

The Effect of *Chlamydia pneumoniae* on the Expression of Peroxisome Proliferator-Activated Receptor- γ in Vascular Smooth Muscle Cells

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Purpose: This study was designed to investigate the change of peroxisome proliferator-activated receptor gamma (PPAR γ) after the infection of the human coronary artery smooth muscle cells (HCSMCs) with *Chlamydia pneumoniae* (*C. pneumoniae*) and the effect of PPAR γ agonist on the expression of PPAR γ of *C. pneumoniae*-infected HCSMCs. **Materials and Methods:** To determine the effect of PPAR γ agonist on the proliferation of *C. pneumoniae*-infected HCSMCs, rosiglitazone at various concentrations was applied 1 hour before inoculation of HCSMCs. **Results:** The expression of PPAR γ mRNA in HCSMCs increased from 3 hours after *C. pneumoniae* infection and reached that of noninfected HCSMCs at 24 hours ($p < 0.05$). The expression of PPAR γ protein in HCSMCs also increased from 3 hours after *C. pneumoniae* and persisted until 24 hours as compared with that of noninfected HCSMCs ($p < 0.05$). The pretreatment of HCSMCs with rosiglitazone followed by the infection with *C. pneumoniae* augmented the expression of PPAR γ mRNA and protein ($p < 0.05$) and decreased cell proliferation. **Conclusion:** Our results showed that the expression of PPAR γ increases in response to *C. pneumoniae* infection and rosiglitazone further augmented the expression of PPAR γ . It is suggested that rosiglitazone could ameliorate the chronic inflammation in the vessel wall induced by *C. pneumoniae* by augmenting PPAR γ expression.

Key Words: *C. pneumoniae*, peroxisome proliferator-activated receptor gamma, rosiglitazone, atherosclerosis

Received July 31, 2007

Accepted September 27, 2007

The work was supported by Catholic Medical Center Research Foundation made in the program year of 2005.

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INTRODUCTION

Infection has attracted attention as a novel risk factor for atherosclerosis, which is different from traditional risk factors.¹ Among various infectious agents, *Chlamydia pneumoniae* (*C. pneumoniae*) is the major infectious agent that has been extensively investigated. Seroepidemiological studies showed that the seropositivity of anti-*C. pneumoniae* antibodies is two times higher in patients with cardiovascular diseases than that of healthy population controls.² Furthermore *C. pneumoniae* were isolated from more than half of atherosclerotic lesions.³ *In vitro* studies suggest that *C. pneumoniae* could be detected in circulating leukocytes, and that they infect all atheroma cell types such as endothelial cells, vascular smooth muscle cells (VSMC), and macrophages,⁴⁻⁶ and induce the inflammatory cytokines, procoagulants, matrix metalloproteinases and adhesion molecules.⁷⁻⁹

Peroxisome proliferator-activated receptor- γ (PPAR γ) is a member of the nuclear receptor superfamily that regulates lipid and lipoprotein metabolism and glucose homeostasis. In addition, PPAR γ has been reported to be an important modulator of the inflammatory response of vessel wall not only through metabolic effects but also through their direct action in vascular and inflammatory cells.^{10,11} PPAR γ is expressed in the major cellular constituents of the vessel wall such as endothelial cells, VSMC, and macrophages, and affects development of atherosclerosis.^{12,13} In endothelial cells, PPAR γ agonist inhibits the expression of monocyte chemoattractant protein-1,

vascular cell adhesion molecule-1, and inter-cellular adhesion molecule-1.^{14,15} PPAR γ also interferes with leukocyte chemoattraction and recruitment to atherosclerotic lesions and suppresses the expression of inducible nitric oxide synthase and matrix metalloproteinase-9 (MMP-9) in macrophages. Furthermore, PPAR γ inhibits VSMC proliferation by attenuating the mitogen-induced degradation of p27Kip1, and platelet-derived growth factor-induced VSMC migration by inhibiting MMP-9 expression.^{11,16}

Although a number of studies indicate the role of PPAR γ in the modulation of vascular inflammation, the effect of atheroprone infectious agents on the expression of PPAR γ in vascular cells has not yet been studied. Interestingly, infection increases PPAR γ expression in porcine white blood cells¹⁷ but down-regulates it in murine adipocytes,¹⁸ suggesting that PPAR γ expression is differentially regulated according to cell types. We hypothesized that *C. pneumoniae* infection, which is a clear risk factor for the development of atherosclerosis, may have an effect on the expression of PPAR γ in vascular smooth muscle cells. Therefore, the present study was undertaken to investigate whether *C. pneumoniae* infection could affect the expression of PPAR γ in human coronary artery smooth muscle cells (HCSMC).

