Inhibition of IL-10-induced STAT3 activation by 15-deoxy-A12,14-prostaglandin J2


Objectives. 15-Deoxy-A12,14-prostaglandin J2 (15d-PGJ2) is a natural ligand that activates the peroxisome proliferator-activated receptor (PPAR)γ, a member of the nuclear receptor family implicated in the regulation of lipid metabolism and adipocyte differentiation. Recent data have shown that 15d-PGJ2 exerts anti-inflammatory action via inhibition of the interferon γ (IFN-γ)-induced Jak-STAT signalling pathway. The anti-inflammatory effect of IL-10 is mediated via activated STAT3 (signal transducer and activator of transcription 3). In this study, we investigated whether 15d-PGJ2 inhibit IL-10-induced STAT activation.

Methods. We used western blotting, flow cytometric analysis and a real-time polymerase chain reaction.

Results. 15d-PGJ2 blocked IL-10-induced STAT1 and STAT3 activation in primary human monocytes, macrophages and THP-1 cells. Inhibition was not specific for IL-10, as induction of STAT activation by IFN-γ and IL-6 was also inhibited by 15d-PGJ2. Inhibition of IL-10 signalling was induced within 1 h after pretreatment of 15d-PGJ2. Other PPARγ agonists, such as troglitazone, did not inhibit IL-10 signalling. Treatment with GW9662, a specific PPARγ antagonist, had no effect on 15d-PGJ2-mediated inhibition of IL-10 signalling even at higher concentrations (50 μM), indicating that 15d-PGJ2 affects the IL-10-induced Jak-STAT signalling pathway via an PPARγ-independent mechanism. Actinomycin D had no effect on 15d-PGJ2-mediated inhibition of IL-10 signalling, indicating that inhibition of IL-10 signalling occurs independently of de novo gene expression. Also, inhibitors of extracellular signal-regulated kinase (ERKs) (PD98059), p38 MAPK (mitogen-activated protein kinase) (SB203580) and protein kinase C (PKC) (GF109203X, calphostin C) had no effect on 15d-PGJ2-mediated inhibition of IL-10 signalling. These results show that MAPKs and PKC are not involved in the inhibition of IL-10 signalling.

Conclusions. We showed that 15d-PGJ2 non-specifically inhibits STAT signalling of the anti-inflammatory cytokine IL-10 as well as the proinflammatory cytokine IFN-γ. These findings indicate the possibility that 15d-PGJ2 can have adverse effects in the management of diseases in which IL-10 plays a critical role in the suppression of inflammation.

Key words: 15d-PGJ2, IL-10, STAT3, Monocytes/macrophages.
IL-10 is known to have suppressive effects on macrophages and this effect is mediated via activated STAT3. In the present study, we investigated whether 15d-PGJ2 inhibits intracellular Jak-STAT signalling of the anti-inflammatory cytokine IL-10 and the proinflammatory cytokine IFN-γ.

Materials and methods

Cell isolation and culture

Monocytes were obtained from peripheral blood mononuclear cells by positive selection, using anti-CD14 magnetic beads, as recommended by the manufacturer (Miltenyi Biotech, Auburn, CA, USA). Monocytes and primary macrophages after 3 days of culture in RPMI 1640 medium supplemented with 10% fetal bovine serum and 20 ng/ml macrophage colony-stimulating factor (R&D Systems). THP-1 cells were cultured at 37°C in 5% CO2 using RPMI medium with 10% fetal bovine serum.

Immunoblotting

Total cellular protein was extracted from several conditioned cells using Pro-prep protein extraction solution (Intron, Seoul, Korea). Extracts corresponding to 2 × 106 cells were separated through 7.5% sodium dodecyl sulphate–polyacrylamide gels, transferred to polyvinylidene difluoride membranes by semidy electrophoretic transfer, and incubated with phospho-specific (Tyr705) STAT3 antibody, phospho-specific (Tyr701) STAT1 antibody (Cell Signalling Technology, Beverly, MA, USA), monoclonal STAT3 and STAT1 antibody (Transduction Laboratories, Lexington, KY, USA) and SOCS1 antibody (Zymed Laboratories, San Francisco, CA, USA). Enhanced chemiluminescence (ECL) western blotting detection reagents (Amersham Biosciences) were used for detection.

Flow cytometric detection of the IL-10 receptor on cell surfaces

The binding of IL-10 to cell surface IL-10Rs was measured using a Fluorokine kit according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN, USA). Analyses were done using a FACScan flow cytometer with CellQuest software (Becton Dickinson, San Jose, CA, USA).

Analysis of cell viability

Trypan blue and propidium iodide (PI) exclusion were used to quantify the numbers of viable cells. The stained and unstained cells were counted with a standard light microscope (trypan blue) or flow cytometer (PI).

