Research Paper

Journal of Vascular Research

J Vasc Res 2005;42:509–516
DOI: 10.1159/000088260

Received: January 18, 2005
Accepted after revision: June 21, 2005
Published online: September 9, 2005

Inhibition of Tumor-Necrosis-Factor-α Induced Endothelial Cell Activation by a New Class of PPAR-γ Agonists

An in vitro Study Showing Receptor-Independent Effects

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Key Words
Adhesion molecules · Atherosclerosis · Endothelial cells · Inflammation · PPAR-γ

Abstract
Proinflammatory cytokines and adhesion molecules expressed by endothelial cells (ECs) play a critical role in initiating and promoting atherosclerosis. Agents that oppose these inflammatory effects in vascular cells include peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands, including 15-deoxy-$\delta^{12,14}$-prostaglandin J2 (15d-PGJ2) and synthetic thiazolidinediones. Recently, a new structural class of potent PPAR-γ agonists, 1,1-bis(3′-indolyl)-1-(*p*-substituted phenyl) methanes, has been characterized. The purpose of this study was to evaluate the anti-inflammatory effects of two PPAR-γ-active members of this class, 1,1-bis(3′-indolyl)-1-(*p*-t-butylphenyl)-methane (DIM-C-pPhtBu) and 1,1-bis(3′-indolyl)-1-(*p*-biphenyl)methane (DIM-C-pPhC$_6$H$_5$), in ECs in vitro. Pretreatment of ECs with DIM-C-pPhC$_6$H$_5$, DIM-C-pPhtBu, or 15d-PGJ2 decreased tumor necrosis factor-α (TNF-α)-induced intercellular adhesion molecule (ICAM)-1 expression in a concentration-dependent manner. At a concentration of 10 μ*M*, DIM-C-pPhtBu and DIM-C-pPhC$_6$H$_5$ decreased ICAM-1 expression by 77.5 and 71.3%, respectively, and comparable inhibition (84.4%) was observed for 10 μ*M* 15d-PGJ2 ($p < 0.05$). In contrast, 10 μ*M* ciglitazone and DIM-C-pPhCH$_3$, which exhibits low PPAR-γ agonist activity, were inactive. The two new PPAR-γ agonists and 15d-PGJ2 also inhibited TNF-α-induced interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) production in supernatants of TNF-α-stimulated ECs, whereas ciglitazone and DIM-C-pPhCH$_3$ did not decrease TNF-α-induced expression of these two proteins. This new structural class of PPAR-γ agonists inhibited the expression of ICAM-1 and the production of IL-6 and MCP-1 in TNF-α-activated ECs at lower concentrations than other synthetic PPAR-γ agonists, suggesting the potential clinical utility of 1,1-bis(3′-indolyl)-1-(*p*-substituted phenyl) methanes for decreasing endothelial inflammation.

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Introduction

Adhesion of leukocytes to vascular endothelial cells (ECs) is a critical step in the development of atherosclerosis and involves recruitment of leukocytes to the site of

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tissue injury or lesion formation and their infiltration into the vessel wall. There are several cytokines involved in this process. One important cytokine in this process is the intercellular adhesion molecule-1 (ICAM)-1, which is expressed on ECs and is one of the major cell surface glycoproteins that promote cell adhesion [1, 2]. Although ICAM-1 is constitutively expressed in ECs, levels of this protein can be significantly raised in response to proinflammatory mediators, such as tumor necrosis factor-α (TNF-α) [3], which may further contribute to the role of ICAM-1-mediated atherosclerosis. Specific chemokines, particularly monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6), which are expressed by ECs, also play a major role in the development of atherosclerosis. Another important protein involved in the pathogenesis of atherosclerosis is peroxisome proliferator-activated receptor-γ (PPAR-γ), a ligand-activated nuclear receptor that has an essential role in adipogenesis and glucose homeostasis and is expressed in atherosclerotic plaques [4]. PPAR-γ is also expressed in vessel wall tissues, including ECs [5]. Although the role of PPAR-γ in inflammation, and in particular its role in the activation of ECs, is unclear, it is possible that ligand-dependent activation of PPAR-γ might constitute an effective strategy for managing atherosclerosis.

Recently, we reported the mode of action of 1,1-bis(3′-indolyl)-1-(*p*-trifluoromethylphenyl) methane (DIM-C-pPhCF_3) and related compounds which inhibit cancer cell growth; these chemicals were characterized as a new class of PPAR-γ agonists that resemble the natural ligand 15-deoxy-$\delta^{12,14}$-prostaglandin J2 (15d-PGJ2), in MCF-7 breast, pancreatic, and colon cancer cell lines [6–8]. Given the possible role of PPAR-γ in the pathogenesis of atherosclerosis, we hypothesized that this new structural class of PPAR-γ agonists might also be effective in opposing the inflammation associated with atherosclerosis.

