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PPARα and PPARδ activators inhibit cytokine-induced nuclear translocation of NF-κB and expression of VCAM-1 in EAhy926 endothelial cells

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Abstract

Endothelium injury is a primary event in atherogenesis, which is followed by monocyte infiltration, macrophage differentiation, and smooth muscle cell migration. Peroxisome proliferator-activated receptors (PPARs) are transcription factors now recognized as important mediators in the inflammatory response. The aim of this study was to develop a human endothelial model to evaluate anti-inflammatory properties of PPAR activators. PPAR proteins (α, δ and γ) are expressed in EAhy926 endothelial cells (ECs). Pirinixic acid (Wy-14643), fenofibrate, fenofibric acid, the Merck ligand PPARδ activator L-165041, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, but not rosiglitazone (BRL-49653) inhibited the induced expression of vascular cell adhesion molecule-1 (VCAM-1), as measured by enzyme linked immunosorbent assay (ELISA), and monocyte binding to activated-EAhy926 cells. The PPARδ activator L-165041 had the greatest potency to reduce cytokine-induced monocyte chemotactic protein-1 (MCP-1) secretion. All PPAR activators tested which impaired VCAM-1 expression reduced significantly nuclear p65 amount. These results show that EAhy926 endothelial cells are an adequate tool to substantiate and characterize inflammatory impacts of PPAR activators.

Keywords: Atherosclerosis; Inflammation; PPAR (peroxisome proliferator-activated receptor); NF-κB (nuclear factor-κB); EAhy926 cell

1. Introduction

Vascular endothelial cells play an active role in many immune and inflammatory diseases such as atherosclerosis and rheumatoid arthritis (Russel, 1993). These cells regulate monocyte adhesion to blood vessels and their subsequent migration to underlying tissue. This process is facilitated by endothelial adhesion molecules and chemokine secretion which are regulated by inflammatory mediators such as cytokines (Chapman and Haskard, 1995; Cines et al., 1998). Multiple genes involved in monocyte–endothelial interaction (vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1), for example) contain in their promoters nuclear factor-κB (NF-κB) binding sites (Collins et al., 1995). NF-κB activity is dependent of reactive oxygen intermediate generation and is controlled by a series of associated proteins and inhibitors, among them inhibitor of κB. Thus, the development of therapeutic agents focused on the reduction of chronic inflammation genes (VCAM-1 and MCP-1) represents an attractive area of cardiovascular research for the next decade.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily, which can be activated by fatty acids, metabolites of arachidonic acid and synthetic molecules such as fibrates and thiazolidinediones (Willson et al., 2000). Three PPAR subtypes, designated PPARα, PPARδ and PPARγ, have been described (Desvergne and Wahli, 1999). PPARs promote transcription by forming heterodimers with members of the retinoid X receptor family and binding to specific DNA motifs termed PPAR-response elements (PPRE). PPARα is abundant in liver where it regulates metabolism of fatty acids. PPARδ is the most widely distributed subtype and has been shown to promote proliferation of adipocyte precursor cells (Hansen et al., 2001) and to increase reverse cholesterol transport

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(Oliver et al., 2001). PPARγ is predominantly seen in adipose tissue where it plays a critical role in adipocyte differentiation. PPAR activators have also anti-inflammatory properties by interfering negatively with other transcription factor pathways such as NF-κB, signal transducers and activators of transcription (STAT), and activator protein-1 (AP-1) (Desvergne and Wahli, 1999).

Vascular endothelial cells contain PPARα, PPARδ and PPARγ (Inoue et al., 1998; Xin et al., 1999). PPARα agonists decrease cytokine-induced VCAM-1 endothelial expression and limit the subsequent adherence of monocytes (Marx et al., 1999a; Jackson et al., 1999; Pasceri et al., 2000) by inhibiting NF-κB. In contrast, PPARγ activators appear to have both potential protective and detrimental effects on endothelial cells by respectively inhibiting endothelin-1 release and promoting plasminogen activator inhibitor expression (Delerive et al., 1999; Marx et al., 1999b).

Primary human umbilical vein endothelial cells represent a suitable model to address leukocyte–endothelial cell (EC) interactions and related mechanisms but are difficult to obtain and have a short life-span. These major problems could be overcome using a human endothelial cell line that retains normal adhesive properties. The aim of this investigation was to develop an endothelial cell model to evaluate anti-inflammatory properties of PPAR activators. We show that PPAR proteins (α, δ and γ) are expressed in EAhy926 endothelial cells and are functional since PPARα and PPARδ activators inhibit tumor necrosis factor-α (TNF-α) induced endothelial inflammation (VCAM-1 expression, monocyte adhesion and MCP-1 secretion). Moreover, our study demonstrates that PPARs anti-inflammatory effects are mediated at least in part by interfering negatively via the NF-κB signaling pathway.