Analysis of mRNA levels

Total cellular RNA was isolated using TRIzol reagent (Gibco BRL) according to the instructions of the manufacturer. For reverse transcription–polymerase chain reaction (RT-PCR), RNA was treated with RNase-free DNase (Gibco BRL), and complementary DNA (cDNA) was prepared by RT with a random hexamer primer (Invitrogen) and M-MuLV reverse transcriptase (Gibco BRL). cDNA was amplified by PCR using PCR PreMix (Bioneer, Seoul, Korea) and a pair of primers specific for the genes of interest. Oligonucleotide primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), AAG GTG TGG TGA AGA G and CCT TCT CCA TGG TGA AGA C; SOCS3, CAC TCT TCA GCT CGT TGG TGA AGA C; PCR products were analysed on 1% agarose gel. The PCR product of GAPDH was used as a control for sample loading. Also, we performed real-time PCR using the iCycler iQ thermal cycler and detection system (Bio-Rad Laboratories). mRNA amounts were normalized relative to GAPDH mRNA.

Results

15d-PGJ2 inhibits IL-10-induced STAT activation in monocytes and macrophages

Phosphorylation of STAT3 and STAT1 is induced rapidly by cytokine treatment (20 ng/ml), within several minutes. To determine the effects of 15d-PGJ2 on cytokine-induced STAT phosphorylation, the cells were pretreated with 10 μM 15d-PGJ2 for 2 h, followed by stimulation with the indicated cytokine for 10 min. IL-10 and IL-6 activated predominantly STAT3, and IFN-γ activated predominantly STAT1 in primary human monocytes. Pretreatment for 2 h with 15d-PGJ2 inhibited STAT3 and STAT1 phosphorylation of all cytokines in primary human monocytes, macrophages and THP-1 cells (Fig. 1A, B and C). Inhibition of IFN-γ-induced STAT1 phosphorylation was similar to that reported by Chen et al. [13], who showed that 10 μM of 15d-PGJ2 suppresses IFN-γ signalling in murine macrophages. To exclude the possibility that cell death may contribute to the decreased STAT phosphorylation with 15d-PGJ2 treatment, trypan blue and PI exclusion was used to quantify the numbers of viable cells. Treatment with 15d-PGJ2 for up to 6 h resulted in a less than 10% decrease in the number of viable cells, so we could exclude this possibility (data not shown). In myeloid cells, IL-10 activates predominantly STAT3, and activated STAT3 is mainly involved in negative regulation of macrophage activation, so we focused primarily on the inhibition of 15d-PGJ2 on IL-10-induced STAT3 phosphorylation. We examined the kinetics of inhibition of STAT3 phosphorylation. To determine the time course of 15d-PGJ2 inhibitory effects on IL-10-induced STAT3 phosphorylation, primary macrophages and THP-1 cells were incubated with 10 μM 15d-PGJ2 for various times. Pretreatment with 15d-PGJ2 for 20 min inhibited IL-10-induced STAT3 phosphorylation (Fig. 2A), suggesting that 15d-PGJ2 stimulates a direct, rapid inhibitory pathway or induces an inhibitor within 20 min for inhibition of IL-10-induced STAT3 phosphorylation. 15d-PGJ2 mediated the inhibition of IL-10-induced STAT3 phosphorylation in a dose-dependent manner (Fig. 2B).

Inhibition of IL-10-induced STAT3 phosphorylation by 15d-PGJ2 was not mediated by changes in IL-10 receptor expression

We also determined whether 15d-PGJ2 inhibited proximal events in the IL-10 signalling pathway. Cell surface expression of IL-10 receptors was measured by flow cytometry using biotinylated Fluorokine kit according to the instructions of the manufacturer (R&D Systems). THP-1 cells supplemented with 10% fetal bovine serum and 20 ng/ml macrophage colony-stimulating factor (R&D Systems). THP-1 cells were cultured at 37°C in 5% CO2 using RPMI medium with 10% fetal bovine serum.

The inhibitory actions of 15d-PGJ2 on IL-10-induced STAT3 phosphorylation occur via a PPARγ-independent mechanism

Because of difficulty obtaining sufficient monocytes from individual patients, other experiments were done on THP-1 cells instead of peripheral blood-derived monocytes. Previous studies have demonstrated that 15d-PGJ2 exerts an anti-inflammatory action via a PPARγ-independent mechanism [3], and inhibition
of IFN-γ-induced Jak-STAT signalling by 15d-PGJ₂ is also independent of PPARγ [12, 13]. We examined whether PPARγ mediates the inhibitory action of 15d-PGJ₂ on IL-10-induced STAT3 phosphorylation. First, we used the potent, irreversible and selective PPARγ antagonist GW9662 to prevent activation of PPARγ [14]. GW9662, at concentrations that blocked PPARγ activity, did not affect the inhibitory effect of 15d-PGJ₂ on IL-10-induced STAT3 phosphorylation (Fig. 4A). Secondly, we compared the inhibitory effects of different PPARγ agonists on IL-10-induced STAT3 activation. 15d-PGJ₂ inhibited IL-10-induced STAT3 phosphorylation, but other PPARγ agonist, such as troglitazone, did not inhibit IL-10-induced STAT3 phosphorylation in THP-1 cells (Fig. 4B).

