Tautomycetin suppresses the TNFα/NF-κB pathway via inhibition of IKK activation

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Abstract. TNFα activated NF-κB and associated regulatory factors including IKK are strongly implicated in a variety of hematological and solid tumor malignancies. We show that tautomycetin (TC) specifically inhibits activation of NF-κB among the three TNFα effectors (NF-κB, JNK and caspase). TC inhibited T-loop phosphorylation of IKKα and IKKß, thereby preventing degradation of the NF-κB inhibitor, IκBα. Co-immunoprecipitation experiments revealed that the catalytic subunit of PP1 (PP1C) was involved in the IKK complex. Pull-down analysis using recombinant GST-TNFα, showed that PP1C was recruited to TNFR1 together with IKK complex, RIP and TAK1 upon stimulus. These results suggest that the PP1 positively regulates the TNFα-induced NF-κB pathway at the level of IKK activation. Thus, TC might be used therapeutically to suppress the TNFα/NF-κB pathway.

Introduction

TNFα induces trimerization of TNFR1 and triggers three signaling pathways leading respectively to activation of the transcription factor NF-κB, c-Jun N-terminal kinase (JNK), and caspases (1-3). Two sequential signaling complexes function in TNFα-induced cascades (3-6). The first (complex I) consists of TNFR1, TRADD, RIP and TRAF2, which trigger IKK and JNK activation. Activated IKK phosphorylates the NF-κB inhibitor IκBs (IκBα, IκBß and IκBε) and targets them for degradation by the ubiquitin-proteasome pathway, enabling NF-κB to enter the nucleus and activate a subset of TNFα target genes. Subsequently, complex I dissociates and TRADD and RIP associate with FADD and caspase-8, forming complex II. FADD via its death effector domain mediates recruitment and activation of caspase-8, leading to release of its active form, which then activates caspase-3 and caspase-7, which execute the apoptotic process.

In these cascades, NF-κB is known to promote cell survival (3-5,7). When NF-κB is activated during complex I formation, it blocks complex II-mediated caspase-8 activation through induction of caspase-8 inhibitory proteins [c-FLIP (also called CASH), Casper, CLARP, FLAME, I-FLICE, MRIT and Usurpin] and an adaptor protein with a pseudocaspase domain that specifically inhibits caspase-8. NF-κB activation promotes cell survival, whereas prolonged JNK activation enhances TNFα-induced death (3,4), indicating that the balance between NF-κB and JNK activities determines the outcome of TNFα signaling. Constitutively actived NF-κB contributes to tumorigenesis by up-regulating gene expression, which promotes growth and survival of cancer cells (8-11). The mechanism of its activation is not fully understood, however, significance of TNFα/IKK/NF-κB pathway has been revealed. Anti-TNFα antibody inhibits constitutive activation of NF-κB in head and neck squamous cell carcinoma and can suppress proliferation of this tumor (9). The MUC1 oncoprotein is aberrantly overexpressed in most human carcinomas and MUC1 is associated with constitutive activation of NF-κB through enhancement of TNFα-induced IKK activation (10). Surprisingly, tumor suppressor TSC1 is phosphorylated and inactivated by the activated IKK following stimulus with TNFα. The IKK-mediated TSC1 suppression activates TOR pathway, enhances angiogenesis and results in tumor

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Abbreviations: JNK, c-Jun N-terminal kinase; CHX, cycloheximide; DMSO, dimethyl sulfoxide; GST, glutathione S-transferase; NBD/γBD, NEMO/γ binding domain; OA, okadaic acid; PBS, phosphate-buffered saline; PP2Cß, Ser/Thr protein phosphatase type 2Cß; PP6, protein phosphatase type 6; PP2A, Ser/Thr protein phosphatase type 2A; PP1, Ser/Thr protein phosphatase type 1; TC, tautomycetin

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development. Thus, NF-κB and IKK have been proposed to be therapeutic targets in cancer (11).