2. Materials and methods

2.1. Antibodies and reagents

Primary monoclonal antibodies to human VCAM-1 (clone 1G11), intercellular cell adhesion molecule-1 (ICAM-1) [clone 84H10], vascular endothelial-cadherin (VE-cadherin) [clone TEA 1/31], platelet endothelial cell adhesion molecule-1 (PECAM-1) [clone 1F11], very late antigen (VLA-4) [clone HP2/1], VLA-5 (clone SAM1) were purchased from Beckman Coulter Immunotech. Primary antibodies against NF-κB complex (rabbit polyclonal antibody to p65 clone C-20, rabbit polyclonal antibody to inhibitor of κB clone C-21) and against PPARα (clone N-19), γ (clone N-20), δ (clone N-20) were purchased from TEBU (Transduction Laboratories).

Purified mouse and rabbit immunoglobulins G (IgGs) [Sigma] from normal animals were used as irrelevant antibodies in ELISA experiments.

Secondary peroxidase-linked antibody against mouse (NA 931) and rabbit IgGs (NA 934) were purchased from Amersham and used in ELISA and Western blot experiments.

Human recombinant TNF-α (expressed in *Escherichia coli*, 10^8 U/mg) was purchased from Boehringer.

Fenofibrate, fenofibric acid, Wy-14643, BRL-49653 (rosiglitazone), L-165041 were synthesized or extracted by a Medicinal Chemistry Division of Pierre Fabre Center. 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 was provided by ICN Chemicals.

All culture medium (except for human umbilical vein endothelial cells) and additives came from Gibco BRL.

2.2. Cell culture

The human endothelial cell line EAhy926 was obtained from Dr. E. Dejana (Mario Negri Institute, Milano, Italy) and cultured in Dulbecco's modified eagle's (DME) medium supplemented with 10% fetal bovine serum, antibiotics and in presence of 2% hypoxanthine, aminopterin and thymidine (HAT). This line resulted from the fusion between human umbilical vein endothelial cells and the nonendothelial lung carcinoma A549/8 cell line (Edgell et al., 1983). EAhy926 cells were subcultured weekly at a split ratio 1:10 and were used up to passage 20.

Human umbilical vein endothelial cells were purchased from Clonetics (BioWhittaker), cultured in EGM-2 medium (Clonetics) and used between passage 3 and 4.

Human monocytic cell line U937 was obtained from American Type Culture Collection (ATCC), and maintained in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics.

In each following protocols, endothelial cells were treated with compound or solvent [0.1% dimethylsulfoxide (DMSO)] for 2 h in 2% fetal bovine serum medium prior to the addition of TNF-α for 24 h.

2.3. Endothelial cell viability

Endothelial cell viability was controlled by cell numeration (24-well plate, in duplicate) using a Coulter Counter®.

Lactate deshydrogenase (LDH) release assay (Cytotox 96® Assay, G1780, Promega) was used to measure cell death (96-well plate, in triplicate). Maximum LDH release was evaluated by adding lysis solution during the latest hour of treatment. At the end of treatment, culture mediums were collected and centrifuged (1500 × *g*, 10 min, 15 °C). Supernatants (50 μl) were transferred to enzymatic assay plate for LDH quantification. LDH measurement was performed by adding 50 μl of substrate mix/well. After a 30-min incubation, reaction was stopped by Stop Solution and $O.D._{490\ nm}$ measured on a microplate reader (Molecular Devices). Compound toxicity was then determined by comparison with standard curves of $O.D._{490\ nm}$/percentage of maximum release and calculated as follow: % Cytotoxicity=[{LDH Release (TNF-α + Compound) − LDH Release (TNF-α + DMSO) − Spontaneous LDH Release}/Maximum LDH Release] × 100.

2.4. Adhesion molecule expression

Cell-surface expression adhesion molecule levels were determined by a cell-based ELISA assay on viable endothelial cells. After each incubation step, cellular monolayer integrity was ensured by phase-contrast microscopy. Confluent 96-well plate ECs were pretreated with compounds or solvent and stimulated with TNF-α. Cell monolayers were washed twice with DMEM plus 2% fetal bovine serum containing 0.01% NaN_3 to prevent antibody internalization. Then, primary mouse anti-human antibody against VCAM-1, ICAM-1 or non-immune IgG (100 μl at 4 μg/ml in DMEM 2% fetal bovine serum) was incubated for 1 h at 37 °C. Monolayers were washed twice with DMEM plus 2% fetal bovine serum and incubated for 1 h at 37 °C with a horseradish peroxidase-conjugated anti-mouse antibody (100 μl at 4 μg/ml in DMEM 2% fetal bovine serum). The amount of cell adhesion molecules was quantified through a colorimetric method (lecture at 480 nm) using phosphate-citrate buffer with urea hydrogen tablets and *o*-phenylenediamine dihydrochloride (OPD) as substrates. Specific cell ELISA values expressed as $O.D._{480\ nm}$ (adhesion molecule) − $O.D._{480\ nm}$ (non-specific binding) were determined after a 20-min incubation at room temperature.