Inhibition of IL-10-induced STAT3 activation by 15d-PGJ₂ is independent of de novo gene expression

We investigated the mechanism of 15d-PGJ₂-induced inhibition of IL-10 signalling. The currently known mechanisms for inhibition of the Jak-STAT signalling pathway are via de novo expression of suppressors of cytokine signalling (SOCS) [15] and a rapid, direct inhibitory pathway that is dependent upon protein kinase C (PKC) or mitogen-activated protein kinases (MAPK) [16–18]. The requirement for de novo production of inhibitors of IL-10-induced STAT3 phosphorylation was investigated by using actinomycin D and cycloheximide to inhibit de novo transcription and translation. When we used cycloheximide for blocking de novo protein synthesis, cycloheximide itself inhibited IL-10-induced STAT3 phosphorylation (Fig. 5A). A recent report [19] showed that cycloheximide can inhibit IL-6 signalling, and this inhibition by cycloheximide was dependent on the p38 stress kinase. So we may guess that inhibition of IL-10-induced STAT3 phosphorylation by cycloheximide is mediated by the p38 stress kinase. But we did not address this question. Inhibition of IL-10-induced STAT3 phosphorylation was preserved when de novo gene expression was blocked using actinomycin D (Fig. 5A). These results indicate that 15d-PGJ₂-induced inhibition of IL-10-induced STAT3 phosphorylation is independent of the expression of new genes. The recently reported, important mechanism for inhibition of Jak-STAT signalling by 15d-PGJ₂ is via de novo expression of SOCS [12]. We tested 15d-PGJ₂-induced SOCS3 expression using real-time PCR and SOCS1 expression using western blotting with THP-1 cells. 15d-PGJ₂ did not induce SOCS3 and SOCS1 expression in THP-1 cells (Fig. 5B and C).

Inhibition of Jak-STAT signalling has been proposed to occur by a rapid and direct inhibitory pathway dependent upon MAPKs, phosphatase or PKC. A recent study suggests that 15d-PGJ₂ stimulates the expression of the IL-8 gene in THP-1 macrophages...
through an MAPK signalling pathway [20]. The role of MAPKs, phosphatase or PKC in inhibition of IL-6 signalling was investigated using specific inhibitors of these kinases. Inhibitors of ERKs (PD98059) and p38 (SB203580) had no effect on the IL-10-induced STAT3 phosphorylation by themselves and did not influence 15d-PGJ2-induced inhibition of IL-10 signalling (Fig. 5D), indicating that MAPKs may be not involved in 15d-PGJ2-induced inhibition of IL-10 signalling. An inhibitor of phosphatase (sodium orthovanadate) also did not influence 15d-PGJ2-induced inhibition of IL-10 signalling (data not shown). Inhibitors of PKC (calphostatin and GF109203X) by themselves suppressed IL-10-induced STAT3 phosphorylation, so we could not definitively rule out the possibility of the involvement of PKC in 15d-PGJ2-induced inhibition of IL-10 signalling (data not shown).

Discussion

We investigated the modulation of cytokine-induced STAT phosphorylation by 15d-PGJ2 in primary human monocytes, macrophages and THP-1 cells. Previous reports have shown that 15d-PGJ2 inhibits IFN-γ-induced STAT phosphorylation in murine macrophages and rat astrocytes [12, 13]. Our results
showed that not only does 15d-PGJ2 inhibit IFN-γ-induced STAT phosphorylation, but it also inhibits IL-10- and IL-6-induced STAT phosphorylation in monocytes. Because of the anti-inflammatory effects of PPARγ agonists, PPARγ has been suggested as a therapeutic target for the treatment of inflammatory diseases [21]. In vivo studies using 15d-PGJ2 and other PPARγ agonists showed that these agents had therapeutic efficacy, and resulted in significant improvement of disease activity [22, 23]. However, in our study, we showed that 15d-PGJ2 inhibits non-specifically the Jak-STAT signalling of the anti-inflammatory cytokine IL-10 as well as proinflammatory cytokines. These findings indicate the possibility that 15d-PGJ2 can have adverse effects in the control of diseases in which IL-10 plays a critical role in the suppression of inflammation. So caution should be exercised before 15d-PGJ2 is used in the treatment of inflammatory diseases.

 Previous reports have demonstrated that inhibition of STAT1 phosphorylation by 15d-PGJ2 is PPARγ-independent [12, 13]. So we investigated the involvement of PPARγ in the inhibition of STAT phosphorylation by 15d-PGJ2. Consistent with previous reports, our data suggest that 15d-PGJ2 inhibits IL-10-induced...
STAT3 phosphorylation. Troglitazone, a synthetic PPAR\gamma agonist, did not inhibit IL-10-induced STAT3 phosphorylation, and the inhibition of IL-10-induced STAT3 phosphorylation by 15d-PGJ\(_2\) was not reversed by GW9662, a selective PPAR\gamma antagonist.

There are several different mechanisms of inhibition of cytokine-induced Jak-STAT signalling. Our results demonstrate that 15d-PGJ\(_2\) generally inhibits the signalling of several cytokines, including anti-inflammatory cytokines. Therefore, further study is required before 15d-PGJ\(_2\) and related agents are used for the treatment of inflammatory diseases.

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References