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Comparison of PPARδ and PPARγ in inhibiting the pro-inflammatory effects of C-reactive protein in endothelial cells

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Abstract

Background: Inflammation associated with endothelial cell dysfunction is a key step of atherogenesis. C-reactive protein (CRP), used to serve as a nonspecific clinical inflammation marker, has now emerged as a new marker for cardiovascular diseases. Recently, PPARδ has revealed benefits for dealing with inflammation. The relationship between CRP-induced inflammation and PPARδ agonist remains unclear.
Methods: Human umbilical vein endothelial cells (HUVECs) were separated into the following groups: 25 μg CRP alone for 15 hours; CRP-treated with 1 μM PPARδ(L-165041) or 10 μM PPARγ(troglitazone) agonists, and untreated HUVECs. This research focused on the CRP underlying signaling pathways and the effects of PPAR agonists on monocyte attachment to endothelial cells.
Results: Levels of interleukin-6 (IL-6) and IL-8 increased by CRP were both significantly attenuated by pretreatment with PPARδ or PPARγ agonists, but the needed dose of PPARδ to reach the same effect was less than PPARγ agonist. After incubation with CRP, immunoblotting showed a significant increase in NF-κB activation and CD32 receptor. These changes were associated with a significant increase of MCP-1 and VCAM-1 expression. PPARδ treatment not only decreased these pro-inflammatory effects in HUVECs but also significantly attenuated monocyte adhesion to endothelial cells in less dosage than PPARγ.
Conclusions: The results suggest that PPARδ attenuated CRP-induced pro-inflammatory effects may through CD32 and NF-κB pathway. PPARδ may serve as a more potent therapeutic target than PPARγ in atherosclerosis or inflammatory therapy.

Keywords: PPAR; CRP; Endothelium dysfunction; Inflammation; Atherosclerosis

1. Introduction

Atherosclerosis is one of the most common causes of human mortality and morbidity, especially in industrialized societies. The pathogenesis of atherosclerotic lesions is complicated. Recently, C-reactive protein (CRP) has been identified as a new marker for coronary artery disease [1]. CRP is used clinically as a marker of acute inflammation or

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infection. The role of CRP in the process of atherosclerosis has been the focus of many researches recently. Chronic inflammation results in endothelial dysfunction and facilitates the interactions between oxidized LDL, macrophages, and normal cellular elements of the arterial wall, leading to atherosclerotic processes [2–4]. This paradigm of the atherosclerotic hypothesis is compatible with the above mentioned observation about CRP as a predictor of coronary artery disease, although the detailed processes involved remain unknown.

The peroxisome-proliferator activated receptors (PPARs) are members of the nuclear receptor gene family that are ligand-activated transcription factors [5]. PPARs form obligate heterodimers with the 9-cis-retinoid X receptor (RXR) and bind to specific sites, termed PPREs in the promoter region of target genes. There are three closely related members of PPAR subtypes: PPARα, PPARγ, and PPARδ [6,7]. Both PPARα and PPARγ have been well characterized for their roles in lipid and carbohydrate metabolism, using specific marketed drugs such as the thiazolidinediones, PPARγ ligands prescribed for the treatment of Type 2 diabetes, and the fibrates, PPARα ligands prescribed for their lipid-modulating properties in clinical use [8]. In contrast, the biological role and function of PPARδ remain relatively unclear.

Several lines of evidences suggested biological roles for PPARδ in epidermal maturation, cell differentiation, and pathogenesis of skin wound healing [9]. PPARδ knockout led to embryonic lethality with placental defects and growth retardation in a small number of surviving mice [10]. Deletion of PPARδ from foam cells increased the availability of inflammatory suppressors, which in turn reduced atherosclerotic lesions [11]. On the other hand, activation of PPARδ stimulates β-oxidation and triglyceride utilization in adipocytes and myocytes. Synthetic PPARδ agonists promote cholesterol accumulation in human macrophages and increase serum HDL while lowering triglyceride levels in obese animal models [12,13]. These data collectively implicate PPARδ as being involved in lipid homeostasis and atherogenesis.

