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15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 induces PPARγ- and p53-independent apoptosis in rabbit synovial cells

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ABSTRACT

A ligand of peroxisome proliferator-activated receptor γ (PPARγ), 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) induces apoptosis in various cells. However, the mechanism appears to be complex and cell-type specific. We investigated the mechanism of 15d-PGJ_2-induced apoptosis of rabbit synovial cells. Exposure to 15d-PGJ_2 resulted in DNA fragmentation accompanied by caspase-3 and -9 activations in the cells, suggesting occurrence of mitochondria-mediated apoptosis. Although the exposure also induced remarkable increase in p53 protein, its transcriptional activity was rather reduced, suggesting non-necessity of p53 in 15d-PGJ_2-induced apoptosis. Covalent binding of 15d-PGJ_2 to cellular proteins including p53 resulted in their insolubilization. N-acetylcysteine inhibited not only the 15d-PGJ_2-induced apoptotic events but also the protein insolubilizations *via* its interaction with 15d-PGJ_2. The studies using a PPARγ-agonist and -antagonist showed noninvolvement of PPARγ in 15d-PGJ_2-induced apoptosis. The pre-exposure to pro-inflammatory cytokines did not affect the cytotoxicity of 15d-PGJ_2 in synovial cells.

Taken together, these results show that 15d-PGJ_2 induces a mitochondria-mediated apoptotic pathway in p53- and PPARγ-independent manners.

1. Introduction

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is produced from a sole precursor, PGD_2 through a series of reactions combined with dehydration and isomerization *in vivo* [1,2] and has been detected in human specimens including urine, cells, and synovial fluid in the articular cavity [1–3]. As a ligand, 15d-PGJ_2 can activate peroxisome proliferator-activated receptor γ (PPARγ), which is a transcription factor that is involved in the differentiation of adipocytes [4,5]. 15D-PGJ_2 is also known to differentially affect the functions of several transcription factors because nuclear factor κB (NFκB) and activator protein-1 (AP-1) are inactivated by its covalent binding [6–8]. In addition, 15d-PGJ_2 was shown to inactivate some transcriptional co-activators such as p300/CBP-associated factor (PCAF), p300, and CREB-binding protein (CBP) through insolubilization [9]. These findings suggest that 15d-PGJ_2 affects the expression of some genes by regulating relevant transcription factors and/or co-activators.

A number of studies over the last 10 years have demonstrated that 15d-PGJ_2 possesses anti-proliferative activity *via* the induction of cell death [10–12]. 15D-PGJ_2 was shown to induce apoptosis independently of PPARγ activation in most of these studies [13,14].

Osteoarthritis (OA) is characterized by synovial inflammation, excessive synovial cell proliferation, and articular cartilage breakdown [15]. Since these pathological changes observed in synovial tissue are associated with clinical symptoms and also reflect joint degradation in OA, synovial tissue is a considerable target of therapy to alleviate the symptoms of OA [16–18]. In OA, synovial cells overproduce diverse pro-inflammatory factors such as IL-1β, TNF-α, matrix metalloproteinases (MMPs), PGE_2, and nitric oxide (NO), and these factors aggravate OA symptoms [16,17]. Since the productions of these pro-inflammatory factors are directly or indirectly regulated by a transcription factor NFκB [19], NFκB is an important target for improvement of OA. 15D-PGJ_2 has been paid attention as a candidate for chemotherapeutic agent of OA and RA (rheumatoid arthritis), because it inhibits NFκB [16,20–22].

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Kawahito et al. demonstrated in advance that 15d-PGJ_2 ameliorated chronic inflammation and pannus formation in rats with adjuvant-induced arthritis and also showed that 15d-PGJ_2 induced apoptosis in a PPARγ-dependent manner in synovial cells derived from RA patients *in vitro* [23]. 15D-PGJ_2 has been reported to induce apoptosis in various cells in a caspase-dependent [3,10,11] or caspase-independent manner [12,24]. In the synovial apoptosis, however, the involvement of caspase remains unclear [23]. In contrast, a report showed that 15d-PGJ_2 did not induce apoptosis but suppressed the NF-κB inhibitor-induced apoptosis of synovial cells derived from OA patients [25]. In addition, other two groups independently reported that 15d-PGJ_2 suppressed the cytokine-induced inflammation of synovial cells derived from OA or RA patients *in vitro*, instead of induction of apoptosis [26–28]. Among these, two reports exhibited that the IL-1β-induced PGE_2 synthase-1 and COX-2 (cyclooxigenase-2) productions was attenuated by 15d-PGJ_2 [26,27], whereas another showed that the TNF-α-induced MMP 13-expression was suppressed by 15d-PGJ_2 through suppression of NF-κB [28]. In all of these studies, synovial cells of arthritic patients were used [23,25–28]. These facts suggest that these synovial cells had been exposed to multiple combinations of pro-inflammatory stimuli such as TNF-α and IL-1β for an unfixed duration *in vivo*. The indefinite pre-exposure may affect the susceptibility of the synovial cells to 15d-PGJ_2 *in vitro* and may cause dissimilarities among the conclusions described in above reports. Therefore, in order to exclude the influence of the pre-exposure, we used HIG-82, which is a synovial cell line established from the articular synovium of healthy rabbit in the present study. HIG-82 cells can express inducible enzymes such as COX-2 and iNOS (inducible NO synthase) in response to mechanical loading [29], suggesting that NF-κB signaling pathway is functionally conserved. By employing this cell line, we demonstrated that 15d-PGJ_2 induced cell death in a PPARγ-independent manner. 15d-PGJ_2-induced death was accompanied by the activation of caspases-3, -7, and -9 and an accumulation of p53 protein, which suggests the occurrence of mitochondria-mediated apoptosis. However, 15d-PGJ_2-induced p53 accumulation did not contributed to the apoptosis, because p53 lost its transcriptional activity. The loss of function in p53 was associated with formation of 15d-PGJ_2-p53 conjugatesand its insolubilization. Thus, 15d-PGJ_2 induced apoptosis through an alternative pathway independently of p53.