2.5. MCP-1 assay

The levels of immunoreactive MCP-1 were quantified by ELISA using a commercially available sandwich-type ELISA (Beckman Coulter Immunotech IM3154). Between each incubation step, wells were washed 4 times. Dilutions of cell-free supernatants or MCP-1 standards (100 μl) were incubated in triplicate on MCP-1 antibody coated 96-wells, followed by the addition of a biotinylated MCP-1 antibody. After a streptavidin horseradish conjugate incubation, detection was performed using a substrate solution (Ultra Blue™). Color development was stopped with 2 M citric acid and the intensity of the color was measured at 450 nm.

2.6. Monocyte adhesion assay

U937 cells were labeled with 2′,7′-bis (2carboxyethyl)-5 (6)-carboxyfluorescein acethoxymethyl ester (BCECF/AM, Sigma, 1.5 μg/ml/5×10^6 cells) in RPMI 2% fetal bovine serum for 30 min at 37 °C. Dye loading was stopped by adding cold RPMI 1640, and cells were suspended in this medium containing 2% fetal bovine serum. Confluent endothelial monolayers in 24-well plates were incubated with 1.5×10^6 loaded U937 cells/well for 30 min at 37 °C. U937 suspensions were removed, and non-adherent monocytes were discarded by three successive washes. Cells were then lysed overnight in 0.1% Triton X-100/0.1 mol/l Tris pH 8.0. Following fluorescence measurement (excitation, 500 nm; emission, 530 nm; Perkin Elmer Luminescence Spectrometer 50B), adherent cells were determined by comparison with standard curves of BCECF/AM activity/cell as % adhesion of labeled cells. Data are representative of at least three experiments made in triplicate.

Some U937 cells were treated with saturating amounts of anti-very late antigen-4 (VLA-4) or anti-VLA-5 (irrelevant adhesion molecule) 30 min before addition and during adhesion of U937 as a control of VLA-4/VCAM-1 interaction.

2.7. Assessment of total protein synthesis

EC protein synthesis was determined by [^{35}S] methionine (70%) and [^{35}S] leucine (30%) incorporation. Briefly, six-well plate ECs were treated with compounds and activated in a specific long-term labeling medium (90% methionine cysteine free DMEM (Gibco) containing 2% dialysed fetal bovine serum (Sigma)/10% high glucose DMEM). ECs were pulsed overnight using 0.715 μCi/ml/well (Amersham Pro-Mix; 14.3 mCi/ml). Incorporation of radioactivity into total cellular protein was determined by trichloroacetic acid precipitation using bovine albumin as a carrier protein. Each six-well plate ECs were washed twice with cold phosphate-buffered saline (PBS) before a 30-min ice-incubation with bovine albumin (500 μl/well at 0.1 mg/ml) and 20% trichloroacetic acid (500 μl/well). The precipitates were transferred to glass microfiber filter discs (GF/C, Whatman) on a vacuum manifold and were washed successively with 10% trichloroacetic acid and absolute ethanol. Filter radioactivity was measured in a β scintillation analyser (Packard Tri-Carb 2300TR).

2.8. Western blot analysis of nuclear and cytoplasmic extracts

For NF-κB translocation experiments (p65), EAhy926 cells were treated for 3 h with compounds prior to a 30-min TNF-α activation. For inhibitor of κB-α studies, endothelial cells were stimulated by TNF-α for 5, 10 and 20 min.

After treatment, cells were washed with ice-cold PBS and lysed during 20 min in hypotonic *buffer A* [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol and protease inhibitors (Complete™ EDTA-free Protease Inhibitor Cocktail Tablets, Boehringer Mannheim)]. Each well was scraped and nuclei were pelleted by centrifugation (10 min, $13{,}000 \times g$, 4 °C), and the supernatants removed and assayed for protein content using a modified Bradford Coomassie assay (Pierce) before a − 80 °C storage as cytosolic proteins. Nuclear pellets were resuspended with *buffer B* [20 mM HEPES (pH 7.3), NaCl 420 mM, $MgCl_2$ 1.5 mM, EDTA 0.2 mM, 25% glycerol and protease inhibitors]. The nuclear debris were removed by centrifugation (10 min, $13{,}000 \times g$, 4 °C), and the resulting nuclear extracts were assayed for protein content. Nuclear proteins were finally stored at − 80 °C in aliquots containing 10 μg of protein for further Western blot analysis.

All protein samples (10 μg and 40 μg per lane for respectively nuclear and cytosolic extracts) were resolved on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels

overnight at 60 V and transblotted onto nitrocellulose membrane (Biorad) in 25 mM Tris, 192 mM glycine, SDS 0.1%, 20% methanol for 1 h. Electrophoretic transfer of proteins was checked by Ponceau S staining. Membrane was blocked with 10% low fat dried milk in PBS (blocking buffer). The blot was incubated for 1 h with the appropriate first antibody in blocking buffer with 0.1% Tween-20 and then for 1 h with horseradish peroxidase-labeled secondary antibody (Amersham). Between the various incubation steps, nitrocellulose membrane was washed three times with PBS containing 0.1% Tween-20. Immunoreactive antigens were revealed by enhanced chemiluminescence (ECL) and analysed on a PhosphorImager scanning (Molecular Dynamics). In some experiments, quantifications were performed using the software program Molecular Dynamics ImageQuant (no statistical tests were carried out because of critical steps as protein transfer and immunologic detection).

