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Newly developed PPAR-α agonist (R)-K-13675 inhibits the secretion of inflammatory markers without affecting cell proliferation or tube formation

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Abstract

Peroxisome proliferator-activated receptor-alpha (PPAR-α) is a key regulator of lipid and glucose metabolism and has been implicated in inflammation. The vascular effects of activator for PPARs, particularly PPAR-α, on vascular cells remain to be fully elucidated. Therefore, we analyzed the hypothesis that newly developed (R)-K-13675 decreases the secretion of inflammatory markers without affecting cell proliferation or tube formation. Human coronary endothelial cells (HCECs) were maintained in different doses of (R)-K-13675 under serum starvation. After 20 h, the levels of monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T expressed and secreted (RANTES), interleukin-6 (IL-6) and interferon-gamma (INF-γ) secreted in the medium and nuclear factor kappa B (NFκB) in cell lysate were analyzed using enzyme-linked immunosorbent assays (ELISA). Upon treatment with (R)-K-13675 at 0, 10, 20, 50 and 100 nM, with the inflammatory markers at 0 nM as 100 (arbitrary units), MCP-1 levels were significantly suppressed (94 ± 9, 88 ± 2, 80 ± 5 and 74 ± 11, respectively). RANTES, IL-6 and INF-γ levels were also significantly suppressed (RANTES: 92 ± 2, 74 ± 9, 64 ± 7 and 60 ± 2, respectively, IL-6: 97 ± 2, 89 ± 10, 82 ± 1 and 66 ± 7, respectively, INF-γ: 98 ± 7, 94 ± 3, 76 ± 8 and 64 ± 8, respectively). NFκB levels were also decreased to 91 ± 5, 90 ± 5, 84 ± 7 and 82 ± 8, respectively. In addition, (R)-K-13675 did not affect HCEC proliferation or tube formation at up to 100 nM. Thus, (R)-K-13675 was associated with the inhibition of inflammatory responses without affecting cell proliferation or angiogenesis, and subsequently may induce an anti-atherosclerotic effect.

Keywords: PPAR-α agonist; Subtype selectivity; Inflammatory markers; Cell proliferation; Tube formation

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that play an important role in the regulation of both vascular cell functions and systemic lipid and glucose metabolism, and may lead to the inhibition of atherosclerosis [1,2].

The isoform of PPAR (PPAR-α) affects the initiation and progression of atherosclerosis with an emphasis on atherosclerosis-associated inflammatory responses [3]. In later stages of atherosclerosis, evidence suggests that the activation of PPAR-α inhibits the formation of macrophage foam cells by regulating the expression of genes involved in reverse cholesterol transport, the formation of reactive oxygen species and associated-lipoprotein oxidative modification [3]. PPAR-α is highly expressed in liver, heart, renal proximal tubular cells, skeletal muscle, intestinal mucosa, and brown adipose, which are all tissues that are considered to be metabolically very active [4,5]. In liver tissue, the administration of PPAR-α ligands leads to the activation of PPAR-α and results in a pleiotropic response that includes increased peroxisome proliferation, upregulation of fatty acid oxidation, reduced inflammation, and suppression of apoptosis. In addition, PPAR-α activation interferes with early steps in atherosclerosis by reducing leukocyte adhesion to activated endothelial cells of the arterial vessel wall and inhibiting sub-

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sequent transendothelial leukocyte migration [3]. Based on these reports, PPAR-α has anti-atherosclerotic effect through lipid-lowering-dependent and -independent mechanisms.

A chemokine, monocyte chemoattractant protein-1 (MCP-1), plays a key role in the platelets and monocytes in the pathophysiology of atherosclerosis [6,7]. In addition, regulated on activation, normal T expressed and secreted (RANTES) [7], interleukin-6 (IL-6) [8] and interferon-gamma (INF-γ) [9] are also important. Nuclear factor kappa B (NFκB) [10] as a transcription factor is upstream of these chemokines, and C-C chemokine receptor 2 (CCR-2) [11] is the receptor for MCP-1 in human coronary endothelial cells (HCECs).