We therefore assessed the effects of this new class of PPAR-γ agonists on vascular inflammation by investigating the expression of selected chemokines/cytokines, such as IL-6, MCP-1, and ICAM-1, following EC activation by TNF-α.

Methods

Chemical and Cell Culture

Human umbilical vein ECs (HUVECs, Cascade Biology, Portland, Oreg., USA) were grown in M199 medium (Gibco, Carlsbad, Calif., USA), 15% fetal bovine serum (Sigma, St. Louis, Mo., USA), 0.2 mg/ml heparin, 0.1 mg/ml EC growth supplement (Biomedical Technologies, Stoughton, Mass., USA), 2 m*M* *L*-glutamine, and 1% penicillin/streptomycin until 70% confluent. Cells from passages 2–4 were used in the experiments. The *p*-substituted phenyl DIM analogs containing *p*-t-butyl (DIM-C-pPhtBu), *p*-phenyl (DIM-C-pPhC_6H_5), and *p*-methyl (DIM-C-pPhCH_3) substituents used in the study were >95% pure and were prepared by the condensation of indole with the corresponding *p*-substituted benzaldehydes. DIM-C-pPhC_6H_5 and DIM-C-pPhtBu are active agents, as shown by earlier structure-activity studies, whereas DIM-C-pPhCH_3 is a relatively inactive PPAR-γ agonist [6]. 2-Chloro-5-nitro-N-phenylbenzamide (GW9662) was purchased from Tocris Bioscience (Ellisville, Mo., USA).

Detection of ICAM-1

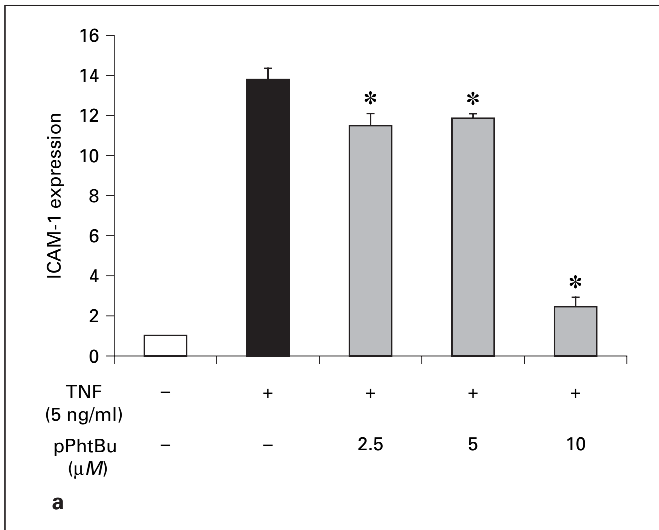
The expression of ICAM-1 on the cell surface was determined in HUVECs cultured in six-well plates pretreated with one of the three different *p*-substituted phenyl DIM analogs or with vehicle (0.1% DMSO) at the concentrations indicated. Their effects were compared with those of other PPAR-γ agonists by preincubating HUVECs with ciglitazone (Biomol, Plymouth Meeting, Pa., USA) or 15d-PGJ2 (Calbiochem, San Diego, Calif., USA) at the same doses. After 6 h, cells were incubated with TNF-α (R&D Systems, Minneapolis, Minn., USA) at a concentration of 5 ng/ml for 12 h according to previous publications. Cells were then detached with 10 m*M* EDTA in 0.5% phosphate-buffered saline, collected by centrifugation, and stained for 30 min on ice in the dark with *R*-phycoerythrin-labeled monoclonal antibody against ICAM-1 (CD54) or with the appropriate *R*-phycoerythrin-labeled isotype IgG (Pharmingen, San Diego, Calif., USA) as a control. The fluorescence intensity of 10,000 gated viable cells was analyzed for each sample on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Diego, Calif., USA) using Cell Quest (Becton Dickinson) acquisition software. All experiments were performed in triplicate. For the inhibition studies with GW9662, the PPAR-γ antagonist was added 30 min before the addition of the DIM compound.