2.9. Statistical analysis

All data were expressed as mean ± S.E.M. Statistical significance was determined with Student–Newman–Keuls multiple comparison procedure applied after a one-way analysis of variance (ANOVA) conducted on data (P value of less than 0.05 was considered significant).

3. Results

3.1. PPARs are expressed in EAhy926 endothelial cells

Primary vascular endothelial cells were known to express PPARα, PPARδ and PPARγ (Inoue et al., 1998; Xin et al., 1999) proteins though, as yet no definitive roles for PPARδ have been described. PPAR isoforms were detected in EAhy926 human endothelial cell line by Western blot experiments (Fig. 1A, B and C). This expression appeared to be rather low and exclusively localised in nuclear fractions (PPARα > PPARγ>PPARδ). To avoid non-specific detection anti-PPAR antibodies specificity was checked using COS-7 cells transfected with human PPAR α, δ and γ (data not shown).

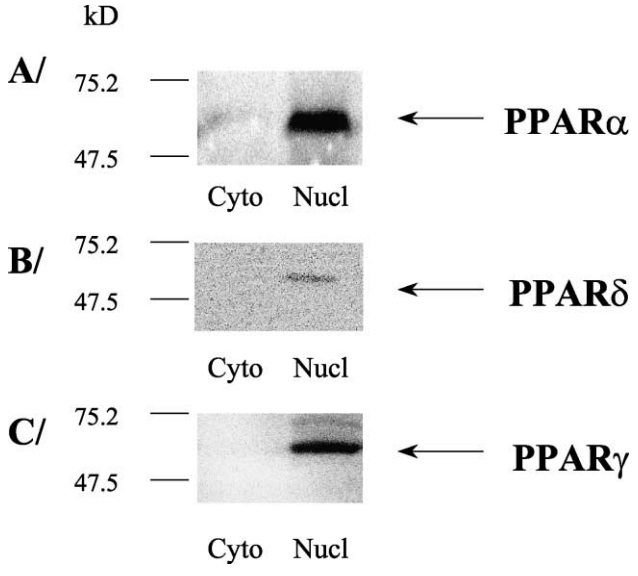

Fig. 1. Expression of PPAR proteins (α, δ and γ) in the nucleus of EAhy926 cells. PPARα (A); PPARδ (panel B); PPARγ (panel C).

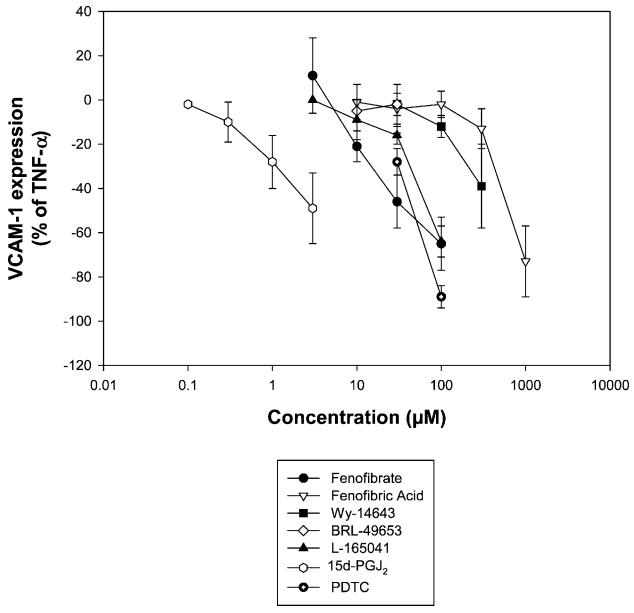

Fig. 2. Effect of PPAR activators on VCAM-1 expression in TNF-α activated EAhy926 cells. Results are representative of three independent experiments and are reported as mean ± S.E.M.

3.2. PPARα and PPARδ activators inhibit VCAM-1 but not ICAM-1 expression

Activation of EAhy926 cells with TNF-α 300 U/ml induced cell-surface expression of VCAM-1 and ICAM-1. Human endothelial cells were treated with PPARα activators (fenofibrate, fenofibric acid and Wy-14643), PPARγ activators (BRL-49653 and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2), PPARδ activator (L-165041) or with the radical-scavenging antioxidant pyrrolidine dithiocarbamate (PDTC) before a 24 h TNF-α activation and subsequent determination of VCAM-1 expression. PDTC was used as a well-known inhibitor of VCAM-1 expression (Weber et al., 1994).