The IKK complex is composed of two highly homologous catalytic subunits α and ß (IKKα and ß) and regulatory subunits (IKKγ/NEMO) (1-5). Despite the fact that IKKα and IKKß have similar structures, their roles in NF-κB activation differ (12-16). For example, deletion of the subunits separately in mouse embryonic fibroblast (MEF) shows that IKKß and IKKγ are required for TNFα and IL-1-induced phosphorylation of IκBα, while IKKα is dispensable (12-14). Moreover, in baculoviral recombinant systems, the IKKß homodimer has an ~30-fold higher activity towards IκBα than the IKKα homodimer (15,16). Thus, IKKß rather than IKKα is essential for NF-κB induction by most proinflammatory stimuli. Many potential phosphorylation sites in proteins of the IKK complex may affect IKK activity. In particular, Ser-177 and -181 in the T-loop of IKKß (Ser-176 and -180 in IKKα) are sites where phosphorylation mediates conformational changes resulting in kinase activation (15). It has been proposed that complex I activates IKK and JNK through activation of the mitogen-activated protein kinase kinase kinase (MAP3K) families including TAK1 (1,5-7). These MAP3Ks are responsible for T-loop phosphorylation. IKKα activity is also regulated by PKB/Akt, mediated phosphorylation at Thr-23 (17). However, mechanisms of TNFα-induced activation of IKK and JNK cascades are not well understood.

The Ser/Thr protein phosphatase type 1 (PP1) is composed of a catalytic subunit (PP1C) and several targeting/regulatory subunits. Thus far, four PP1C isoforms, α, γ1, γ2 and δ have been shown to be widely expressed in mammalian tissues (18-21). Biochemical analysis of bacterially expressed PP1C isoforms of all four types indicates that they have similar properties (22). PP1C is regulated by interaction with diverse subunits that target it to specific subcellular locations, regulate its activity, or define substrate specificity (23,24).

We previously showed that tautomycetin (TC) is a PP1-specific inhibitor and that treatment with 5 μM TC completely inhibits PP1 activity without affecting PP2A activity (25,26). Therefore, it was deemed a significant advantage for *in vivo* analysis of PP1 and PP2A activity that treatment with 5 μM TC and 100 nM OA could differentiate between PP1 and PP2A function in cells. In this study, we used TC to analyze PP1 function in regulating TNFα-induced pathways. We show that TC specifically inhibits activation of NF-κB among three effector pathways (NF-κB, JNK and caspase) following TNFα treatment. TC treatment suppressed activation of IKK activity, resulting in inactivation of the TNFα-induced NF-κB pathway. We show that PP1C physically interacts with the IKK complex and is recruited to TNFR1 together with IKK complex upon TNFα stimulus. This is the first implication of PP1 in its positive regulatory roles in TNFα-activation of NF-κB and TC might be a potential therapeutic reagent to suppress the TNFα/NF-κB pathway.

Materials and methods

Reagents and antibodies. Human TNFα was provided by Peprotech EC Ltd (London, UK). Tautomycetin (TC) was prepared from *Streptomyces griseochromogenes* as described (27,28). Okadaic acid (OA) and cycloheximide (CHX) were obtained from Wako (Osaka, Japan). OA and TC were dissolved in dimethyl sulfoxide (DMSO) and stored at -80˚C. TNFα and CHX were dissolved in water and stored at -80˚C. Specific antibodies to phospho-IKKα/ß (T23) [sc-21660], IKKα [sc-7606], IκBα [sc-371], IKKß [sc-7607], IKKγ [sc-8256 and sc-8330], PP1C [sc-7482], TAK1 [sc-7967] and TRAF2 [sc-876] were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-actin AC-40 [A4700] and anti-Flag M2 [F3165] monoclonal antibodies were from Sigma Chemical Co (St. Louis, MO, USA). Anti-phospho-IκBα (S32) antibody [#9241] and anti-phospho-IKKα/ß (S180/S180) antibody [#2681] were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-RIP antibody was purchased from BD Biosciences (San Jose, USA). Anti-GST monoclonal antibody was prepared as described (26). Specific antibodies to active-JNK, JNK and the Myc-tag were previously described (22). Horseradish peroxidase-conjugated anti-mouse IgG secondary antibody [cat# 18-8877-33] and anti-rabbit IgG secondary antibody [cat# 18-8816-33] were from eBioscience (San Diego, CA, USA).

Mammalian expression vectors. The pNF-κB-Luc reporter plasmid was purchased from Stratagene (Garden Grove, CA, USA). The expression vectors, pCMV-ß-galactosidase, pcDNA3-myc-PP1Cα, pRK7-N-Flag-IKKα and pRK5-C-Flag-IKKß were previously described (29-31).