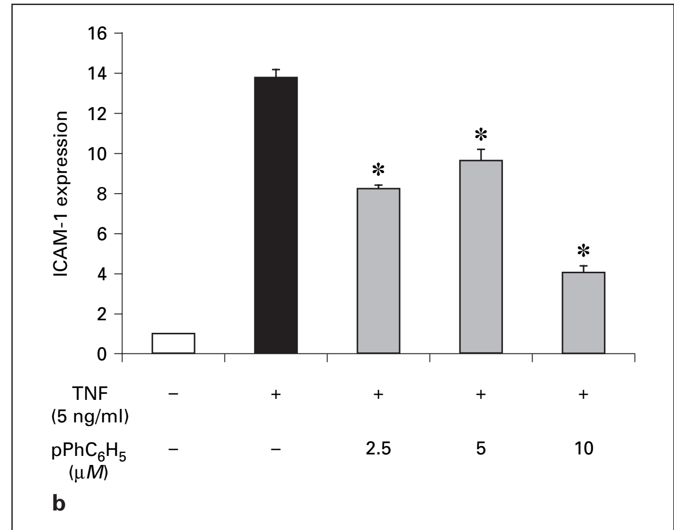
Chemokine and Cytokine Assays

In order to measure chemokine/cytokine levels in the cell supernatant, HUVECs cultured in 24-well plates were preincubated for 6 h with one of the three *p*-substituted phenyl DIM analogs at the concentrations indicated or with vehicle and then stimulated with 5 ng/ml TNF-α. For comparison, HUVECs were also preincubated with ciglitazone or 15d-PGJ2 at the same concentrations and then stimulated with TNF-α at a concentration of 5 ng/ml. Cell culture supernatants were collected 6 and 24 h after the stimulation for analysis of IL-6 and MCP-1, respectively. These time points were selected according to previous reports. The levels of IL-6 and MCP-1 were quantified using commercial ELISA kits (BioSource, Camarillo, Calif., USA) according to the manufacturer's directions. The minimum detectable concentration of the assay was 2 pg/ml for IL-6 and <20 pg/ml for MCP-1. All experiments were performed in at least three replicate determinations for each treatment group. For the inhibition studies with GW9662, the PPAR-γ antagonist was added 30 min before the addition of the DIM compound.

Statistical Analysis

Data are reported as means ± SD. Differences were analyzed by ANOVA followed by the Fisher least significant difference test. A p value of <0.05 was considered significant.