As shown in Fig. 2, cytokine-induced VCAM-1 expression was reduced in a concentration-dependent manner by the three synthetic PPARα activators, the PPARδ activator L-165041 and the natural PPARγ activator 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2. In contrast, BRL-49653 was ineffective. The rank order of potency of PPAR agonists on VCAM-1 cell-surface expression was the following: 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2>fenofibrate>L-165041> Wy-14643>fenofibric acid. Regarding fenofibrate, it can

Table 1
Endothelial inflammation assay: IC_{30} of PPAR activators on VCAM-1 expression and on monocyte adhesion (EAhy926 cells and human umbilical vein endothelial cells)

	EAhy926			HUVEC	
	VCAM-1	ICAM-1	Adhesion	VCAM-1	ICAM-1
	IC_{30}, μM	IC_{30}, μM	IC_{30}, μM	IC_{30}, μM	IC_{30}, μM
Fenofibrate	16	>100	22	100	0
Fenofibric acid	388	0	462	$300 < IC_{30} < 1000$	0
Wy-14643	223	0	$200 < IC_{30} < 300$	$100 < IC_{30} < 300$	>300
BRL-49653	0	0	0	0	0
L-165041	37	0	44	$30 < IC_{30} < 100$	>100
15d-PGJ_2	1	0	1	$3 < IC_{30} < 10$	0

be hypothesised that EAhy926 endothelial cells contain active esterases to metabolize fenofibrate into fenofibric acid, and that fenofibrate was more potent than fenofibric acid due to differences in membrane permeability.

EAhy926 cells activated by TNF-α also expressed intracellular adhesion molecule-1 (ICAM-1) with a similar kinetic as VCAM-1. Nevertheless, ICAM-1 expression was unaffected by any of the compounds tested (Table 1) highlighting the specificity of PPAR activators among inducible adhesion molecules. Moreover, PPAR activators alone neither induced VCAM-1 or ICAM-1 expression nor affected adhesive protein expression or organization at cell to cell junctions (VE-cadherin and PECAM-1, data not shown) under resting and activated EAhy926 endothelial cells. Cell viability in all conditions tested was over 90% and all compounds did not affect total protein synthesis (Table 2) demonstrating that pharmacological activity was not related to toxic effects of agents at these relative high concentrations.

Table 2
Cell viability and total protein synthesis: effect of PPAR activators

Treatment	Cell viability (percentage of TNF-α)	Protein synthesis (percentage of TNF-α)
TNF-α 300 U/ml	100	100
TNF-α + fenofibrate 30 μM	>95	92 ± 12
TNF-α + fenofibrate 100 μM	90	85 ± 15
TNF-α + Wy-14643 30 μM	>95	96 ± 7
TNF-α + Wy-14643 300 μM	>95	82 ± 7
TNF-α + L-165041 30 μM	>95	100
TNF-α + L-165041 100 μM	>95	100
TNF-α + 15d-PGJ_2 3 μM	>95	100
TNF-α + PDTC 100 μM	>95	100
TNF-α + cycloheximide 1 μM	>95	51 ± 10

Values are mean ± S.E.M., $n = 3$.
Cell viability was determined by LDH release assay.
TNF-α 300 U/ml: 20% decrease of total protein synthesis (as compared to resting condition).

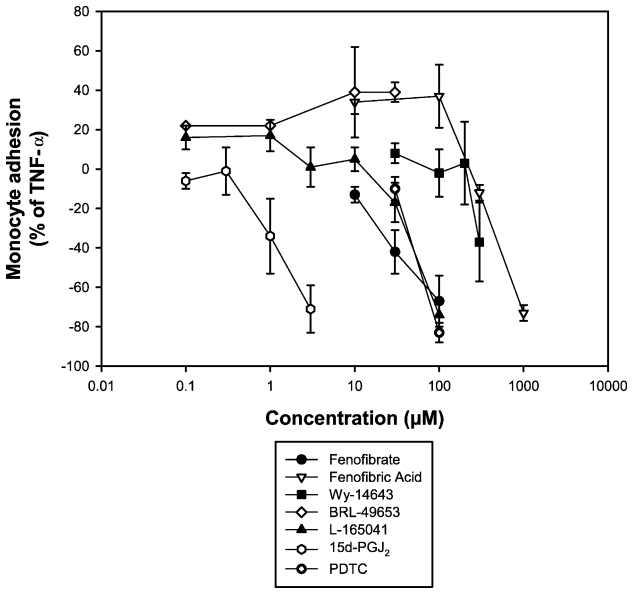

Fig. 3. Effect of PPAR activators on monocyte adhesion on TNF-α activated EAhy926 cells. Results are representative of three independent experiments and are reported as mean ± S.E.M.

Same experiments were performed using primary endothelial cells instead of EAhy926 cells to validate these results. PPAR IC_{30} in inhibiting VCAM-1 expression was comparable in both endothelial cell types (Table 1).