PPAR-α has an anti-atherogenic effect and is expressed in vascular cells such as endothelial cells. Since newly developed (R)-K-13675, a PPAR-α agonist, has 500 times greater selectivity for PPAR-α than PPAR-γ and PPAR-delta (PPAR-δ), and since the vascular effects of activator for PPARs, particularly PPAR-α, on vascular cells remain to be fully elucidated [12,13], we hypothesized that (R)-K-13675 induced anti-inflammation, and inhibited chemokine secretion, cell proliferation and tube formation as key issues in the progression of atherosclerosis.

2. Materials and methods

2.1. Cell culture

HCECs and HC smooth muscle cells (HCSMCs) were purchased from Cambrex (Walkersville, MD). Both cells were cultured in medium with 5% fetal bovine serum (FBS) penicillin/streptomycin, and endothelial cell growth supplement (Takara, Osaka, Japan) on 10-cm diameter cell culture dishes at 37 °C under 5% CO_2 as a standard tissue culture condition. At 70–90% confluence, the medium was changed, and cells were incubated with (R)-K-13675 (PPAR-α selective agonist, kindly provided by Kowa Co., Ltd, Tokyo, Japan) at concentrations of 0, 10, 20, 50 and 100 nM for varying time periods up to 24 h with 0.5% FBS in the media.

2.2. Cell proliferation assay

HCECs or HCSMCs (5×10^3 per well) were placed in a 96-well plate and cultured under 5% FBS conditions. After 48 h, the cells were cultured for an additional 24 h in 0, 0.1, 1, 10 or 100 nM of (R)-K-13675 without FBS at 37 °C and 5% CO_2. After incubation, the cells were stained with CellTiter 96 One Solution Reagent (a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium, inner salt; MTS assay] (Promega, Madison, WI) for 2 h at 37 °C and 5% CO_2, and absorbance at 490 nm was recorded with a 96-well plate reader.

2.3. EC tube formation assay on matrigel

The EC tube formation assay has been described previously [14,15]. Briefly, Matrix gels [16] were purchased from Chemicon International, Inc. (Temecula, CA). The gels were allowed to polymerize in a 96-well plate for 1 h at 37 °C. HCECs were seeded at 1×10^4 per well and grown in medium supplemented with 5% FBS and without endothelial cell growth supplement for 24 h in a humidified 37 °C/5% CO_2 incubator. In the experiments, cells were cultured with 0, 0.1, 10 or 100 nM of (R)-K-13675. After washing, EC tube formation was observed using a light microscope, and pictures were captured with a computer system. In an attempt to automate the procedure, we performed a 'pixel analysis' of the area of EC tube formation [14,15]. The control sample was defined as 100% EC tube formation, and the % increase or decrease in tube formation relative to the control was calculated for each sample.

2.4. Enzyme-linked immunosorbent assays (ELISA) for inflammatory markers

Aliquots of the medium were removed for analysis of MCP-1, RANTES, IL-6, INF-γ by enzyme-linked immunosorbent assay using matched antibodies in a DuoSet ELISA development system (R&D Systems, Inc. Minneapolis, MN). Antibody binding was visualized with horseradish peroxidase-conjugated streptavidin and a tetra methyl benzidine liquid substrate system. At our laboratory, the intra- and inter-assay coefficients of variation were each 5%.

2.5. Preparation of protein extract

After 24 h of incubation with or without (R)-K-13675, HCECs were collected by low-speed centrifugation. The lysates were diluted with immunoprecipitation buffer [2% Triton-X, 300 mM NaCl, 20 mM Tris pH 7.4, 2 mM ethylene diamine tetraacetic acid, 0.4 mM sodium orthovanadate, 0.4 mM phenylmethanesulphonylfluoride or phenylmethylsulphonyl fluoride, 1% Nonidet (N) P-40] for immunoblotting, and buffer RLT for RNA extraction (Qiagen) according to the instruction given by the manufacturer. Total protein was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL).

2.6. NFκB immunoassay

Cell lysate was prepared using radio immuno precipitation assay (RIPA) extraction buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, PBS pH 7.4). Immunoassay for the redox-regulated transcription factor NFκB in HCECs was performed using an EZ-Detect transcription factor kit (Pierce Biotechnology) according to the manufacturer's instructions.