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Role of STAT3-Interacting Protein (STIP1) in Δ^{12}-Prostaglandin J_2-Induced Cell Death

Seong-Mook Kim, Sun Lee, Hwan-Jong Kwak, Bo-Eun Kim, Dong-Jin Kim, In-Kyung Kim, and Seong-Whan Jeong

Department of Biochemistry, College of Medicine, The Catholic University of Korea, Seoul, Korea

Δ^{12}-Prostaglandin J_2 (Δ^{12}-PGJ_2) is one of cyclopentenone prostaglandins. The Δ^{12}-PGJ_2 is known to induce apoptosis of tumor cells, however, it's action mechanism is not clear. It has recently been reported that STAT3 is involved in tumorigenesis. In the present study, we investigated the role of STAT3-interacting protein (STIP1) in the cytotoxicity of Δ^{12}-PGJ_2, since STIP1 was recently reported as a modulator of STAT3 activation by specifically binding to inactive (unphosphorylated) STAT3. The effect of Δ^{12}-PGJ_2 was observed in stably overexpressing Neuro-2A cells transfected with full cDNA of STIP1, and cytotoxicity of Δ^{12}-PGJ_2 in the transfected cells was increased, compared with the vector control cells. The cytotoxicity of Δ^{12}-PGJ_2 treatment was significantly accentuated by pretreatment of the STIP1-transfected cells with protein kinase inhibitor, genistein, and less activation of STAT3 in STIP1-transfected cells was shown, compared with the vector control cells. Expression of bax was also increased in the STIP1-transfected cells. These data suggest that STIP1 inhibits cell growth via inhibition of STAT3 activation in Δ^{12}-PGJ_2 treatment.

Key Words: Δ^{12}-Prostaglandin J_2, STAT3-interacting protein, STAT3, Apoptosis, Bax

INTRODUCTION

It is known that cyclopentenone prostaglandins (PGs), such as PGA_2 and Δ^{12}-PGJ_2, have the inhibitory activity of tumor cell growth (Fukushima et al, 1994; Lee et al, 1995; Ahn et al, 1998). PGA_2 and Δ^{12}-PGJ_2 are enzymatic dehydration products of PGE_2 and PGD_2, respectively. In previous report, we described that human hepatocellular carcinoma cells exposed to Δ^{12}-PGJ_2 showed the morphological characteristics of apoptosis such as chromatin condensation, nuclear fragmentation, and formation of apoptotic bodies (Ahn et al, 1998). Gene expressions of p53, c-myc, and heat shock protein 70 were also modulated by the exposure to Δ^{12}-PGJ_2. These genes and their products play roles as a positive or negative regulator in Δ^{12}-PGJ_2-induced apoptotic cell death (Lee et al, 1995; Ahn et al, 1998; Ahn et al, 1999; Ahn et al, 2002). However, the precise mechanisms of Δ^{12}-PGJ_2-induced apoptosis are not fully understood.

In cDNA library screening to isolate new genes involved in oxidative stress-induced apoptosis, we cloned a gene that was down-regulated by the exposure to hydrogen peroxide: The gene was STAT3-interacting protein (STIP1) reported by Collum et al (2000) who showed that STIP1 is potential scaffold protein that promotes the interaction between Janus kinases and their STAT3. The preferential association of STIP1 with inactive (unphosphorylated) STAT3 suggests that it may contribute to the regulation of STAT3 activation.

One of the mechanisms of Δ^{12}-PGJ_2-induced apoptosis is the inhibition of NF-κB activation via activation of peroxisome proliferator-activated receptor-γ (PPAR-γ) as a Δ^{12}-PGJ_2 receptor (Kim et al, 2003). Various tumor cells show higher level of STAT3 activation (Coffer et al, 2000; Song & Grandis, 2000). Ricote et al (1998) reported that PPAR-γ and STAT3 are reciprocally regulated by each other, suggesting that STAT3 activation may lead to the inactivation of PPAR-γ. Therefore, STIP1 may contribute to the tumor cell apoptosis by the inhibition of STAT3 inhibitory to PPAR-γ.

In the present study, we examined the role of STIP1 in Δ^{12}-PGJ_2-induced cell death, and found that overexpression of STIP1 gene in neuroblastoma cells inhibited the activation of STAT3 and increased the cytotoxicity of Δ^{12}-PGJ_2.