Increasing evidences had shown that agonists of PPARγ and PPARδ modulate lipid metabolism in the cell, decreasing the inflammatory effects [14]. However, the relationship between PPAR and CRP-induced inflammation in endothelial cells still needs to be determined. On the other hand, the specific CRP receptor on endothelial cells is still not well understood. Low- and high-affinity binding of CRP to human immunoglobulin G (IgG) Fcγ receptor I (CD64) and Fcγ receptor II (CD32) on immune cells has been reported [15]. In recent studies, anti-CD32 and anti-CD64 antibodies resulted in significant reduction in CRP-induced inflammation *in vitro* and *in vivo* [16,17]. The purpose of this study is to investigate the CRP-induced inflammatory signaling pathways through its major receptor and the effects of PPARδ agonist in damaged endothelial cells. This study provides important insights into the molecular mechanism of PPAR agonist's attenuation of inflammation and may serve as therapeutic targets in order to decrease endothelial dysfunction and atherogenesis.

2. Materials and methods

2.1. Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) were cultured in Endothelial Cell Growth Medium (Cambrex Bio Science, MD, USA) containing 12% FBS, 100 μg/mL penicillin, and 100 μg/mL streptomycin. The experiments were performed using HUVECs after 2–4 passages. High-purity recombinant human CRP was purchased from Calbiochem (Calbiochem, CA, USA). The purity of CRP preparations was confirmed by SDS-PAGE. The preparation displayed a single band. Presence of endotoxin in CRP was ruled out by limulus amebocyte lysate (LAL) test (Sensitivity 0.03 EU/mL; Pyrotell, MA, USA). CRP at 25 μg/mL was used for the experiments. The effects of 10 μM PPARγ agonist (Troglitazone) and 1 μM PPARδ agonist (L-165041) from Sigma were evaluated by pre-treating HUVECs 1 h before co-incubation with CRP.

2.2. RNA and protein extraction

Total cellular RNA was isolated from HUVECs using the single-step acid guanidinium thiocyanate/phenol/chloroform extraction method. For preparation of total protein extracts, HUVECs were homogenized in modified RIPA buffer (50 mmol/L tris [pH 7.4], 1% IGEPAL CA-630 (Sigma), 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/mL aprotinin, leupetin, and pepstatin). The samples were centrifuged at 14,000 g for 15 min, and the resulting supernatants were collected as total protein extracts. For preparation of nuclear-protein extracts, the cells were treated with 200 μL buffer A (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 25 μg/mL leupeptin, and 1 mM PMSF). After 5 min incubation on ice, 25 μL buffer B (buffer A + 0.1% NP-40) was added and the samples were centrifuged at 1850 g for 10 min at 4 °C. The crude nuclear pellets were resuspended in 50 μL buffer C (20 mM HEPES [pH 7.9], 0.45 M NaCl, 1.0 mM EDTA). After thoroughly mixing at 4 °C for 30 min, the samples were centrifuged at 14,000 rpm for 30 min. The resulting supernatants were the nuclear extracts for NF-κB immunoblot assay. The purity of the nuclear and cytosolic extracts was examined by Western blotting with nucleolin and actin antibodies (Santa Cruz Biotechnology, Inc., CA, USA). The protein concentrations were determined by a BioRad protein assay kit.

2.3. Reverse Transcription (RT) and polymerase chain reaction

For reverse transcription, 1 μg of RNA was incubated with 200 U of Moloney-murine leukemia virus reverse transcriptase in a buffer containing a final concentration of 50 mmol/L Tris/HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 20 U of RNase inhibitor, 1 μmol/L poly $(dT)_{12-18}$ oligomer (Life Technologies, MD,USA), and 0.5 mmol/L of each dNTP at a final volume of 20 μL. The reaction mixture was incubated at 42 °C for 1 h and then at 94 °C for 5 min to inactivate the enzyme. The produced cDNA was used to generate PPARδ DNA product by polymerase chain reaction (PCR) with PPARδ forward primer (5′-ACGCTATCCGTTTGGTCG-3′); reverse primer (5′-CTCACGGGTGACAAAGCC-3′). Agarose gel electrophoresis was used to confirm the size of the PCR product. The size of the PCR product for PPARδ was 395 bp and sequence was confirmed.

2.4. Flow cytometric analysis

Cells were harvested, washed and resuspended in phenol red-free Hanks' balanced salt solution (HBSS). After preincubation of 5×10^5 cells for 30 min on ice, cells were washed and then incubated with anti-CD32 antibody (Santa Cruz Biotechnology Inc., CA, USA) dilution for 30 min on ice. Following this, cells were washed and treated for another 30 min with 50 μL of fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG (Santa