Pioglitazone, a PPAR-γ Agonist, Downregulates Cytokine Production and AP-1 DNA-Binding Protein in Human Peripheral Blood T Cells – Potential Implication in the Treatment of Atherosclerotic Disease

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Background: Atherosclerotic cardiovascular diseases are known to be inflammatory ones. In atherosclerotic lesions, T lymphocytes resulted in the vascular inflammatory reactions by producing many proinflammatory cytokines. Peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonists, insulin sensitizers, exhibit anti-inflammatory effects in various types of cells and may have anti-atherosclerotic actions.

Aim: To determine whether pioglitazone exhibits anti-inflammatory actions in vitro, we examined the effects of pioglitazone in human peripheral T cells.

Methods: The purified drug “pioglitazone” was prepared in solution with different concentrations. Human peripheral blood T cells were purified from the buffy coat of whole blood. For cell activation, PMA and ionomycin were used. Cytokines were measured by ELISA, and activator protein-1 (AP-1) DNA binding activity was detected by electrophoretic mobility shift assays in activated T cells which were either pretreated with pioglitazone or not.

Results: We demonstrated that pioglitazone inhibited the production of IL-2, IL-4, IFN-γ, and TNF-α from activated T cells. Molecular investigation indicated that pioglitazone downregulated the activity of AP-1 DNA binding protein in stimulated T lymphocytes.

Conclusion: In this in-vitro study, pioglitazone might have had some anti-inflammatory effects on human peripheral T lymphocytes via regulation of both cytokine production and AP-1 DNA binding activity.

Key Words: Activator protein-1 • Cytokine • Pioglitazone • T lymphocyte

INTRODUCTION

Atherosclerotic cardiovascular diseases have had major impact on the health of people in recent years,1,2 and much evidence has pointed toward coronary atherosclerosis belonging to one kind of inflammation. Though the mechanisms were not thoroughly clear, some inflammatory processes were identified in the earliest historical observations regarding the development of atherosclerosis, suggesting that both cellular and humoral pathways were involved.3-6 Many reports also showed that several inflammatory cytokines, appearing on either systemic or local responses, were implicated in the processes of atherosclerosis.7-13 From the clinical exploration, the markers indicating increased or decreased systemic inflammation were directly associated with the risk of atherosclerosis, which could support the impor-
tant role of inflammation in the pathogenesis of atherosclerosis.

Earlier, in 1985, T cells were found to exist in human atherosclerotic plaques. Particularly, T lymphocytes could be pulled toward the sites of atherosclerosis, resulting in vascular inflammatory reactions. Otherwise, these T cells could produce many inflammatory or pro-inflammatory cytokines, acting synergistically, to damage the normal physiological reactions of vessel wall, such as modulation of macrophage and vascular smooth muscle cells. Together with monocytes, T lymphocytes can go through the arterial intima at an early stage. Later, they can recognize antigens presented by inflammatory cells to transform atherosclerotic plaque from stable lesions to unstable situations. In turn, these activated cells act as both initiators and regulators of immune activities to control the functions of other cells in the atherosclerotic plaques.

The peroxisome proliferator-activated receptor-gamma (PPAR-γ) is a nuclear transcription factor. It is highly expressed in all major cell types participating in atherosclerotic process to regulate transcription of a variety of genes encoding proteins involved in glucose and lipid metabolism. Pioglitazone is a PPAR-γ agonist and is used extensively in the treatment of insulin resistance and type 2 diabetes mellitus. Besides being insulin sensitizers, PPAR-γ ligands have been shown to inhibit inflammatory and proliferative process. In hypercholesterolemic mice, PPAR-γ ligands reduced atherosclerosis. In diabetic patients treated with coronary stent placement, PPAR-γ ligands also inhibited instant neo-intimal formation.

To determine whether pioglitazone exhibits anti-inflammatory actions in vitro, we examined the effects of pioglitazone in human peripheral T cells. We demonstrated that pioglitazone effectively inhibited cytokines production from simulated T cells and the underlying mechanisms might involve the downregulation of activator protein-1 (AP-1) signaling pathway.