MATERIALS AND METHODS

Cell lines

Human coronary artery smooth muscle cells (HCSMC) were purchased from Clonetics and cultured in SmBm media (smooth muscle cell basal media, Clonetics, Walkersville, MD, USA). Passages between 5 and 10 were used.

C. pneumoniae

C. pneumoniae AR-39 strain was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The organisms were inoculated into shell vials containing McCoy cells (ATCC, Manassas, VA, USA), and then shell vials were centrifuged at 1500g for 1 hour and incubated in RPMI 1640 medium containing 1 μ g/mL

cycloheximide (Sigma, St. Louis, MO, USA). After 48 hours, the infected cells were disrupted by ultrasonication, and culture supernatants and cell debris were removed by centrifugation at 800g for 10 minutes. The organisms were then concentrated by a high-speed centrifugation at 30,000g for 30 minutes. The bacterial pellets were resuspended in sucrose phosphate glutamate transport medium and stored at -70°C until use. The organisms resuspended in RPMI 1640 medium were used for experiments. The number of infectious *C. pneumoniae* was determined as inclusion forming units per mL by counting chlamydial inclusions formed in McCoy cells with fluorescein isothiocyanate-conjugated *C. pneumoniae*-specific monoclonal antibody (DAKO, Copenhagen, Denmark).

Infection of HCSMC with *C. pneumoniae*

HCSMCs were trypsinized and plated at a density of 4×10^5 cells/well on 6-well plates. Once confluent, the medium was then changed to serum-free medium for 24 hour to achieve synchronous growth arrest. HCSMCs were inoculated with *C. pneumoniae* at a multiplicity of infection (MOI) of 2, and the plates were centrifuged at 500g for 1 hour at room temperature. Multiple inclusion bodies in HCSMCs were observed after 48 hours of *C. pneumoniae* infection.

Cell proliferation assay

HCSMCs were plated at a density of 5×10^3 cells/well on 96-well plates and inoculated with *C. pneumoniae* by the method described above. Cell proliferation was determined using XTT proliferation assay kit (JBI, Deagu, Korea) 6 hours, 24 hours and 48 hours after inoculation. As a control for cell proliferation, noninfected HCSMCs were used. To determine the effect of a PPAR γ agonist on the proliferation of *C. pneumoniae*-infected HCSMCs, rosiglitazone (GlaxoSmithKline Corporate, King of Prussia, PA, USA) at various concentrations was applied 1 hour before inoculation.

Real time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from HCSMCs using

TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total extracted RNA was subjected to reverse transcription using an onestep RT kit (Invitrogen). Expressions of PPAR γ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were examined in real time PCR using an SYBR Green dye. Primers used were as follows: human PPAR γ sense, 5'-TCTCTC CGTAATGGAAGACC-3', human PPAR γ anti-sense, 5'-CCCCTACAGAGTATTACG-3'; GAPDH sense, 5'-TGCACCACCAACTGCTTAGC-3', GAPDH antisense 5'-GGCATGGACTGTGGTCAT GAG-3'. To examine the changes in the expression of PPAR γ mRNA according to time elapse after *C. pneumoniae* infection in HCSMCs, relative values were compared at various time intervals.

Western blot analysis

HCSMCs were seeded at 4×10^5 cells/well in 6-well plates and incubated with *C. pneumoniae*. After various time intervals, cells were harvested, lysed, and sonicated in Tris-lysate buffer [20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM Na₂P₂O₇, 100 mM NaF, 2 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin]. Cell lysate were cleared by centrifugation, and protein concentrations were determined by Bradford protein assay kit (Pierce, Rockford, IL, USA). Proteins were resolved on a 9% sodium dodecylsulfate-polyacrylamide gel, and electrophoretically transferred onto a nitrocellulose membrane. The membrane was incubated with anti-PPAR γ monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). HRP-conjugated anti-mouse IgG (Amersham Bioscience, Piscataway, NJ, USA) was used as a secondary antibody. The antigen-antibody complexes were detected using enhanced chemiluminescence kit (Amersham Bioscience). Quantification of the protein band was done by densitometry.