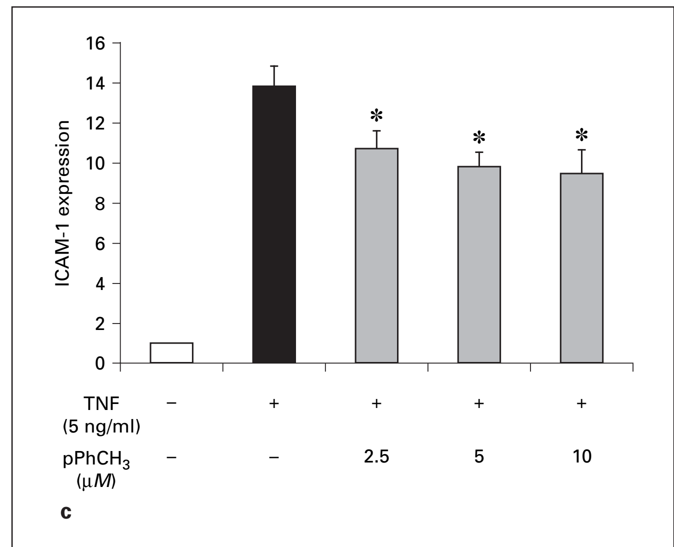


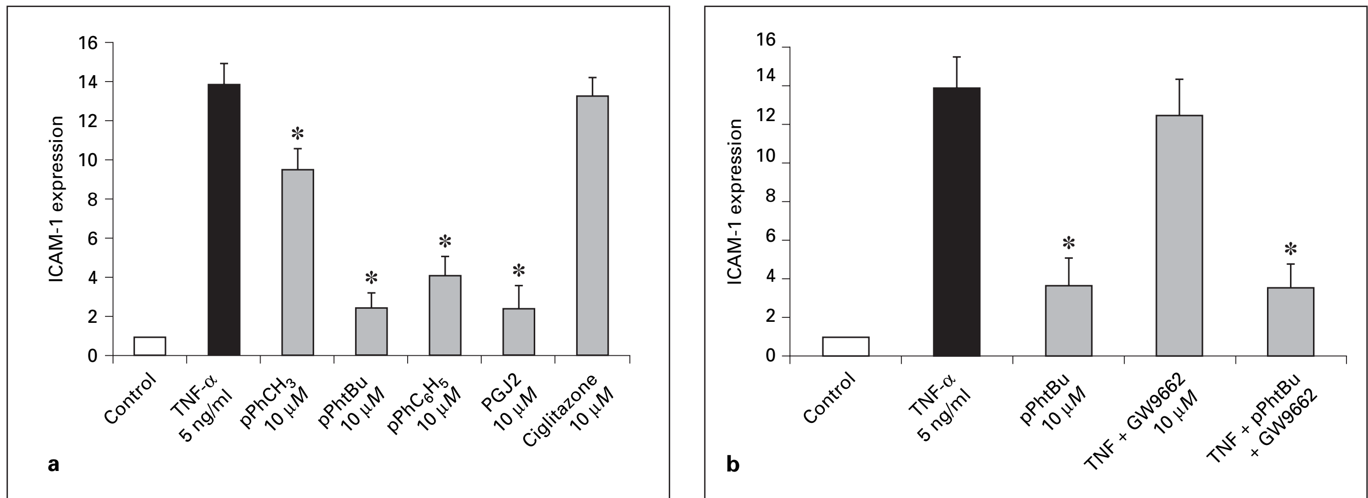
Fig. 1. Effects of three members of the new class of PPAR-γ agonists on the TNF-α-induced expression of ICAM-1 (median of intensitivity/control) in HUVECs. Cells were pretreated with DIM-C-pPhtBu (**a**), DIM-C-pPhC$_6$H$_5$ (**b**), or DIM-C-pPhCH$_3$ (**c**) at the concentrations shown for 6 h and then incubated with 5 ng/ml TNF-α for 12 h. Cell surface expression of ICAM-1 was measured by FACS. Data are expressed as the mean ± SD of a representative experiment performed in triplicate. * $p < 0.05$.

Results

Effect of p-*Substituted Phenyl DIM Analogs on ICAM-1 Expression in HUVECs*

HUVECs expressed low basal levels of ICAM-1. Similarly, treatment with different concentrations (up to 10 μ*M*) of one of the three *p*-substituted phenyl DIM analogs, ciglitazone, or 15d-PGJ2 did not induce apoptosis or change the baseline expression of ICAM-1 (data not shown). In contrast, incubation of HUVECs with 5 ng/ml TNF-α for 12 h significantly increased the expression of ICAM-1. Conversely, pretreatment of HUVECs with DIM-C-pPhtBu (fig. 1a) decreased the expression of ICAM-1 in a concentration-dependent manner. In particular, 10 μ*M* DIM-C-pPhtBu maximally reduced the expression of ICAM-1 by 77.5%. DIM-C-pPhC$_6$H$_5$ had a similar effect (fig. 1b), with a maximal reduction in ICAM-1 expression of 71.3% observed for a dose of 10 μ*M* ($p < 0.05$). However, pretreatment with 10 μ*M* DIM-C-pPhCH$_3$ (fig. 1c) induced only a small, but significant 32% decrease in the expression of TNF-α-induced ICAM-1. This order of potency – DIM-C-pPhtBu ≅ DIM-C-pPhC$_6$H$_5$ > DIM-C-pPhCH$_3$ – parallels the relative PPAR-γ agonist activities of these compounds observed in transactivation assays [6].

On the basis of these results, we chose 10 μ*M* as the concentration for the comparison experiments examining other PPAR-γ agonists. These experiments showed