MATERIALS AND METHODS

Preparation of pioglitazone

The purified drug “pioglitazone” was dissolved in dimethyl sulfoxide (DMSO) to make a 100 mM stock concentration. As the experiment was performed, the pioglitazone stock solution was diluted by DMSO to make 1 μM, 5 μM and 10 μM. The defined concentrations were added in culture media with T cells to test its effects on our designed methods. The final concentrations of DMSO as a vehicle were consistently less than 0.05%, which absolutely did not interfere with interpretation of the results.

Preparation of peripheral blood T cells

Human peripheral blood T cells were purified according to our previous report. Briefly, buffy coat from blood bank (Taipei, Taiwan) was mixed with Ficoll-Hypaque. After centrifugation, the layer of mononuclear cells was collected. After lysis of red blood cells, the peripheral blood mononuclear cells were laid on Petri dishes to remove adherent cells and then incubated with antibodies including L243 [anti-DR; American Type Culture Collection (ATCC), Rockville, MD, USA], OKM1 (anti-CD 11b; ATCC) and LM2 (anti-Mac1; ATCC) for 30 min at 4°C. The cells were then washed with medium containing 10% fetal bovine serum and incubated with magnetic beads conjugated with goat anti-mouse IgG (R&D, Minneapolis, MN, USA). The antibody-stained cells were then removed with a magnet. Following a repeat of the above procedures, the T cells were obtained with a purity of > 98% as determined by the percentage of CD3+ cells in flow cytometry (Beckton Dickinson, Mountain View, CA, USA).

Cell treatment and stimulation

For cell activation, the following stimuli were used: PMA (Phorhol 12-myristate13-acctate, sigma. St. Louis, MO) at 10 or 50 ng/ml; ionomycin (Sigma) at 1 μM. The cells were incubated with the stimuli for defined time points after addition of drugs for defined time, and the cells or supernatant were collected for further analysis.

Cytokine production assay

The determination of cytokine concentrations, including IL-2, IL-4, INF-γ and TNF-α, was performed according to manufacturer’s instruction (R&D, St. Paul, MN, USA). Briefly, a 96-well flat-bottom plate was coated with anti-cytokine (IL-2, IL-4, INF-γ or TNF-α) mAb (100 μl at 4 mg/ml) in phosphate-buffered saline (PBS) pH 7.3 at room temperature overnight. Then the
plate was washed with PBS containing 0.05% Tween 20 (PBS-T) three times. After this, the plate was incubated with a blocking solution containing 1% bovine serum albumin, 5% sucrose and 0.05% NaN₃ in PBS for more than one hour. After a wash with PBS-T, the collected 100 μl supernatant was then added into each well for 24 hours. After that, the plates were washed with PBS-T three times and then incubated with biotinylated anti-cytokine detection antibodies (100 μl at 12.5 ng/ml) for 2 hours at room temperature. Following the wash, 100 μl of streptavidin horseradish peroxidase (1:2000 dilution of a 1.25 mg/ml solution) was added and incubated for 20 minutes at room temperature. After a triple wash, 100 μl of substrate solution containing 1:1 mixture of H₂O₂ and tetramethylbenzidine was added and incubated for another 20 minutes at room temperature. The reaction was ceased by adding stop solution and the cytokine concentrations were measured with a microplate reader (Dynatech, Chantilly, VA, USA).

Nuclear extract preparation
Nuclear extracts were prepared according to our published work.²² Briefly, the treated cells were left at 4 °C in 70 μl of buffer A (10 mM HEPES, pH 7.9; 10 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol (DTT), 1 mM PMSF, and 3.3 μg/ml aprotinin) for 15 minutes with occasional gentle vortexing. The swollen cells were centrifuged at 15,000 rpm for 3 minutes. After removal of the supernatant (cytoplasmic extract), the pelleted nuclei was washed with 70 μl buffer A and subsequently, the cell pellets were resuspended in 25 μl buffer C (20 mM HEPES, pH 7.9; 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, and 3.3 μg/ml aprotinin) and incubated at 4 °C for 30 minutes with occasional vigorous vortexing. Then the mixtures were centrifuged at 15,000 rpm for 20 minutes and the supernatants were used as the nuclear extracts.