3.3. PPARα and PPARδ activators inhibit monocyte binding

Functional relevance of PPAR effects on VCAM-1 expression was confirmed by an *in vitro* adhesion assay using

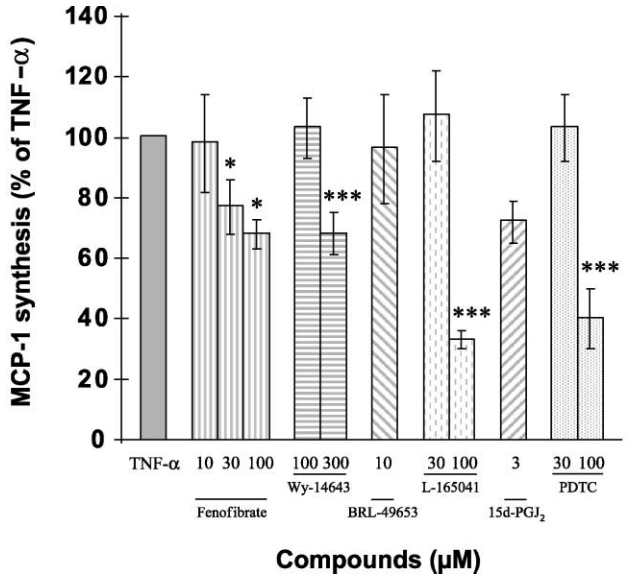

Fig. 4. Effect of PPAR activators on MCP-1 secretion in TNF-α activated EAhy926 cells. Results are representative of three independent experiments and are reported as mean ± S.E.M. * $P < 0.05$ (versus TNF-α); * * * $P < 0.001$ (versus TNF-α).

U937 monocytes. U937 cell line expresses VLA-4 which is the receptor for VCAM-1 ligand, but not CD11b (Mac-1), the counter receptor for ICAM-1. Activation of EAhy926 cells with TNF-α (300 U/ml) enhanced U937 adhesion up to 30% of monocyte added (about 2–3% in resting condition). This stimulation of monocyte binding was inhibited in a