Recombinant protein. A plasmid encoding a glutathione S-transferase (GST) fusion protein of human mutant IκBα amino acids (1-53) was constructed by PCR. Primers designed based on the human IκBα were: sense, 5'-GGGAATTCCAT GTTCCAGGCGGCCGAGCG-3' and anti-sense, 5'-CCCGC GGCCGCTCAGTGGCGGATCTCCTGCAGC-3'. cDNA was amplified by PCR using wild-type human IκBα cDNA as template. The PCR product was digested with *Eco*RI and *Not*I and ligated into *Eco*RI and *Not*I-digested pGEX-6P-3. The expression vector, pGEX-4T3-hTNFα encoding a glutathione S-transferase (GST) fusion protein of human TNFα (amino acids 77-233) was kindly provided by Zhijian J. Chen (University of Texas Southwestern Medical Center, USA) (1). Induction and purification of the GST fusion protein in *E. coli* were performed according to the manufacturer's protocol (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Cell culture, transfection and treatment with phosphatase inhibitors. HeLa and COS-7 cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 1.9 g/l sodium bicarbonate, 100 μg/ml streptomycin and 20 U/ml penicillin G at 37˚C under 5% CO_2. 293-T cells were cultured as described (26). For transient transfections cells were transfected using Fugene-6 (Roche Diagnostics Inc., Mannheim, Germany) according to the manufacturer's recommendation. Cells were treated with 100 nM OA and 5 μM TC for 5 h, respectively as described (26).

Luciferase assay. 293-T cells in 35-mm dishes were co-transfected with 1 μg pNF-κB-Luc and pCMV-ß-galactosidase.

Four hours later, cells were harvested into new dishes and cultured for 16-34 h, treated with or without phosphatase inhibitors for 5 h and then stimulated with 5-10 ng/ml TNFα. Luciferase activity was measured with Picagene (Toyo Ink, Tokyo, Japan) and the Luciferase Assay System (Promega, Madison, WI, USA). ß-Galactosidase activity was measured with the Beta-Glo™ Assay System (Promega). Chemiluminescence was determined by liquid scintillation counting (Beckman Coulter, Fullerton, CA, USA) and with a microplate luminometer, Veritas™ (Promega). ß-Galactosidase activities and protein concentration were used to normalize transfection efficiency and cell number. Protein concentration was measured by the modified method of Bradford using bovine serum albumin (BSA) as a standard (26).

Caspase activity assay. HeLa cells in 12-well plate were treated with or without phosphatase inhibitors for 4 h, treated with 20 μg/ml CHX for 1 h for suppression of caspase inhibitory proteins and then stimulated with 10 ng/ml TNFα. Cells were washed with phosphate-buffered saline (PBS) on ice and lysed by sonication in Casp-lysis buffer (PBS containing 0.5% Triton X-100 and 0.1% SDS). Lysates were centrifugated at 20,000 x g for 10 min and the supernatants were used for caspase activity assay. Total activities of caspase-3 and caspase-7 were measured using the Caspase-Glo™ 3/7 Assay (Promega) according to the manufacturer's insructions. Caspase-8 activity was measured with the Glo™ 8 assay (Promega). Chemiluminescence was determined by the microplate luminometer, Veritas™ .

Immunoblot analysis. Cells were sonicated in lysis buffer A [20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.5% deoxycholate, 10% glycerol, 137 mM NaCl, 5 mM EDTA, 50 mM ß-glycerophosphate, 2 mM orthovanadate, 20 mM NaF, 1 mM DTT, 0.5 mM benzamidine, 10 μg/ml leupeptin and 10 μg/ml aprotinin] and extracts were prepared by centrifugation at 20,000 x g for 10 min. Immunoblot analysis was performed as described (26). Signals were detected either with the Enhanced Chemiluminescence reagent (NEL105, Perkin Elmer) using X-ray film or a luminescence image analyzer, LAS-1000plus (Fujifilm, Tokyo, Japan).

Activity and phosphorylation of IKK. Cells were sonicated in lysis buffer A, then the IKK complex in cell extracts was immunoprecipitated with an anti-IKKγ. IKK activity was determined by incubating GST-IκBα (1-53) with immunoprecipitates in 40 μl of kinase reaction mixture [20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 20 mM ß-glycerophosphate, 0.4 mM benzamidine, 5% glycerol and 100 μM (γ-^{32}P) ATP)] for 20 min at 31-35˚C. Proteins in the reaction mixture were subjected to SDS-PAGE and immunoblotting. The phosphorylation level of GST-IκBα (1-53) was quantified by autoradiography with a Fluoro image analyzer, FLA-3000G (Fujifilm). Total IKKß levels were monitored by immunoblot and quantified with anti-IKKß antibody using the LAS-1000 plus. IKK kinase activity was calculated as intensity of phospho-GST-IκBα (1-53) divided by amount of IKKß.