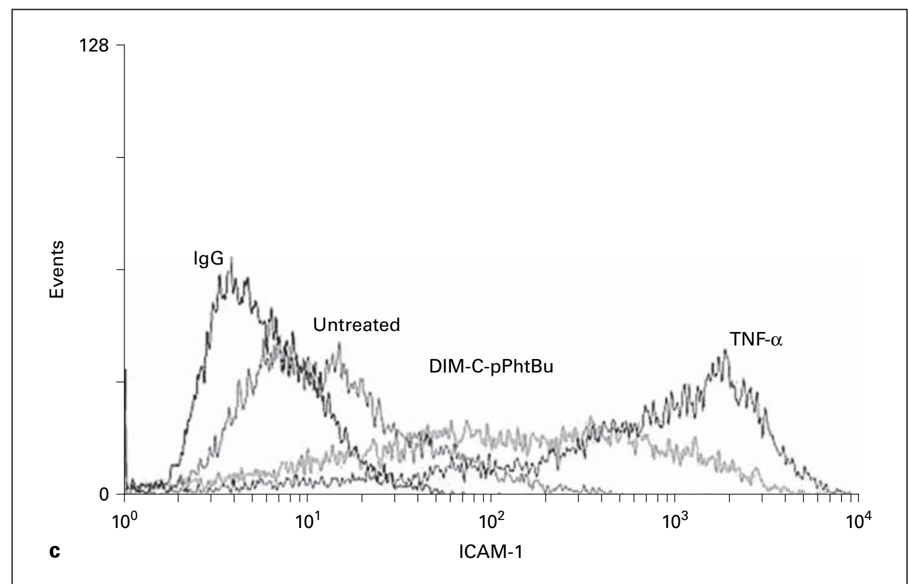


Fig. 2. Comparison of the effects of different PPAR-γ agonists and GW9662 on TNF-α-induced expression of ICAM-1 (median of intensitivity/control) in HUVECs. **a** Inhibition of TNF-α-induced ICAM-1: Cells were pretreated with 10 μ*M* DIM-C-pPhCH$_3$, DIM-C-pPhtBu, DIM-C-pPhC$_6$H$_5$, 15d-PGJ2, or ciglitazone for 6 h and then incubated for 12 h with 5 ng/ml TNF-α. Cell surface expression of ICAM-1 was measured by FACS. **b** Effects of GW9662. Experiments were carried out as described in **a**; however, 10 μ*M* GW9662 was added 30 min prior to DIM-C-pPhtBu. Data are expressed as the mean ± SD of a representative experiment performed in triplicate. * $p < 0.05$. **c** A representative histogram of the FACS analysis experiment using DIM-C-pPhtBu.

that pretreatment with 15d-PGJ2 was associated with a significant (i.e. 84.4%) reduction in ICAM-1 expression compared with the untreated TNF-α-stimulated HUVECs. However, pretreatment with 10 μ*M* ciglitazone had no inhibitory effect on TNF-α-induced ICAM-1 expression in HUVECs (fig. 2a). Previous studies showed that the PPAR-γ-active DIM compounds and thiazolidinediones exhibit comparable activity in PPAR-γ-dependent transactivation assays (≥ 5 μ*M*) whereas the DIM compounds were more active in functional growth inhibition/differentiation induction assays [6, 7]. Previous studies with other structural classes of PPAR-γ agonists indicate that many of their anti-inflammatory effects in ECs are PPAR-γ independent [9]. The results illustrated in figure 2b show that the inhibition of TNF-α-induced ICAM-1 expression by DIM-C-pPhtBu was not reversed by the PPAR-γ antagonist GW9662. This suggested that the anti-inflammatory activity of the PPAR-γ-active DIMs is receptor independent, and this parallels with previous results obtained with 15d-PGJ2 [9]. Figure 2c shows a representative histogram of the FACS analysis experiment using DIM-C-pPhtBu.

Effects of PPAR-γ Agonists on IL-6 and MCP-1 Production by TNF-α-Stimulated HUVECs

To determine the effects of the three *p*-substituted phenyl DIM analogs on TNF-α-induced chemokine/cytokine production in HUVECs, cells were pretreated for 6 h with