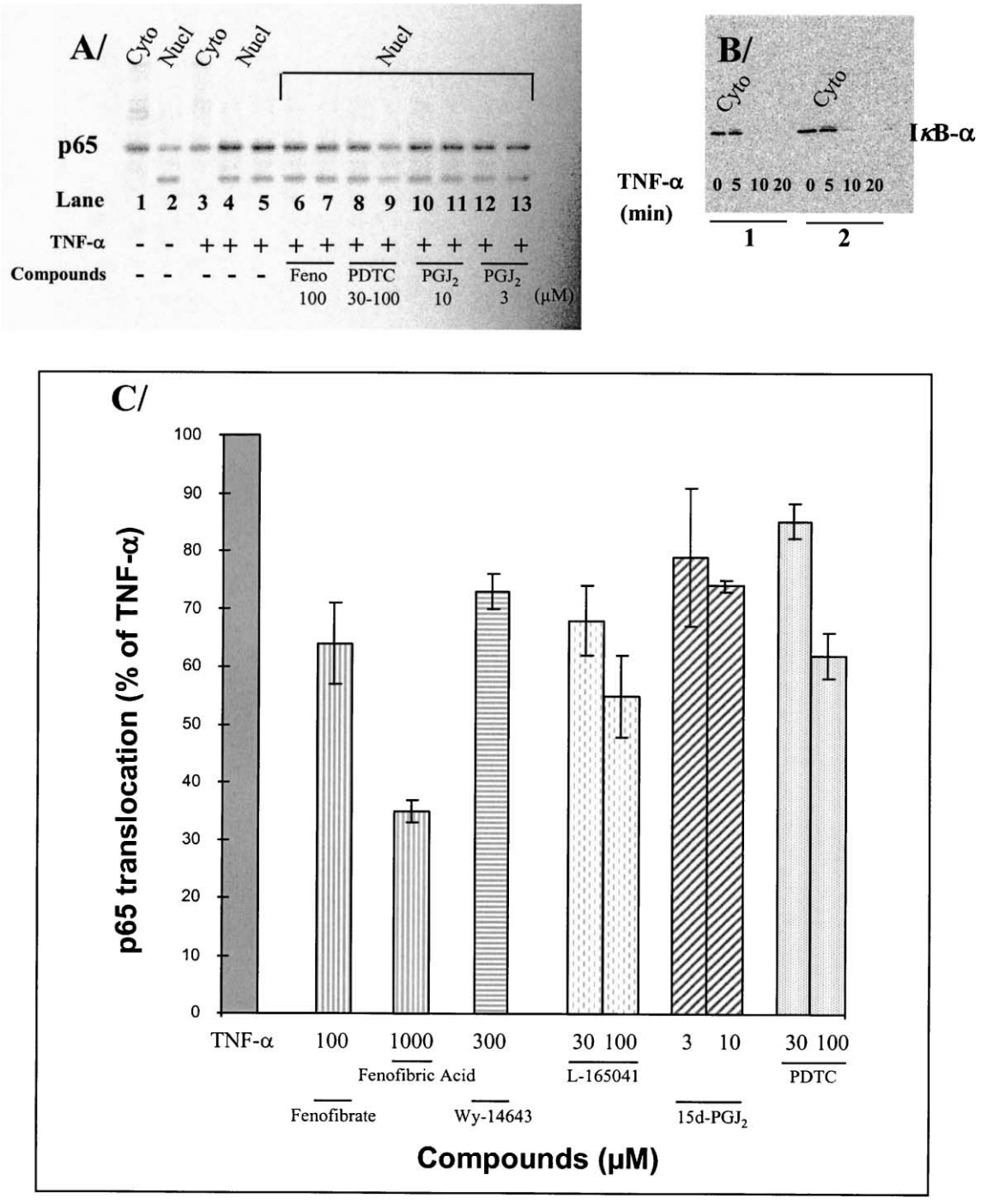

Fig. 5. Effect of PPAR activators on cytokine-induced NF-κB translocation. (A) Western blot analysis of NF-κB (p65) in nuclear (nucl) and cytoplasmic (cyto) fractions of EAhy926 cells. Cells were pre-treated for 3 h with compounds before further TNF-α activated for 30 min. Lanes 1 and 2 (basal): p65 cyto (lane 1) and p65 nucl (lane 2); Lanes 3 to 13 (TNF-α): p65 cyto (lane 3); p65 nucl (lanes 4–5); p65 nucl fenofibrate 100 μM (lanes 6–7); p65 nucl PDTC 30 μM (lane 8) and 100 μM (lane 9); p65 nucl 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 10 μM (lanes 10–11) and 3 μM (lanes 12–13). (B) Western blot analysis of inhibitor of κB-α (IκB-α) in cytoplasm in EAhy926 cells. Cells were TNF-α activated for 5, 10 and 20 min (20 μg [condition 1] or 40 μg [condition 2] of cytoplasmic proteins). (C) Quantification of nuclear p65 in treated and activated EAhy926 cells. Three independent Western blot experiments were performed and the results obtained expressed as percentage of TNF-α p65 translocation.

concentration dependent manner with PPARα activators (fenofibrate, fenofibric acid and Wy-14643), PPARδ activator (L-165041) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (Fig. 3). These results were paralleled by those on VCAM-1 expression (Table 1) and were comparable in both EAhy926 cells and human umbilical vein endothelial cells (data not shown).

As described, VCAM-1 dependence of U937 cell adhesion was further confirmed by monocyte pre-incubation with blocking anti-VLA-4 monoclonal antibody versus anti-VLA-5 irrelevant monoclonal antibody. A strong inhibition in monocyte binding ($82 \pm 6\%$) was only obtained using anti-VLA-4 monoclonal antibody, whereas anti-VLA-5 monoclonal antibody treatment promoted adhesion consequently to "outside in" signal mediated by β_1 integrins.

3.4. PPAR activators down regulate cytokine-induced MCP-1

To investigate putative anti-migratory effects of PPAR activators, endothelial MCP-1 was quantified using a sandwich ELISA assay. EAhy926 endothelial cells were able to produce MCP-1 at physiological levels as compared to primary endothelial cells (Parry et al., 1998). MCP-1 production was very low in resting EAhy926 cells ($13 \pm 3 \times 10^{-4}$ pg/cell) and strongly enhanced after activation with TNF-α to reach 20×10^{-2} pg/cell (150 fold increase).

MCP-1 secretion was inhibited by 30% under treatment with PPARα and the natural 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 at high concentrations (Fig. 4). In contrast, PPARδ activator (L-165041) at 100 μM decreased up to 70% this chemokine secretion ($P < 0.001$ by Student–Newman–Keuls multiple comparison procedure).

3.5. PPAR activators inhibit NF-κB translocation to the nucleus

Nuclear translocation of NF-κB played a key role in vascular biology since several genes implicated in monocyte recruitment exhibited in their promoter binding sites for NF-κB (Collins, 1993). To determine whether the inhibitory effects of PPAR activators were due to an inhibition of nuclear translocation of NF-κB, Western blot analysis of NF-κB p65 protein levels in the nuclei were performed in EAhy926 cells. As shown in Fig. 5, TNF-α induced a strong translocation of p65 from the cytoplasm to the nucleus (panel A, lanes 1 to 5) and a rapid proteolysis of inhibitor of κB-α which became undetectable after a 20-min cytokine activation (panel B).

Immuno-bands of p65 were quantified using a PhosphorImager and the results obtained were expressed as percentage of TNF-α translocation (Fig. 5, panel C). As shown in Fig. 5, panel C, cytokine-induced translocation of p65 to the nucleus was decreased by pre-treatment with PPARα activators (fenofibrate, fenofibric acid and Wy-14643), PPARδ activator (L-165041) and endogenous 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2. All PPAR activators tested which inhibited VCAM-1 expression and monocyte adhesion to endothelial cells reduced nuclear p65 amount. In contrast, the specific and potent γ activator BRL-49653 was devoid of activity (data not shown). Thus, these results demonstrated that NF-κB signaling pathway was involved at least in part in PPAR anti-inflammatory effects in EAhy926 cells.