Phosphorylation status of T-loop of IKKα/ß and Thr-23 of IKKα were assessed by immunoprecipitation with anti-IKKγ antibody followed by immunoblot with anti-phospho-IKKα/ß (S180/S181) and anti-phospho-IKKα/ß (T23) antibodies, respectively.

Co-immunoprecipitation. Transfected 293-T cells were lysed in 425 μl/60 mm plate co-IP buffer (50 mM Tris-HCl, pH 7.5, 4 mM EDTA, 5% glycerol, 0.1% Triton X-100, 1 mM benzamidine, 50 mM ß-glycerophosphate, 2 mM orthovanadate and 0.1% ß-mercaptoethanol) containing 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor and 10 μg/ml aprotinin. Cell lysates were centrifuged at 20,000 x g for 10 min. Three hundred and eighty μl of supernatant was first incubated with anti-Flag M2 monoclonal antibody (20 μg) or anti-Myc-tag monoclonal antibody (10 μg) for 30 min at 4˚C and then with 10 μl of Protein G-Sepharose 4 Fast Flow (Amersham Pharmacia Biotech). After incubation for 1.5 h, beads were washed twice with 1 ml of the co-IP buffer. Immunoprecipitates were resuspended in 45 μl of 1.25 x Laemmli SDS sample buffer, boiled for 5 min, separated on SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). Flag- and myc-tagged proteins were detected by immunoblot with the respective antibodies. The procedure used to co-immunoprecipitate the endogenous IKK complex and PP1C was slightly modified. Briefly, 4 μg specific antibody against IKKα was used for immunoprecipitation and normal mouse IgG antibody (4 μg) (sc-2025, Santa Cruz Biotechnology) was used as negative control and the incubation time with G-Sepharose was 3 h.

GST-TNFα pull-down assay. The pull-down assay was performed as described (1). 293-T cells in the 10-cm dishes were treated with 1 μg/ml GST-TNFα for different lengths of time (for t = 0, GST-TNFα was added after the cells were lysed). The cells were suspended in lysis buffer B [20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 10% glycerol, 25 mM ß-glycerolphosphate, 0.5 mM dithiothreitol (DTT), 1 mM Na_3VO_4, 0.5 mM benzamidine, 10 μg/ml leupeptin] and then centrifugated at 16,000 x g for 20 min at 4˚C. Five mg proteins of cell lysates were incubated with 100 μl of glutathione sepharose at 4˚C for 1 h. The resins were washed twice with the co-IP buffer (50 mM Tris-HCl, pH 7.5, 4 mM EDTA, 5% glycerol, 0.1% Triton X-100, 1 mM benzamidine, 50 mM ß-glycerophosphate, 2 mM Na_3VO_4, and 0.1% ß-ME) at 6,000 rpm for 2 min at 4˚C. GST pulled-down protein complexes were prepared by the treatment of the resins with SDS sample buffer, boiled for 5 min, separated on SDS-PAGE and transferred to a nitrocellulose membrane. The proteins were detected by immunoblot with the respective antibodies.

Results

TC suppresses TNFα-induced NF-κB activation. To determine whether PP1 functions in TNFα-induced NF-κB activation, the effect of pretreatment of 293-T cells with TC on activation of reporter plasmid containing 5 tandem repeat NF-κB responsive elements was analyzed. As reported, 100 nM OA, a PP2A inhibitor, enhanced basal and TNFα-induced NF-κB-dependent luciferase activity (Fig. 1A). In contrast, 5 μM TC dramatically decreased TNFα-induced NF-κB-dependent luciferase activity (Fig. 1A). As shown in Fig. 1B, TC