Statistics

Statistical analysis was performed using the Statistical Package for Social Sciences (version 10, SPSS Inc., Chicago, IL, USA). Values of continuous variables are expressed as mean \pm standard deviation. Comparisons of data were made using

Student t-test. *P*-values less than 0.05 were considered as indicative of statistical significance.

RESULTS

C. pneumoniae stimulates HCSMCs proliferation

HCSMC proliferation was compared between *C. pneumoniae*-infected and uninfected-HCSMCs, according to time intervals such as 6, 24, and 48 hours after infection at a MOI of 2. As shown in Fig. 1, HCSMCs proliferation was stimulated after inoculation with *C. pneumoniae* in a time-dependent manner. At 48 hours, XTT assay showed that *C. pneumoniae* significantly increased the number of HCSMCs compared to the uninfected control (Fig. 1).

Rosiglitazone suppresses *C. pneumoniae*-induced proliferation of HCSMCs.

To explore whether PPAR γ activation directly affects *C. pneumoniae*-induced HCSMCs proliferation, the proliferation rate was compared between *C. pneumoniae*-infected HCSMCs and rosiglitazone-treated *C. pneumoniae*-infected HCSMCs. Treatment of HCSMCs with 10 and 50 μ M rosiglitazone significantly decreased the *C. pneumoniae*-induced proliferation of HCSMCs from 5 hours after *C.*

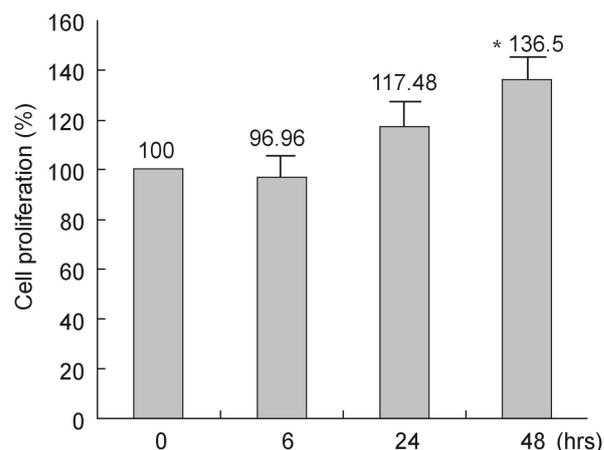


Fig. 1. Cell proliferation assay of HCSMCs infected with *Chlamydia pneumoniae*. Cell proliferation was assessed by XTT proliferation assay kit (JBI, Daegu, Korea). Changes in cell proliferation are expressed by a percentage of the mean value of control (uninfected HCSMCs). **p* < 0.05.

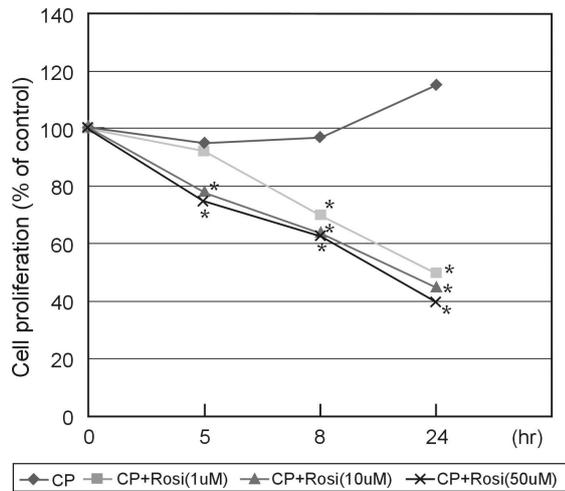


Fig. 2. Dose-dependent effects of rosiglitazone on the proliferation of HCSMCs infected with *Chlamydia pneumoniae* during 24 hours. * $p < 0.05$.

pneumoniae infection and from 8 hours even at 1 μ M concentration (Fig. 2).

***C. pneumoniae* upregulates the expression of PPAR γ mRNA expression in HCSMCs**

To determine whether *C. pneumoniae* activates PPAR γ mRNA in HCSMCs, cells were left untreated or stimulated with *C. pneumoniae*, and cDNA was then prepared from each HCSMCs after 3-, 6-, and 24-hour exposure to *C. pneumoniae*. The expression of PPAR γ mRNA was evaluated by real time RT-PCR. The levels of PPAR γ mRNA were significantly increased in HCSMCs 3 hours after *C. pneumoniae* inoculation, and remained elevated until 6 hours after infection as compared with uninfected control. At 24 hours, the level of PPAR γ mRNA in HCSMCs infected with *C. pneumoniae* returned to the level of uninfected control (Fig. 3).