METHODS

Materials

Rat PC12 and mouse Neuro-2A (neuroblastoma) cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, LipofectAMINE and G418

Corresponding to: Seong-Whan Jeong, Department of Biochemistry, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Socho-gu, Seoul 137-701, Korea. (Tel) 82-2-590-1178, 1182, (Fax) 82-2-596-4435, (E-mail) swjeong@cmc.cuk.ac.kr, swjeong@catholic.ac.kr

ABBREVIATIONS: Δ^{12}-PGJ_2, Δ^{12}-prostaglandin J_2; STIP1, STAT3-interacting protein; PPAR-γ, peroxisome proliferator-activated receptor-γ.

were obtained from Life Science (Grand Island, NY): Fetal bovine serum and horse serum were from HyClone (Logan, UT): Δ^{12}-PGJ_2 was from BioMol (Plymouth Meeting, PA): H_2O_2, cisplatin, and mouse anti-β-actin antibody were from Sigma (St. Louis, MO): RNAzol was from Tel-Test (Friendswood, TX): Terminal transferase, mRNA isolation kit, nylon membranes, blocking reagent, digoxigenin (DIG)-11-dUTP, CDP-STAR and anti-DIG antibody conjugated with alkaline phosphatase were from Boehringer Mannheim (Indianapolis, IN): The PCR-select cDNA subtraction kit was from Clontech (Palo Alto, CA): pBluescript II KS vector was from Stratagene (La Jolla, CA): The plasmid DNA isolation kit was from Promega (Madison, WI): The DNA sequencing kit was from USB (Cleveland, Ohio): The pCR3.1 expression vector was from Invitrogen (Carlsbad, CA): The enhanced chemiluminescence kit was from Amersham (Buckinghamshire, England): Mouse anti-STAT3 and anti-bax antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA): Rabbit anti-phospho-STAT3 antibody was from Cell Signaling Technology (Beverly, MA). Unless specified otherwise, all other reagents were from Sigma.

Cell culture

PC12 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 5% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 10% CO2/ 90% air. Neuro-2A cells were cultured in the above medium, lacking horse serum, at 37°C in 5% CO2/95% air.

Preparation of PC12 cDNA libraries and screening

Total RNA was isolated from either 800 μM H_2O_2-treated or -untreated PC12 cells collected 8h after the treatment using RNAzol solution, according to the manufacturer's (Tel-Test) manual. Messenger RNA was purified from total RNA using an mRNA isolation kit, and double-stranded cDNA was synthesized from the mRNA using a PCR-select cDNA subtraction kit. After endonuclease digestion with RsaI, the cDNA was poly(A)-tailed at both ends using terminal transferase, and amplified by PCR with the AL1 primer (5'-ATTGGATCCAGGCCGCTCTGGACAAAATATG AATTCTTTTTTTTTTTTTTTTTTT-3') containing an EcoRI site in the 5' end. Plasmid cDNA libraries from the H2O2-treated and -untreated PC12 cells were prepared by ligation of the cDNAs digested with EcoRI into pBluescript II KS vector. Clones expressed specifically by the H_2O_2-treated PC12 cells were isolated by a differential screening method (Sargent, 1987). Colonies of master plates were transferred to nitrocellulose sheets after bacterial transformation of the cDNA libraries, and screened by hybridization with a ^{32}P-labeled cDNA probe prepared from the cDNA library of H_2O_2-treated PC12 cells. Colonies from H_2O_2-treated PC12 cells showing specifically increased signals in the cDNA library were selected, and the plasmid DNA was isolated using a plasmid DNA isolation kit. Sequences of the clones were determined by DNA sequencing kit and matched by searching in GenBank.

Northern blot analysis

Total RNA from PC12 and Neuro-2A cells, treated with H_2O_2 and Δ^{12}-PGJ_2 in serum-free DMEM, was separated on 1% agarose gels containing 7% formaldehyde, and transferred to nylon membranes. After UV cross-linking, the membranes were incubated in prehybridization solution containing 5× SSC (20× SSC; 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 50% formamide, 7% SDS, and 2% blocking reagent at 50°C for 2 h. Hybridization was done in prehybridization solution containing DIG-labeled cDNA probe corresponding to 1~324 nucleotide of mouse STIP1 cDNA (GenBank Accession No., AF291064) at 50°C for 15 h. The membrane was washed three times in 2× SSC containing 0.1% SDS at room temperature for 10 min, and again three times in 0.2× SSC containing 0.1% SDS at 55°C for 10 min. The hybridization signals were visualized by chemiluminescent detection using anti-DIG antibody conjugated with alkaline phosphatase and the substrate, CDP-STAR.