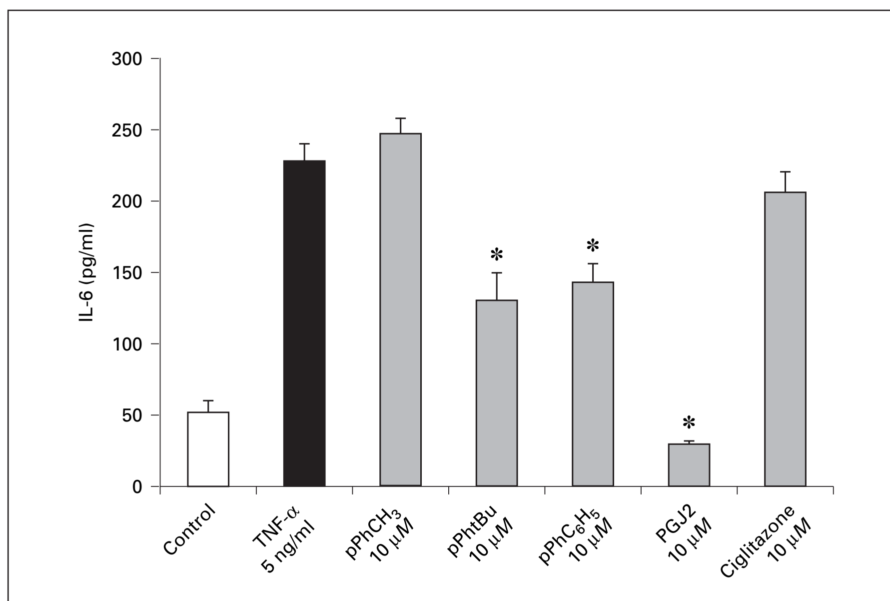
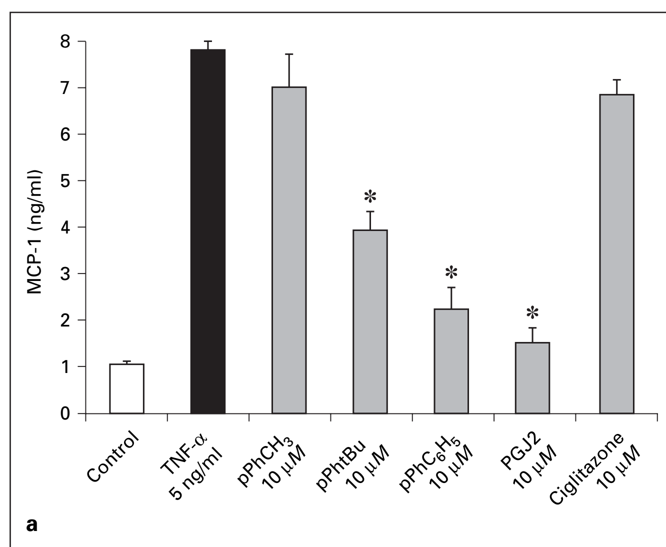


Fig. 3. Effects of different PPAR-γ agonists on IL-6 production in HUVECs stimulated with TNF-α. HUVECs were seeded in 24-well plates. After 2 days, the cells were first pretreated with different PPAR-γ agonists at a dose of 10 μ*M* for 6 h and then incubated with 5 ng/ml TNF-α for 6 h. IL-6 concentrations in the culture supernatants were measured by ELISA. Data are expressed as the mean ± SD of a representative experiment performed in triplicate. * $p < 0.05$.



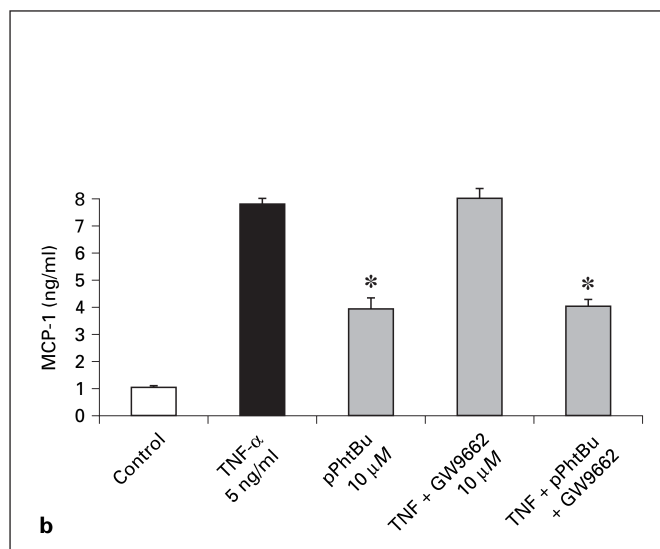


Fig. 4. a Effects of different PPAR-γ agonists on MCP-1 production in HUVECs stimulated with TNF-α. HUVECs were seeded in 24-well plates. After 2 days, the cells were first pretreated with different PPAR-γ agonists at a dose of 10 μ*M* for 6 h and then incubated with 5 ng/ml TNF-α for 24 h. MCP-1 concentrations in the culture supernatants were measured by ELISA. **b** Effects of GW9662. Experiments were carried out as described in **a**; however, 10 μ*M* GW9662 was added 30 min prior to DIM-C-pPhtBu. Data are expressed as the mean ± SD of a representative experiment performed in triplicate. * $p < 0.05$.

one of the three analogs at the concentrations indicated or with vehicle and then stimulated with 5 ng/ml TNF-α for the indicated time before the assays were performed. As expected, the levels of IL-6 were markedly increased (>4-fold) in response to TNF-α stimulation for 6 h (from 52.8 ± 7.5 pg/ml at baseline to 228 ± 12.7 pg/ml, $p < 0.05$; fig. 3). In contrast, pretreatment of cells with 10 μ*M* DIM-C-pPhtBu or DIM-C-pPhC_6H_5 inhibited TNF-α-induced IL-6 production, with IL-6 levels of 130.3 ± 19.3 and 143.4 ± 12.2 pg/ml, respectively, in the treatment

groups. Pretreatment with DIM-C-pPhCH_3 did not significantly inhibit TNF-α-induced IL-6 production.