***C. pneumoniae* stimulates the expression of PPAR γ protein in HCSMCs**

To investigate whether *C. pneumoniae* infection affects PPAR γ protein expression in HCSMCs, cells were stimulated with *C. pneumoniae* infection for 3, 6, and 24 hours. Cell lysates were analyzed by Western blot using a anti-PPAR γ monoclonal

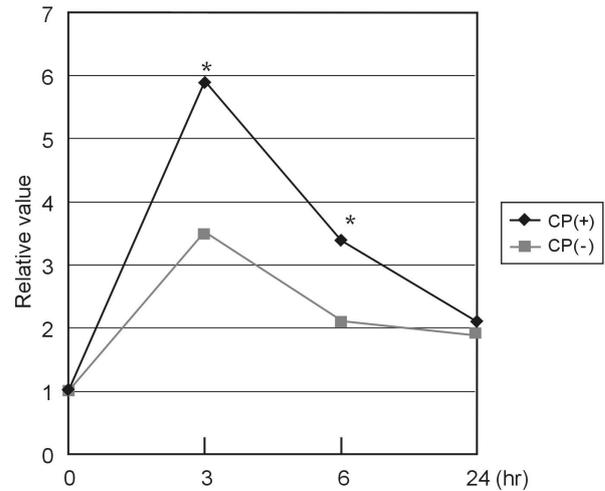


Fig. 3. Real time RT-PCR of PPAR γ in HCSMCs infected with *Chlamydia pneumoniae*. Relative value was arbitrarily defined as corrected for GAPDH mRNA value. CP represents *Chlamydia pneumoniae*. * $p < 0.05$ vs CP(-).

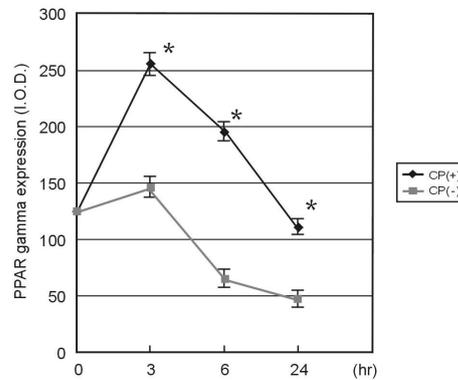
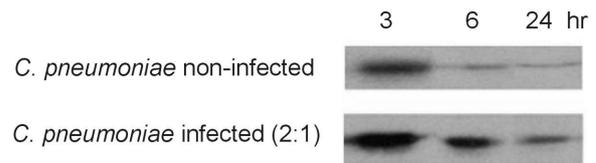


Fig. 4. Western blot analysis of PPAR γ in HCSMCs infected with *Chlamydia pneumoniae*. After 3, 6, and 24 hours, cells were subjected to 10% SDS-PAGE and blotted with anti-PPAR γ monoclonal antibody. Expression levels of PPAR γ were quantified by densitometry. * $p < 0.05$ vs CP (-).

antibody. The levels of PPAR γ protein were already increased in HCSMCs after 3-hour exposure to *C. pneumoniae* as compared with uninfected HCSMCs, and the status of elevation was maintained until 24 hours (Fig. 4).

PPAR γ activator further increases the expression of PPAR γ protein in *C. pneumoniae*-infected HCSMCs

The observation that *C. pneumoniae* has a stimulatory effect on PPAR γ protein in HCSMCs prompted us to investigate the potential synergistic effect of rosiglitazone on the expression of PPAR γ in HCSMCs. Thus, HCSMC cells were divided into untreated control, *C. pneumoniae*-inoculated, rosiglitazone (20 μ M)-treated, and co-treated with both rosiglitazone and *C. pneumoniae*. Changes in the expression of PPAR γ in each HCSMCs were evaluated by Western blot 24 hours after treatment. As compared with untreated control, both rosiglitazone and *C. pneumoniae* significantly increased the expression of PPAR γ protein in HCSMCs. Interestingly, co-treatment of HCSMCs with rosiglitazone and *C. pneumoniae* resulted in an additive effect on the expression of PPAR γ protein (Fig. 5).

DISCUSSION

The present study shows that *C. pneumoniae* could upregulate the expression of PPAR γ mRNA

and protein in HCSMCs, and PPAR γ activator further increased its expression in concert with *C. pneumoniae*. In addition, rosiglitazone significantly attenuated *C. pneumoniae*-induced proliferation of HCSMCs. Our results suggest that PPAR γ plays a role in the development of atherosclerosis induced by *C. pneumoniae*. It is suggested that rosiglitazone, a PPAR γ agonist, could prevent the development of atherosclerosis through its anti-inflammatory and anti-atherogenic effects.