Electrophoresis mobility shift assay (EMSA)
Oligonucleotides containing AP-1 (5’-CGCTTGATGACTCAGCCGAA-3’; 3’-GCGAACATGGCCTT-5’) binding site were purchased and used as the DNA probes (Promega, Madison, WI, USA). The DNA probes were radiolabeled with [γ-³²P]ATP using the T4 kinase (Promega). For the binding reaction, the radiolabeled AP-1 probe was incubated with 5 μg of nuclear extracts prepared from treated cells. The specificity of binding was also examined by competition assays with the 100-fold molar excess of unlabeled oligonucleotides. If competitor DNAs were added, they were preincubated for 10 minutes before the addition of the radiolabeled probes. The binding buffer contained 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol and 2 ng poly(dI-dC). The reaction mixture was left at room temperature to proceed binding reaction for 20 minutes. The final reaction mixture was analyzed in a 6.6% non-denaturing polyacrylamide gel with 0.5X Tris-borate/EDTA (TBE) as an electrophoresis buffer.

Statistics
The results were expressed as means ± SD. A paired or unpaired Student’s t-test was used to determine the significance of differences; a value of P less than 0.05 was considered statistically significant.

RESULTS
Pioglitazone inhibited IL-2 production from activated T cells
We examined the effect of pioglitazone on cytokine production from activated T cells. As expected, incubation of cells with PMA and ionomycin significantly increased IL-2 protein secretion. Human peripheral blood T cells were pretreated with different concentrations of pioglitazone for 2 hours and then stimulated with PMA and ionomycin for another 24 hours. We showed that pioglitazone significantly inhibited IL-2 production from activated T cells at concentration of 1-10 μM in a dose-dependent manner (Figure 1).

Pioglitazone inhibited interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) productions from activated T cells
To examine whether the effects of PPAR activators extended beyond IL-2 to other proinflammatory cytokines, we performed similar experiments measuring IFN-γ and TNF-α proteins expression of human T cells. Human peripheral blood T cells were pretreated with various concentrations of pioglitazone for 2 hours and
then stimulated with PMA and ionomycin for another 24 hours. We demonstrated that pioglitazone effectively inhibited with IFN-γ and TNF-α production in activated T cells at concentration of 1-10 μM in a dose-dependent manner (Figures 2, 3).

**Pioglitazone inhibited IL-4 production from activated T cells**

Since Th1 cytokines, including IL-2, IFN-γ and TNF-α expression, were suppressed by pioglitazone, we examined the effects of pioglitazone on Th2 cytokine production in activated T cells. We performed similar experiments measuring IL-4 protein expression of human T cells. Human peripheral blood T cell were pre-treated with different concentrations of pioglitazone for 2 hours and then stimulated with PMA and ionomycin for another 24 hours. We showed that pioglitazone effectively inhibited IL-4 production in activated T cells at concentration of 1-10 μM in a dose-dependent manner (Figure 4).

**Pioglitazone downregulated AP-1 DNA-binding activity in activated T cells**

Pioglitazone inhibited Th1 and Th2 cytokine pro-
duction effectively in activated T cell. In order to study the intracellular signaling pathway which pioglitazone suppressed, we examined the AP-1 DNA-binding activity in stimulated T cells pretreated with pioglitazone. Human peripheral blood T cells were pretreated with various doses of pioglitazone for 2 hours and then stimulated or not with PMA + ionomycin for one hour. The nuclear extracts from treated cells were analyzed by EMSA. Pioglitazone inhibited AP-1 DNA-binding activity in dose-dependent manner (Figure 5A). For competition assays, cell lysates were preincubated with a 100-fold molar excess of unlabeled AP-1 wild type or mutant oligonucleotide (5'-CGC TTG ATG ACT TGG CCG GAA-3'; 3'-GCG AAC TAC TGA ACC GGC CTT-5'). The wild-type AP-1 caused complete disappearance of the band but mutant type did not, indicating the specificity of AP-1 (Figure 5B).