Preparation of stably transfected Neuro-2A cells and measurement of cell viability

The insert 2.5 kb cDNA, corresponding to full length of mouse STIP1 cDNA (GenBank Accession No., AF291064), was ligated into cytomegalovirus (CMV) promoter-containing expression vector (pCR3.1) which was digested with HindIII/XbaI. The plasmid DNA was isolated using a plasmid DNA isolation kit after confirming the sequence and direction by DNA sequencing. The plasmid DNA was transfected into Neuro-2A cells using LipofectAMINE. After 2 days of culture, the transfected cells were selected by addition of 1 mg/ml G418, changing medium every 4 days for two weeks. The stable cells were maintained in 0.2 mg/ml G418. Effective expression of the genes was confirmed by comparing them with mock vector-transfected control cells by Northern hybridization analysis. To examine the role of the gene, various concentrations of Δ^{12}-PGJ_2 or cisplatin in serum-free DMEM were added to each well after 16 h culture of 1×10^4 cells seeded into 96-well plates and, after further culture for 16 h, cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay. The cell viability assay was also performed, following pretreatment with tyrosine kinase inhibitor, genistein (5 μM), 1 h before the additions of various concentrations of Δ^{12}-PGJ_2. Cell viability was expressed as a percentage of that observed in untreated cells.

Western blot analysis

The stably transfected cells were incubated with the various concentrations of Δ^{12}-PGJ_2 or cisplatin in serum-free DMEM for 8 h. After the incubation, the cells were harvested and washed with phosphate-buffered saline, and lysed with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS). The lysates equivalent to 20 μg protein were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes. After blocking with 5% skim milk for 2h, the membrane was incubated with primary antibody against STAT3, phosphorylated STAT3, bax, or β-actin protein. Anti-mouse or anti-rabbit IgG antibody conjugated with peroxidase was used as secondary antibody, and protein signals were visualized using an enhanced chemiluminescence kit.

Statistics

Data are presented as mean±SEM. Statistical significance of differences was determined by unpaired t test. Dif-

ferences were considered significant at $p<0.05$.

RESULTS

Isolation of a gene showing decreased expression after H_2O_2 treatment

It is well known that PC12 cells are induced to undergo apoptosis by H_2O_2 treatment. cDNA libraries prepared from 800 μM H_2O_2-treated and -untreated PC12 cells were used to isolate genes related to apoptosis of the cells by a differential screening method. Three clones showed distinct signal to the probe prepared with the cDNA library from H_2O_2-treated cells, and cDNA of the clones was sequenced and searched in GenBank. One of them was found to be identical to STAT3-interacting protein (STIP1) gene (GenBank Accession No., AF291064). As shown in Fig 1A, the expression of the STIP1 gene was decreased in a time-dependent manner by the treatment of PC12 cells with 500 μM H_2O_2.

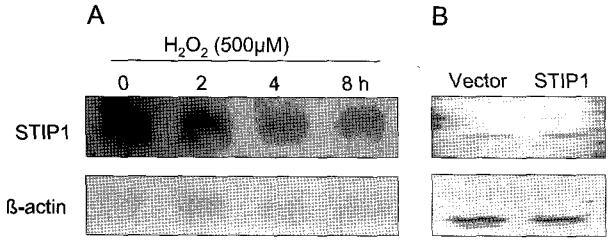

Fig. 1. Northern blot analysis. A, Decrease of STAT3-interacting protein (STIP1) gene expression in H_2O_2-treated PC12 cells. Total RNAs from PC12 cells treated with 500 μM H_2O_2 for the indicated time were separated on 1% agarose gels and transferred to nylon membranes. The membranes were incubated with a DIG-labeled probe corresponding to the 324 bp N-terminal sequence of STIP1 cDNA. The hybridization signals were visualized by chemiluminescent detection, using anti-DIG antibody conjugated with alkaline phosphatase and the substrate, CDP-STAR. The β-actin probe was used to confirm the equal loading in agarose gel electrophoresis. B, Effective expression of STIP1 gene in stably transfected Neuro-2A cells. Full length of STIP1 2.5 kb cDNA in expression vector was transfected into Neuro-2A. The transfected cells were selected by addition of 1 mg/ml G418, changing medium every 4 days for two weeks. Effective expression of the genes was determined by comparing them with mock vector-transfected control cells in Northern hybridization analysis, as described above.