2. Materials and methods

2.1. Reagents

15D-PGJ_2, biotinylated 15d-PGJ_2, troglitazone, and GW9662 were purchased from Cayman Chemical, USA. Three synthetic substrates of caspases [30], Ac-DEVD-MCA, Ac-LEHD-MCA, and Ac-IETD-MCA, and a pan-caspase inhibitor z-VADfmk [31,32] were purchased from PEPTIDE Institute Inc., Japan. N-acetylcysteine (NAC), L-N^G-nitroarginine methyl ester (L-NAME), α-tocopherol, and ascorbic acid were from Nacalai Tesque Inc., Japan. Bilirubin was from Wako Pure Chemicals Co., Japan. Resveratrol and glutathione (GSH) were from Sigma Aldrich Japan. Pifithrin α (Pft. α) was from Enzo Life Sciences Inc. Recombinant human TNF-α, murine TNF-α, and murine IL-1β were obtained from PeproTech. Inc., USA.

2.2. Antibodies

Mouse monoclonal anti-p53 (DO-1) and rabbit polyclonal anti-PPARγ (H-100) antibodies were purchased from Santa Cruz. Mouse monoclonal anti-human β-actin and anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Applied Biological Materials (ABM), Inc. Canada and Applied Biosystems Inc., Japan, respectively. Goat polyclonal anti-human PARP1 (poly(ADP-ribose) polymerase 1), mouse monoclonal anti-polyubiquitin, and rabbit polyclonal anti-biotin antibodies were obtained from R & D Systems Inc., Enzo Life Sciences Inc., and BETHYL Lab. Inc., USA, respectively. The horseradish peroxidase-conjugated goat polyclonal anti-rabbit IgG antibody and horseradish peroxidase-conjugated rabbit polyclonal anti-murine IgG antibody were purchased from DAKO Cytomation.

2.3. Cell culture

A cell line of rabbit synovial fibroblasts (HIG-82) was obtained from the American Type Culture Collection (ATCC) and maintained in a culture medium (Ham's F-12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS)) at 37 °C in a humidified CO_2 incubator under 5% CO_2 [29].

2.4. Treatment of cells with 15d-PGJ_2

HIG-82 cells were seeded at 5×10^5 cells/2 ml of the culture medium in a 12-well plate and cultured at 37 °C in a CO_2-incubator for 24 h. Since albumin in FBS has been shown to attenuate the effects of 15d-PGJ_2 on cells by its binding to the PG [33], the culture medium was changed to 2 ml of FBS-free medium (OPTI-MEM1; GIBCO BRL, Canada) immediately prior to the treatment. Biotinylated 15d-PGJ_2 was used instead of 15d-PGJ_2 to examine the binding of 15d-PGJ_2 to cellular proteins. In some experiments, cells were pretreated with the indicated reagents prior to the treatment with 15d-PGJ_2 as described in figure legends.

2.5. Cytotoxicity assay

Cytotoxicity of 15d-PGJ_2 was determined by a colorimetric assay using Cell Counting Kit-8 (CCK-8) (DOJINDO Molecular Technologies, Inc, Japan). HIG-82 cells (2×10^4 cells/100 μl of OPTI-MEM1) were cultured in a 96-well plate for 24 h and then treated with 15d-PGJ_2 at the indicated concentration or for the indicated time. After treatment, 10 μl aliquots of CCK-8 were added into the wells, and incubated for 1 h in a CO_2^- incubator. Absorbance was measured at 450 nm using a plate reader (SpectraMax M2e HK, Molecular Devices).

2.6. Reporter plasmids

Reporter plasmids, pp53-TA-luciferase (pp53-TA-luci), pNFκB-TA-luciferase (pNFκB-TA-luci), pRSV-®-galactosidase (pRSV®gal), and p®-galactosidase-basic (p®gal-basic) were obtained from Clontech Laboratories. Inc. A reporter plasmid for PPARγ was constructed as follows; a responsive element free plasmid, pTAL-luciferase (Clontech) was digested with Nhe I and Xho I and then purified. The digested plasmid was ligated with a DNA consisting of 5′-CTAGCAGGTCAAAGGTCACTAGTAGGTCAAAGGTCAC-3′ and 3′-GTCCAGTTTCCAGTGATCATCCAGTTTCCAGTGAGCT-5′) (underlined sequences are positions of PPAR-response element [34]). After ligation, the plasmid was propagated in *E. coli* JM109 and then purified. The constructed plasmid was designated as pPPRE-luciferase (pPPRE-luci).

2.7. Transfection of reporter genes and the luciferase reporter assay

Plasmid carrying luciferase gene (100 ng) was mixed with either 10 ng of pRSV®gal or 50 ng of pβgal-basic. In some cases, a p53 expression vector, pCMV-p53WT (pCMV-Neo-Bam/p53; a gift from by Dr. B. Vogelstein), was added to the above mixture of reporter

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