C. pneumoniae could infect and proliferate within endothelial cells, vascular smooth muscle cells, and macrophages, and evoke inflammatory response in these cells.⁴⁻⁹ Animal model studies have shown that *C. pneumoniae* could infect vessel wall and cause arterial inflammation, which leads to the initiation and development of atherosclerotic lesions.^{19,20} In contrast, PPAR γ has an important effect of inhibiting growth and migration of all vascular cells, and reducing vessel inflammation, which leads to attenuation of atherosclerosis in the vessel wall.^{12,13,16,21-23} However, the influence of *C. pneumoniae* infection on the PPAR γ in atherosclerosis-related vascular cells has not yet been studied. Since PPAR γ is expressed in all vascular cells and has significant effects on the protection from vessel inflammation, it is of value to demonstrate the effect of *C. pneumoniae* on the PPAR γ in vascular cells.

Few studies have evaluated the association between infection and PPAR γ . Leininger et al. reported that *in vivo* lipopolysaccharide challenge caused a dynamic increase in PPAR γ protein expression in peripheral white blood cells.¹⁷ In tissues of *Helicobacter pylori*-induced atrophic gastritis or gastric carcinoma, the expression of PPAR γ was upregulated,²⁴ and Huang B et al. showed that the expression of PPAR γ was upregulated in the endothelial cells in mice infected with *C. pneumoniae*.²⁵ In the present study, we also observed that *C. pneumoniae* stimulated the expression of PPAR γ in vascular smooth muscle cells. This observation was contrary to the expectation that *C. pneumoniae* might suppress the expression of PPAR γ because of their counterbalancing actions on all the vascular cells and vessel walls. However, the mechanisms of *C. pneumoniae*-induced PPAR γ activation in HCSMCs are not clear. *C. pneumoniae* is a Gram-negative

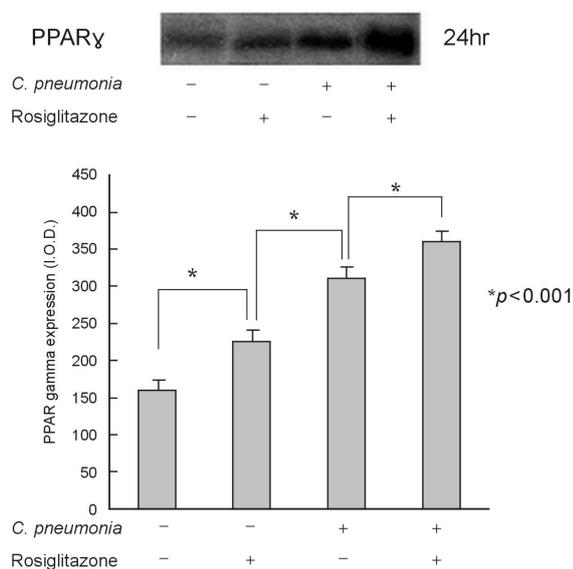


Fig. 5. Western blot analysis of PPAR γ in HCSMCs infected with *Chlamydia pneumoniae* and/or treated with rosiglitazone (20 μ M). After 24 hours, cells were subjected to 10% SDS-PAGE and blotted with anti-PPAR γ monoclonal antibody. Expression levels of PPAR γ were quantified by densitometry. * $p < 0.05$ vs CP(-).

bacterium, and its cell wall is rich in lipopolysaccharides, which can induce hepatocytes and macrophages to secrete prostaglandin (PG) D₂.^{26,27} 15d-PGJ₂, a metabolite of PGD₂, is a ligand for PPAR γ and can induce the PPAR γ expression. Further study is needed to elucidate in detail the mechanism of *C. pneumoniae*-induced PPAR γ upregulation *in vitro*.

The increase of PPAR γ expression may act as a counter-regulatory mechanism of attenuating the negative effect of *C. pneumoniae* in HCSMCs. Rosiglitazone suppressed *C. pneumoniae*-induced proliferation of HCSMCs and further elevated the expression of PPAR γ protein. It might be a novel mechanism of PPAR γ activators to be protective against the atheroprone *C. pneumoniae*. Further studies are required to clarify the association and influence between infection and PPAR γ in vascular cells as well as in animal models.

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