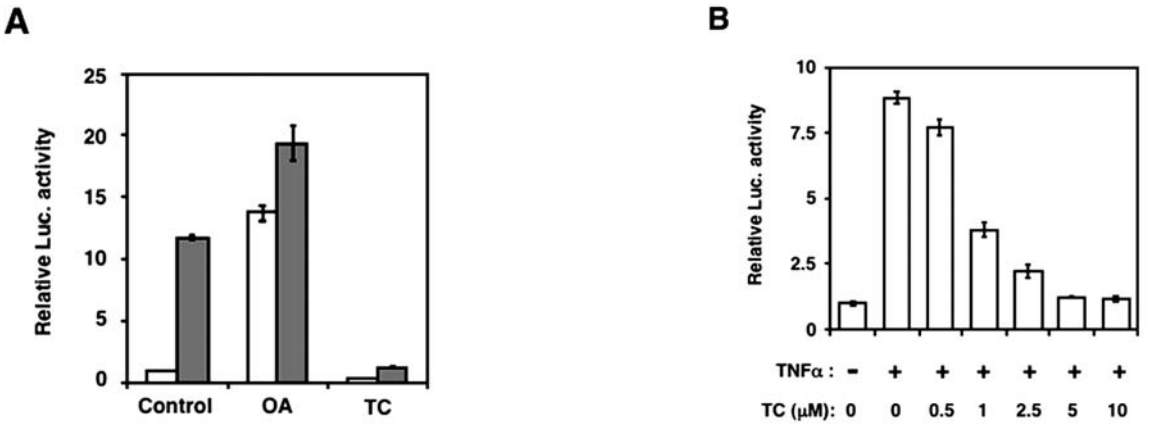

Figure 1. Effect of OA and TC on TNFα-induced NF-κB activation. (A) Transfected 293-T cells were treated with vehicle (control), 100 nM OA or 5 μM TC and then incubated for 5 h with (closed bars) or without (open bars) 5 ng/ml TNFα. (B) Transfected 293-T cells were incubated with the indicated dose of TC and then stimulated for 4 h with 10 ng/ml TNFα. Shown is luciferase activity relative to that seen in cells without TNFα or TC treatment. Data are means from four independent experiments.

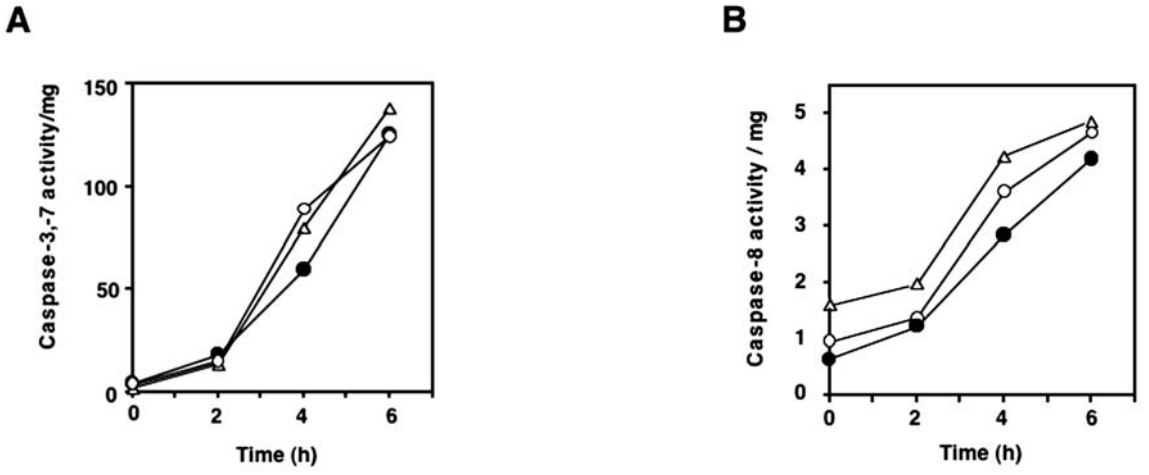

Figure 2. Effect of OA and TC on TNFα-induced caspase activation. HeLa cells were treated with vehicle (open circles), 100 nM OA (open triangles) or 5 μM TC (closed circles) for 4 h and then incubated in the presence of 20 μg/ml CHX for 1 h. Treated cells were stimulated for the indicated periods with 10 ng/ml TNFα. (A) Combined caspase-3 and caspase-7 activity. (B) Caspase-8 activity. Data represent three independent experiments.