A similar pattern was observed in the production of MCP-1 by HUVECs. Specifically, treatment of these cells with TNF-α for 24 h significantly induced (>7-fold) MCP-1 production (from 1.05 ± 0.07 ng/ml at baseline to 7.8 ± 0.19 ng/ml, $p < 0.05$; fig. 4a). However, pretreatment of cells with 10 μ*M* DIM-C-pPhtBu resulted in a significant inhibition of TNF-α-induced MCP-1 production to 3.9 ± 0.41 ng/ml ($p < 0.05$). DIM-C-pPhC_6H_5 also strongly inhibited the TNF-α-induced production of MCP-1 in HUVECs. Specifically, MCP-1 levels were decreased to 2.2 ± 0.49 ng/ml, whereas DIM-C-pPhCH_3, a relatively inactive PPAR-γ agonist, did not affect the TNF-α-induced levels of MCP-1.

In order to compare the effects of these PPAR-γ agonists with those of other known PPAR-γ agonists, HUVECs were pretreated for 6 h with 10 μ*M* 15d-PGJ2 or ciglitazone and then stimulated with 5 ng/ml TNF-α for the indicated times before the chemokine/cytokine assays were performed. 15d-PGJ2 significantly ($p < 0.05$) inhibited TNF-α-induced IL-6 production (to 29.8 ± 1.6 pg/ml), whereas ciglitazone only weakly affected IL-6 production (to 207 ± 13 pg/ml, $p = 0.17$; fig. 3). TNF-α-induced MCP-1 production in HUVECs was also significantly ($p < 0.05$) inhibited after cells were pretreated with 15d-PGJ2 (to 1.5 ± 0.3 pg/ml), whereas ciglitazone only slightly inhibited MCP-1 synthesis in HUVECs (to 6.8 ± 0.2 pg/ml, $p = 0.01$; fig. 4a). The results illustrated in figure 4b show that the inhibition of TNF-α-induced MCP-1 expression by DIM-C-pPhtBu was not reversed by the PPAR-γ antagonist GW9662. These results again suggested that the anti-inflammatory activity of the PPAR-γ-active DIMs is receptor independent and this parallels with previous results obtained with 15d-PGJ2 [9].

Discussion

ECs are primary cellular targets for the actions of proinflammatory cytokines, such as TNF-α, which are produced predominantly by activated macrophages [10]. The binding of TNF-α to the p55 TNF receptor may lead to EC activation. The TNF-α-mediated inflammatory response involves the induction of cell adhesion molecules, including ICAM-1 (CD54) and VCAM-1 (CD106) [11, 12]. The interactions of inflammatory cells with other cells via ICAM and VCAM are a necessary first step in atherogenesis [13]. Once they adhere to the endothelium, inflammatory cells migrate into the subendothelial space, attracted by MCP-1 [14].

In response to several atherogenic stimulants such as oxidized low-density lipoprotein and IL-1β, MCP-1 is induced in ECs and promotes the transmigration of monocytes through the endothelial barrier, which is thought to be the earliest and most significant event in the formation of atherosclerotic lesions [15, 16]. A major role for MCP-1 in atherogenesis is supported by the observation that disruption of the MCP-1 gene markedly reduced the development of atherosclerosis in low-density-lipoprotein receptor-deficient or apolipoprotein-B-overexpressing mice [14, 17]. IL-6 is a circulating cytokine secreted by numerous cells, including activated macrophages, lymphocytes, and ECs. It might therefore play a key role in the development of coronary disease through a number of different mechanisms [18].

PPAR-γ is a member of the nuclear receptor superfamily of ligand-activated transcription factors [19–21]. PPAR-γ is highly expressed in tumors and cancer cell lines, and agonists for this receptor inhibit tumor growth [6, 22–24]. PPAR-γ is also highly expressed in adipose tissue and in other tissues, including ECs [5]. Further, PPAR-γ has been identified in atherosclerotic plaques, and the ligand-dependent activation of PPAR-γ inhibits monocyte activation [25]. Previous studies by our group and others have shown that PPAR-γ agonists, such as 15d-PGJ2 and the thiazolidinedione class of insulin-sensitizing drugs, can modulate the expression of many proinflammatory cytokines [4, 25], chemokines [26], and adhesion molecules [27] in macrophages and other cell types, including ECs. These effects result from the targeting of multiple pathways and include inhibition of NFκB-dependent responses [28]. However, some studies [29, 30] have not shown modulation of the inflammatory process by PPAR-γ agonists, and this may be due, in part, to the variable doses and structures of PPAR-γ agonists used in these studies. It is also now evident that many of these effects might be PPAR-γ independent. In order to address this point, we have carried out studies using the PPAR-γ antagonist GW9662 (fig. 2b, 4b), and the results show that GW9662 does not inhibit the anti-inflammatory activity of DIM-C-pPhtBu in the ICAM-1 and MCP-1 assay procedures. These results are consistent with previous data on other structural classes of PPAR-γ agonists which also induce receptor-independent responses in ECs [9]. Moreover, results obtained for the PPAR-γ-active DIM compounds in cancer cells also show induction of both receptor-dependent and -independent responses [6–8].