Measurement of Δ^{12}-PGJ$_2$-induced cytotoxicity in STIP1-transfected cells

The decreased STIP1 gene expression in PC12 cells treated with H_2O_2 suggested that the gene might have a role in the process of cell death. Therefore, a Neuro-2A cell line was chosen to further characterize the role of STIP1 gene in Δ^{12}-PGJ$_2$-induced cytotoxicity. Full size of STIP1 cDNA was cloned in CMV promoter-containing expression vectors and stably transfected into Neuro-2A cells. Northern hybridization analysis confirmed the effective expression of the genes in the transfected cells (Fig. 1B). The amount of STIP1 mRNA in the transfected cells was significantly increased, compared with that of vector control cells. As seen in Fig. 2A, the STIP1-transfected cells showed a slight but significant increase of cytotoxicity by Δ^{12}-PGJ$_2$ treatment, as compared with that of vector control cells. To compare the action mechanism of Δ^{12}-PGJ$_2$, the cells were treated with cisplatin, an apoptosis-inducing agent (Fig. 2C). The pattern of cytotoxicity in the cells was different from that of Δ^{12}-PGJ$_2$ treatment. The cytotoxicity of Δ^{12}-PGJ$_2$ treatment in the STIP1-transfected cells was significantly accentuated by pretreatment with protein kinase inhibitor, genistein (Fig. 2B). These data suggest that the STIP1 is inhibitory to the activation of STAT3.

Protein expression pattern in Δ^{12}-PGJ$_2$ treatment

The increase of cytotoxicity in STIP1-transfected cells suggests that STIP1 contributes to the modulation of STAT3 activation in Δ^{12}-PGJ$_2$ treatment. To confirm the possibility, Western blot analysis was performed with protein lysates prepared from the transfected cells after treatments with Δ^{12}-PGJ$_2$ (Fig. 3). Less activation of STAT3 in the STIP1-transfected cells was detected than in the vector control cells. The expression of STAT3 protein was increased in the STIP1-transfected cells, possibly due to compensatory response to the inhibitory effect of STIP1.

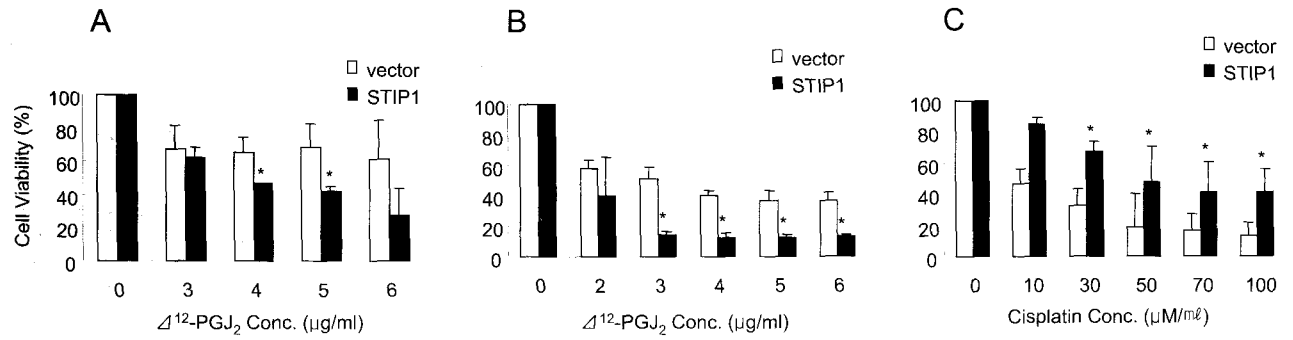

Fig. 2. Cytotoxicity assay in STAT3-interacting protein (STIP1)-transfected Neuro-2A cells treated with Δ^{12}-prostaglandin J$_2$ (Δ^{12}-PGJ$_2$). Transfected Neuro-2A cells with the full length of STIP1 2.5 kb cDNA and control vector were treated with the indicated concentration of Δ^{12}-PGJ$_2$ (A) and cisplatin (C) in serum-free DMEM for 16 h. Cell viability was determined by MTT reduction assay. The cell viability assay was also performed, following pretreatment with tyrosine kinase inhibitor, genistein (5 μM), 1 h prior to the additions of various concentrations of Δ^{12}-PGJ$_2$ (B). Data represent mean±SEM of four separate experiments. *$p<0.05$, compared with the cells treated with the same concentrations in control vector-transfected cells.