decreased NF-κB activation induced by TNFα in a dose-dependent manner. In a separate experiment we confirmed that treatment of 293-T cells for 5 h with 5 μM TC and 100 nM OA resulted in complete and specific inhibition of PP1 and PP2A, respectively (data not shown). These results showed that inhibition of PP1 by TC blocks NF-κB activation following TNFα stimulation. Binding of TNFα to TNFR1 results in rapid activation of NF-κB, followed by complex II-dependent caspase-8, and -3/7 activation. To determine whether caspase activation is also affected by TC and OA, HeLa cells were incubated with either OA or TC, treated with CHX to block induction of caspase inhibitor proteins (3,4,32) and then assayed for caspase activities. As shown in Fig. 2A, neither TC nor OA had any effect on caspase-3/7 activation. Furthermore, TC or OA treatment also had no effect on the upstream enzyme caspase-8 (Fig. 2B). Treatment of HeLa cells for 4 h with either 5 μM TC or 100 nM OA resulted in complete and specific inhibition of PP1 or PP2A, respectively (data not shown). These data suggest that the PP1 and PP2A targets in the TNFα signaling pathway are not downstream of complex II but rather are downstream of complex I.

TC suppresses TNFα-induced IκBα phosphorylation but not JNK activation. Since pathways downstream of complex I bifurcate into respective NF-κB and JNK pathways, we compared the effects of OA and TC on phosphorylation/degradation of IκBα and activation of JNK in 293-T cells (Fig. 3). As reported, 100 nM OA induced degradation of IκBα and hyper-phosphorylation of JNK in 293-T cells, confirming that PP2A is a negative regulator of NF-κB and JNK activation (33,34). Under the same conditions, 5 μM TC dramatically reduced IκBα degradation but had no apparent effect on JNK activation (Fig. 3). These results suggest that target(s) of PP1 that suppress NF-κB activation are present downstream of the bifurcation point and upstream of IκBα.

TC blocks IKK activation. Since IκBα is phosphorylated by IKK, we analyzed the effect of TC and OA on IKK activation following TNFα stimulation. As shown in Fig. 4A, IKK

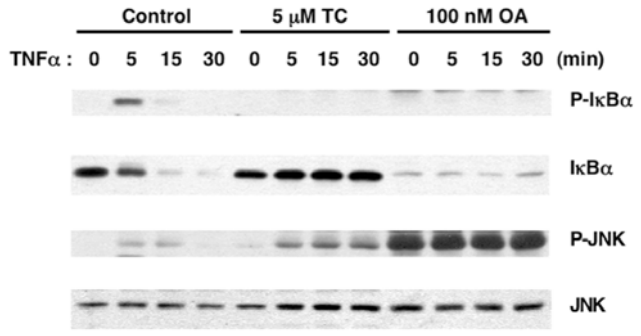

Figure 3. Effect of OA and TC on TNFα-induced IκBα degradation and JNK activation. 293-T cells were treated with vehicle, 5 μM TC, or 100 nM OA for 5.5 h and then stimulated for the indicated periods with 10 ng/ml TNFα. Immunoblots of cell lysates were then analyzed using anti-phospho-IκBα (P-IκBα S32), anti-IκBα, anti-activated-JNK (P-JNK) and anti-JNK antibodies.

activity was increased with a peak at 10-15 min after TNFα stimulation. Under the same conditions, treatment of cells with 5 μM TC inhibited IKK activation by 77% at 10 min. Interestingly, inhibition of PP2A by 100 nM OA resulted in significant reduction (by 87% at 10 min) of IKK activation, indicating that PP2A positively regulates IKK activation but negatively regulates NF-κB activation. We then analyzed TC dose-dependent inhibition of IKK activation after TNFα stimulation (Fig. 4B). The IC_{50} of IKK inhibition by TC was determined to be 0.8 μM. Of note is that TC-inhibition of IKK (Fig. 4B) and NF-κB (Fig. 1B) activation was almost the same in terms of dose and rate. These data indicate that NF-κB inhibition by TC is likely mediated by suppression of IKK activity.

TC attenuates T-loop phosphorylation of IKK. We next asked whether TC inhibits phosphorylation of T-loops at Ser-176/Ser-180 of IKKα and Ser-177/Ser-181 of IKKß. As shown in Fig. 5A, phosphorylation at sites in T-loops of IKKα and IKKß appeared 10 min after TNFα stimulation in 293-T cells, but 5 μM TC blocked phosphorylation to almost basal levels. By contrast, phosphorylation levels at Thr-23 of IKKα which is not in the IKKα T-loop, were not affected by TNFα or TC treatment (Fig. 5B). Phosphorylation of Thr-23 in IKKß was not detected under the same conditions used. Suppression of T-loop phosphorylation induced by TC was also observed in HeLa and COS-7 cells (Fig. 5C). These data suggest that TC negatively regulates IKK activity by inhibiting phosphorylation of IKKα and IKKß T-loops.