4. Discussion

The present study reports the expression of PPARα, δ and γ in the human endothelial cell line EAhy926 and the reduction of cytokine-induced VCAM-1 expression by PPARα and δ agonists through inhibition of NF-κB translocation. This inhibition of VCAM-1 expression decreased adhesion of monocyte-like cells to stimulated ECs.

Natural 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 appears to have the greatest potency to reduce monocyte recruitment than any of the other molecules tested. This difference may be related to the chemical structure of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 which may interact with an unknown cell surface receptor. Moreover, Straus et al. (2000) recently showed that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 inhibits inhibitor of κB kinase (IKK) the enzyme which phosphorylates inhibitor of κB after activation with cytokines and also affects the DNA-binding domains of NF-κB subunits. 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 contains a cyclopentenone ring system (Rossi et al., 2000; Willoughby et al., 2000) which is able at 100 μM (approximatively 30 fold higher than 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2) to inhibit by itself up to 55% TNF-α elicited VCAM-1 expression (data not shown). These results suggest that the potent repressive activity of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 is mediated at least in part by a PPARγ independent pathway in EAhy926 cells as described in other cell types (Moore et al., 2001). This hypothesis can also be supported by the lack of effect of BRL-49653 in VCAM-1 cell-surface expression.

Our results described for the first time that endothelial cells express a functional PPARδ since the PPARδ activator L-165041 can reduce monocyte recruitment by inhibiting VCAM-1 expression and MCP-1 secretion. Despite recent reports (Hansen et al., 2001; Oliver et al., 2001), PPARδ functions are not fully identified and these data suggest that it could play a role in the regulation of vascular inflammation. In common with the other subtypes, PPARδ is a receptor for naturally occurring saturated and unsaturated fatty acids. Among the polyunsaturated fatty acids, arachidonic acid and eicosapentaenoic acid (which has been co-crystalized with PPARδ) bound with affinities in the low micromolar range (Xu et al., 1999). As described for docosahexaenoic acid (Weber et al., 1995), chronic treatment of EAhy926 cells with PPARδ agonist L-165041 reduces VCAM-1 expression more severely (data not shown). It is of interest to point out that in contrast to PPARα activators or the natural 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 which inhibit MCP-1 secretion by 30%, L-165041 at 100 μM decreases TNF-induced MCP-1

secretion up to 70%. In conclusion, these results emphasize the putative anti-inflammatory effects of PPARδ activator which impairs at least two NF-κB-driven genes VCAM-1 and MCP-1.

MCP-1 CC chemokine was clearly detected in atherosclerotic lesions by anti-MCP-1 antibody (Takeya et al., 1993) and *in situ* hybridization (Nelken et al., 1991); its production by various cell types including vascular endothelial cells was enhanced by modified low-density lipoprotein, cytokines and macrophage-colony stimulating factor (Sica et al., 1990). Our results are consistent with Marx et al. (2000) who have shown that specific PPARγ activators (as BRL-49653) do not inhibit MCP-1 secretion by endothelial cells. Moreover, it has been demonstrated that PPARγ agonist treatment of LDL-receptor null mice had no effect on MCP-1 expression (Li et al., 2000; Lee et al., 2000). MCP-1 receptor named CCR2 has been suggested as a PPARγ down-regulated gene on monocyte (Han and Quehenberger, 2000) which may lead to a decreased MCP-1-related chemotaxis upon PPARγ agonists treatment (Kintscher et al., 2000). Despite MCP-1 probably plays a major role on monocyte as compared to endothelial cell, it would be worthwhile to test whether the EAhy926 cell line does express CCR2, even in a minor extent as described for primary endothelial cells. We were able to detect CCR2 expression on EAhy926 endothelial cells, but only by Western blot experiments (data not shown). As a consequence of the low level of CCR2 expression in EAhy926 endothelial cells as compared to monocytes, we did not investigate any PPAR-mediated regulation.

EAhy926 cell line is a continuous, clonable, human endothelial cell line that displays a number of features characteristic of vascular endothelial cells (Edgell et al., 1983). EAhy926 cells exhibit the same adherent properties after cytokine activation as freshly isolated human umbilical vein endothelial cells (Thornhill et al., 1993), retain the capacity to give rise to new capillaries and can be an useful model for *in vitro* study of angiogenic process (Bauer et al., 1992). Moreover, they express all PPAR isoforms and appear to be a valuable and relevant research tool for investigating mechanisms of adhesion molecule expression and inhibition by various physiological classes of drugs (PPAR agonists, antioxidants, prostanoids). EAhy926 cells also produced MCP-1 at levels comparable to those produced by human umbilical vein endothelial cells both under basal and stimulated condition (25×10^{-4} and 20×10^{-3} pg/cell, respectively; Parry et al., 1998) and can be used as a model in compound evaluation.

Thus, we have shown that PPARα and PPARδ activators as exemplified by fenofibrate and L-165041, respectively, are able to inhibit vascular cell adhesion molecule expression *in vitro*, while having no effect on "house-keeping proteins". Nevertheless, each active compound identified has to be carefully evaluated in clinical studies to definitively determine whether it has beneficial effects in treating inflammatory vascular diseases.

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