**DISCUSSION**

The atherosclerotic plaques contain numerous macrophages and T lymphocytes. This suggested that an immune response might take place in the lesions. Cytokines are master regulators of the immune response and are known to regulate and coordinate many stages of atherosclerosis. The helper-T (Th) cells have been categorized on the basis of the pattern of cytokines that they can secrete, resulting in either a cell-mediated immune response (Th1) associated with IL-2, TNF-α, IFN-γ secretion, or a humoral immune response (Th2), associated with IL-4, IL-5, IL-10 and IL-13 secretion. The pro-inflammatory Th1 cytokines, IL-2 and IFN-γ were found in a large portion of atherosclerotic plaques. Subsequent studies have clearly shown a critical pathogenic role for the Th1 response in atherosclerosis. However, the role of Th2 cytokines in atherosclerosis is controversial. They were rarely observed (IL-4 and IL-5) in plaques in previous reports. Deficiency of IL-4 has been associated with a decreased atherosclerotic lesion formation, suggesting a proatherogenic role. With the use of apoE−/− X IL-12−/− and apoE−/− X IL-4−/− mice, it has been shown that Th1 is predominant during the initiation of lesion formation with a switch toward a proatherogenic Th2 response in the chronic phase of plaque development. Our study demonstrated that pioglitazone effectively inhibited both Th1 (IL-2, TNF-α and IFN-γ) and Th2 (IL-4) cytokine production in activated human T lymphocytes. This implicated that pioglitazone had anti-inflammatory effects and might suppress the possible progression of atherosclerotic disease.

According to recent studies, a group of transcription factors as well as some kinases are involved in vascular injury and atherosclerosis. Many cytokine genes are regulated cooperatively by a transcription factor complex consisting of AP-1. AP-1 consists of homodimers or heterodimers of Fos, Jun or ATF subunits which recognize DNA response elements. Many pro-inflammatory genes, including those encoding TNF-α, IL-2, IL-6, E-selectin, MMPs (metalloproteinases)-1,-9,-12 and -13, are regulated by the AP-1 pathway. While IFN-γ exerts its effects on cells mainly through JAK-STAT (signal transducer and activator of transcription) signaling pathway, it may activate Ap-1 DNA binding that requires c-Jun but is independent of JAK1 and STAT1. From this point, AP-1 might be an important modulator in inflammatory diseases. In this study, the AP-1 DNA binding activity in stimulated human peripheral T lymphocytes was blocked by pioglitazone in a dose-dependent manner. Blocking of AP-1 signaling pathway might have played a role when pioglitazone suppressed the cytokines production.

![Image](https://via.placeholder.com/150)
A variety of functions have been attributed to PPAR-γ in different cell types. Several studies have reported potentially anti-inflammatory effects of PPAR-γ ligands in monocytes, macrophages and other type of cells. However, most of these effects are mediated via PPAR-γ independent mechanisms. Chawla and colleagues showed that PPAR-γ ligands have anti-inflammatory effects that are independent of PPAR-γ in embryonic stem cell-derived macrophages that were stimulated with lipopolysaccharide and IFN-γ. These PPAR-γ independent mechanisms interfere with nuclear factor-kappa B and AP-1, phosphatase 2A, extracellular signal-regulated kinase (ERK) and c-Jun NH(2)-terminal kinase (JNK) activity via a process termed transrepression. This may occur in large part via PPAR-γ mediated stabilization of the nuclear receptor corepressor (NCoR) complex. Our data showed one of the possible PPAR-γ independent pathways, AP-1 signaling, through which pioglitazone might exert its anti-inflammatory effects. However, there is limitation of this study in that we could not exclude the possibility of PPAR-γ-dependent pathways participating in the anti-inflammatory action of pioglitazone.

The clinical effects on cardiovascular outcomes have been reported for pioglitazone treatment in prospective randomized trials. In meta-analysis of these studies, pioglitazone was associated with a significantly lower risk of death, myocardial infarction, or stroke among diabetic patients. Beyond the benefit of improved glucose and lipid metabolism by pioglitazone, its anti-atherogenic effects may directly related to the anti-inflammatory ability by different mechanism. However, this study was restricted to in vitro observation of the possible effect of pioglitazone on cytokines and AP-1 DNA binding proteins. In the future, we can perform transfection assay to determine in-vivo effects of pioglitazone in the Jurkat T cells line for promoter activity and conduct the clinical evaluation of possible cytokines production in patients who are taking pioglitazone.

In conclusion, our study provides evidence that PPAR-γ agonist, pioglitazone, might have in vitro anti-inflammatory effects on human peripheral T lymphocytes. These effects could be through inhibition of AP-1 DNA binding activity, one of the mechanisms to regulate the production of cytokines.

ACKNOWLEDGEMENTS

Supported by Tri-Service General Hospital (TSGH-C98-22). Also, thanks to Takeda Pharmaceuticals, Taiwan, for the kind gift (pioglitazone).

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