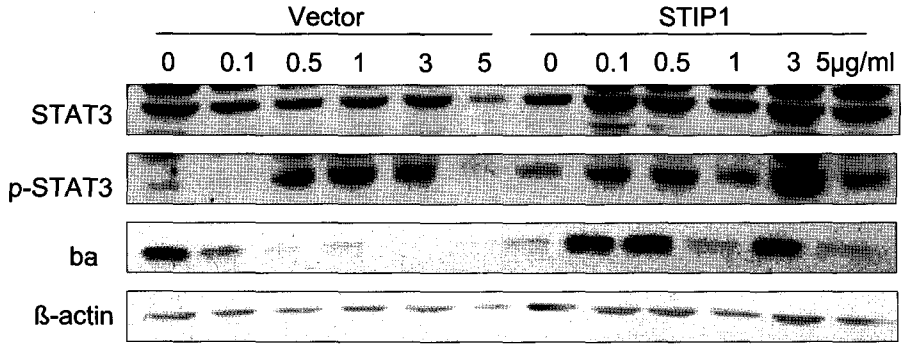

Fig. 3. Expression pattern of proteins in STAT3-interacting protein (STIP1)-transfected Neuro-2A cells treated with Δ^{12}- prostaglandin J_2 (Δ^{12}-PGJ_2). The transfected cells were incubated with various concentrations of Δ^{12}-PGJ_2 in serum-free DMEM for 8 h. Protein lysates were prepared with RIPA buffer. The lysates equivalent to 20 μg protein were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and were transferred to nitrocellulose membranes. The membrane was incubated with primary antibody against STAT3, phosphorylated STAT3, bax, or β-actin protein. Anti-mouse or anti-rabbit IgG antibody conjugated with peroxidase was used as secondary antibody, and protein signals were visualized using an enhanced chemiluminescence kit.

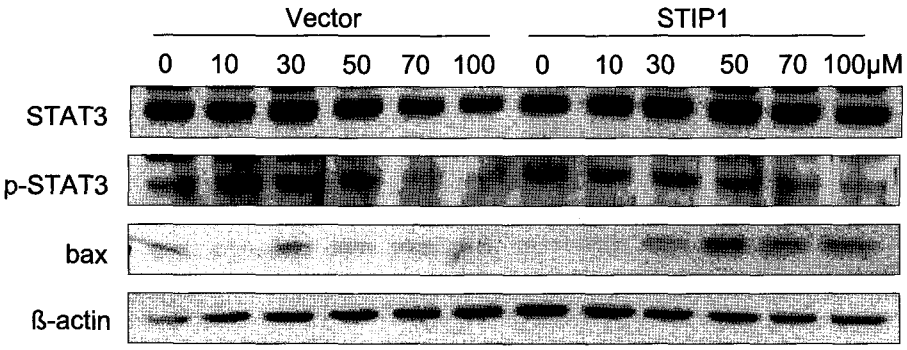

Fig. 4. Expression pattern of proteins in STAT3-interacting protein (STIP1)-transfected Neuro-2A cells treated with cisplatin. The transfected cells were incubated for 8 h with various concentrations of cisplatin in serum-free DMEM. Expressions of STAT3, phosphorylated STAT3, bax, and β-actin protein were examined by Western blot analysis, as described in Fig. 3.

Several Bcl-2 family proteins were examined to identify the target proteins involved in Δ^{12}-PGJ_2-induced cytotoxicity. The bax, one of apoptosis-inducing proteins, was significantly increased in the STIP1-transfected cells. The cells treated with cisplatin showed protein expression patterns different from that of Δ^{12}-PGJ_2 treatment (Fig. 4). Expression of STAT3 and bax and activation of STAT3 in both cells were not changed.

DISCUSSION

It has been known that Δ^{12}-PGJ_2, a cyclopentenone PG, induces apoptotic death of tumor cells (Lee et al, 1995; Ahn et al, 1998). In the apoptosis, expression of p53, c-myc, and heat shock protein 70 were modulated by Δ^{12}-PGJ_2 (Lee et al, 1995; Ahn et al, 1998; Ahn et al, 1999; Ahn et al, 2002). However, the precise mechanisms of Δ^{12}-PGJ_2-induced apoptosis are not fully understood.

In cDNA library screening to isolate new genes involved in H_2O_2-induced apoptosis in PC12 cells, we have cloned a gene, STIP1. The STIP1 is preferentially associated with inactive (unphosphorylated) STAT3 (Collum et al, 2000), which suggests that STIP1 serves as an inhibitory protein in STAT3 activation.