Interactions between the PPAR-γ and NFκB signaling pathways result in the downregulation of proteins involved in the inflammatory process. However, our study primarily focused on TNF-α induction of adhesion molecules/cytokine protein expression in ECs and the role of this new class of PPAR-γ agonists in modulating these phenomena. It is possible that some of the observed responses may include interactions with the NFkB pathway, and this will be investigated in future studies.

15d-PGJ2 and the thiazolidinediones represent two important classes of PPAR-γ ligands, and previous studies in our laboratory have shown that PPAR-γ activators markedly decrease the expression of adhesion molecules in activated human ECs. Moreover, short-term treatment with the PPAR-γ agonist, troglitazone, significantly inhibited macrophage homing to atherosclerotic plaques [27]. Figure 2 demonstrates that 10 μ*M* 15d-PGJ2 significantly inhibited TNF-α-induced ICAM-1 expression and IL-6 and MCP-1 secretion in ECs, whereas ciglitazone was inactive at this concentration.

In this study, we investigated a new class of PPAR-γ agonists as inhibitors of TNF-α-induced responses in ECs and compared their potencies to 15d-PGJ2 and ciglitazone. The compounds selected for this study consisted of two potent (DIM-C-pPhtBu and DIM-C-pPhC_6H_5) and one less active (DIM-C-pPhCH_3) analog, as demonstrated in previous structure-activity relationship studies in cancer cell lines [6]. We found that both DIM-C-pPhtBu and DIM-C-pPhC_6H_5 inhibited TNF-α-induced ICAM-1 expression (fig. 1a, b) and IL-6 and MCP-1 production (fig. 3, 4a) in ECs and that their potencies were comparable to those of 15d-PGJ2. In contrast, DIM-C-pPhCH_3 (fig. 1c, 3, 4a) exhibited lower activity, which is consistent with the observations made in structure-activity studies of these compounds as inducers of PPAR-γ-dependent transactivation [6].

As indicated above, treatment of HUVECs with different concentrations (up to 10 μ*M*) of the *p*-substituted phenyl DIM analogs, ciglitazone, or 15d-PGJ2 for 12 h did not induce apoptosis. However, this does not exclude the possibility of activation of apoptotic and non-apoptotic cell death pathways using different cell culture conditions, i.e. at longer incubation times and/or higher concentrations of PPAR-γ agonists. This study focused on the initial TNF-α-PPAR-γ agonist crosstalk and does not exclude induction of other downstream effects of these new compounds, including modulation of NFκB-dependent activities.

PPAR-γ agonists such as the thiazolidinediones are used for treatment of type 2 diabetes and have potential for clinical applications in cancer chemotherapy and treatment of endothelial inflammatory processes. Previous studies in our laboratory have demonstrated that DIM compounds are well tolerated in animal studies and exhibit potent anticarcinogenic activity [6, 31–33]. Moreover, in vitro studies in cancer cell lines suggest that these compounds induce PPAR-γ-dependent and -independent responses in cancer cell lines which are more potent than those of thiazolidinediones [6, 7], and this is comparable to results of this study where PPAR-γ-active DIM compounds are more active than ciglitazone as inhibitors of TNF-α-induced responses.

Results of this study demonstrate that inhibition of TNF-α-induced MCP-1 in HUVECs by the DIM compounds was PPAR-γ independent. Current studies with these compounds and other PPAR-γ agonists are focused on the mechanisms of their PPAR-γ-independent responses and the development of improved drugs that will target critical pathways linked to their anti-atherogenic activity.

Acknowledgments

This work was supported by the Disaster Relief and Emergency Medical Services (DREAMS) project from the US Department of the Army to J.T.W and E.T.H.Y. and by the NIH grant ESO-9106 to S.H.S.

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