have demonstrated that the p38

MAPK signaling pathway, and to a minor extent ERK1/2, control IL-8 mRNA stability (13, 35–37). Here, we also demonstrated that the induction of p38 and ERK1/2 by PPAR $\delta$  agonists resulted in an increase in IL-8 mRNA stability and therefore IL-8 production that could be partially abolished by inhibiting the MAPK signaling pathway. A comparable mechanism has already been described for a PPAR $\alpha$  agonist. Ren *et al.* demonstrated that the activation of PPAR $\alpha$  led to reduced mRNA stability and therefore a decrease of nephrin expression in kidney epithelial cells (23). Interestingly, PPAR $\delta$  agonists activated Akt but failed to influence IL-8 expression by this signaling pathway.

Taken together, we demonstrated that in nonstimulated quiescent HUVECs, treatment with PPAR $\delta$  agonists resulted in an NF $\kappa$ B-dependent transcriptional and posttranscriptional MAPK signaling pathway-dependent induction of IL-8 expression. These novel findings reveal that the effects of PPAR $\delta$  agonists may depend on the activation status of endothelial cells and that this status is responsible for a more pro- or antiinflammatory action. These findings may influence the development of PPAR $\delta$  agonists for clinical use.

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