One of the mechanism in Δ^{12}-PGJ_2-induced apoptosis is the inhibition of NF-κB activation via activation of PPAR-γ (Kim et al, 2003). Various tumor cells show higher level of STAT3 activation (Coffer et al, 2000; Song & Grandis, 2000), and Ricote et al (1998) suggested that STAT3 activation leads to the inactivation of PPAR-γ. Therefore, the STIP1 inhibitory to STAT3 activation might contribute to apoptosis of tumor cells.

STATs comprise a family of transcription factors. STAT3 and STAT5 have a role as an oncogene in tumorigenesis, whereas STAT1 has a role as a suppressor. If the STIP1 has a role in inhibition of STAT3 activation, the growth inhibitory activity of Δ^{12}-PGJ_2 can be modulated by the level of STIP1. To examine the possibility, we prepared the STIP1-transfected stable cells using neuroblastoma cells,

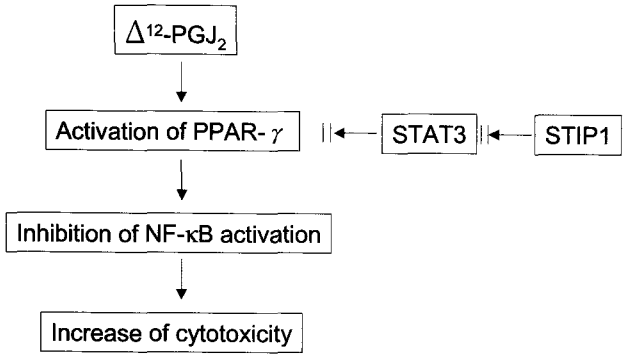

Fig. 5. Proposed model for STAT3-interacting protein (STIP1) in Δ^{12}-prostaglandin J$_2$ (Δ^{12}-PGJ$_2$)-induced cytotoxicity.

Neuro-2A. The STIP1-transfected cells showed a slight but significantly increased cytotoxicity by Δ^{12}-PGJ$_2$ treatment, as compared with that of vector control cells. If the inhibition of STAT3 activation by STIP1 is directly involved in cytotoxicity by Δ^{12}-PGJ$_2$, agents that modulate STAT3 phosphorylation would be suspected to lead to changes of the cytotoxicity pattern. In the present study, the cytotoxicity by Δ^{12}-PGJ$_2$ treatment was significantly accentuated by pretreatment of the STIP1-transfected cells with protein kinase inhibitor, genistein. These data suggest that the STIP1 is inhibitory to the activation of STAT3, which leads to increase of PPAR-γ activation and eventually inhibition of NF-κB activation (Fig. 5). When the cells were treated with an apoptosis-inducing agent cisplatin, the pattern of cytotoxicity was different from that of Δ^{12}-PGJ$_2$ treatment, implying different action mechanisms between Δ^{12}-PGJ$_2$ and cisplatin.

The increase of cytotoxicity in STIP1-transfected cells suggests that STIP1 contributes to the modulation of STAT3 activation by Δ^{12}-PGJ$_2$ treatment. To confirm the possibility, Western blot analysis was performed with protein lysates prepared from the STIP1-transfected cells after treatments with Δ^{12}-PGJ$_2$. Less activation of STAT3 in STIP1-transfected cells was shown than with the vector control cells. Several Bcl-2 family proteins were examined to identify the target proteins involved in Δ^{12}-PGJ$_2$-indueced cytotoxicity. The bax, one of apoptosis-inducing proteins, was significantly increased in STIP1-transfected cells. It has been reported that the inhibition of STAT3 activation increases bax expression in mycosis fungoides tumor cells (Nielson et al, 1999) and ischemic preconditioning of rat heart (Hattori et al, 2001). However, it is not clear whether STAT3 acts as a direct suppressor of bax expression. It seems highly likely that the effector molecule of Δ^{12}-PGJ$_2$ is bax which is modulated by STIP1/STAT3 interactions. The cells treated with cisplatin showed protein expression patterns different from that of Δ^{12}-PGJ$_2$ treatment, suggesting that the action mechanism of Δ^{12}-PGJ$_2$ and cisplatin is different.

In summary, we found that STIP1 exhibits inhibitory activity of cell growth via the inhibition of STAT3 activation in Δ^{12}-PGJ$_2$ treatment. These data suggest that STIP1 might be a new tumor therapeutic target. However, further studies are needed to clarify whether the inhibition of STAT3 activation induces activation of PPAR-γ and subsequently inhibits NF-κB activation in Δ^{12}-PGJ$_2$ treatment.

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