Interaction of PP1C with the IKK complex in the presence or absence of TC. To determine how PP1 positively regulates IKK activity, we asked whether PP1 physically associates with IKKs. We co-transfected 293-T cells with Flag-IKKα or Flag-IKKß and Myc-PP1Cα and undertook immunoprecipitation using an anti-Flag (Fig. 6A) or an anti-Myc (Fig. 6B) antibody. As shown in Fig. 6A, Myc-PP1C was detected in the Flag-IKKα and the Flag-IKKß immunoprecipitates. Conversely, Fig. 6B indicates that Flag-IKKα and Flag-IKKß were detected in the Myc-PP1C immunocomplex. These data show that overexpressed Myc-PP1C in cells interacts with Flag-IKKα and Flag-IKKß.

We then asked whether endogenous PP1C interacts with the endogenous IKK complex. We found that complexes immunoprecipitated with IKKα contained not only IKKß and IKKγ but also PP1C (Fig. 7A). Levels of PP1C associated with the IKK complex were not affected by TNFα stimulation (Fig. 7A). Inhibition of PP1 activity by TC treatment also did not affect complex formation between PP1C and IKKs (Fig. 7B). These results suggest that PP1C physically interacts with the IKK complex in cells.

Interaction of PP1C with TNFR1. The binding of trimeric TNFα to its receptor leads to the rapid recruitment of several signaling proteins such as RIP, TAK1 and IKK complexes to the receptor complex (1). To determine whether PP1C is also recruited to the receptor complex, we stimulated the 293-T cells with GST-TNFα and then isolated the TNFR1 complex by using glutathione sepharose. As shown in Fig. 8, GST-

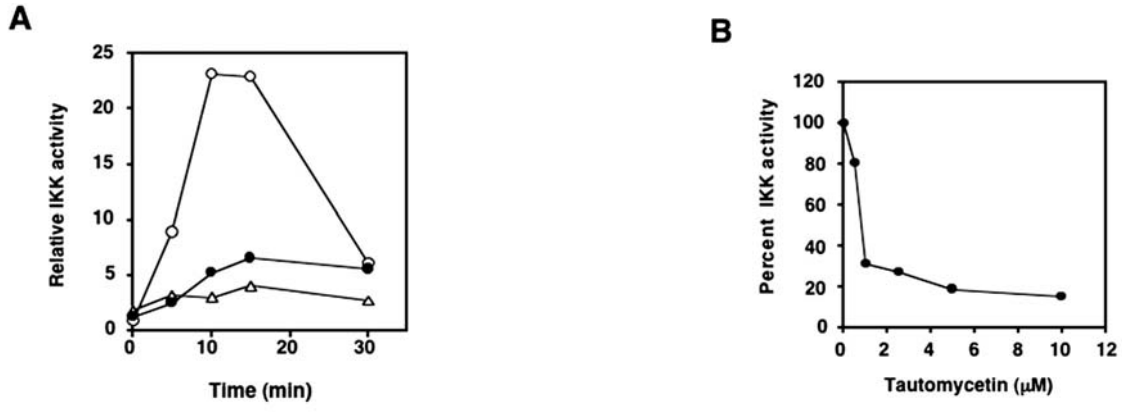

Figure 4. Effects of OA and TC on IKK activation in 293-T cells. (A) 293-T cells were treated with vehicle (open circles), 100 nM OA (open triangles) or 5 μM TC (closed circles) for 5 h and then stimulated for the indicated periods with 40 ng/ml TNFα. IKK kinase activity was quantitated by dividing levels of radiolabeled-phospho-GST-IκBα (1-53) by levels of IKKß, determined by autoradiography using a Fluoro image analyzer and immunoblotting with an anti-IKKß antibody, respectively. IKK activity is expressed as fold activation relative to vehicle-treated sample at Time 0. (B) 293-T cells were treated with vehicle (Dose 0) or TC at the indicated dose for 5 h, stimulated for 10 min with 10 ng/ml TNFα and analyzed for IKK activity. IKK activity is calculated in a manner identical to that described in A. IKK activity is expressed as a percentage of that seen in the vehicle-treated control. Data are representative of three independent experiments.

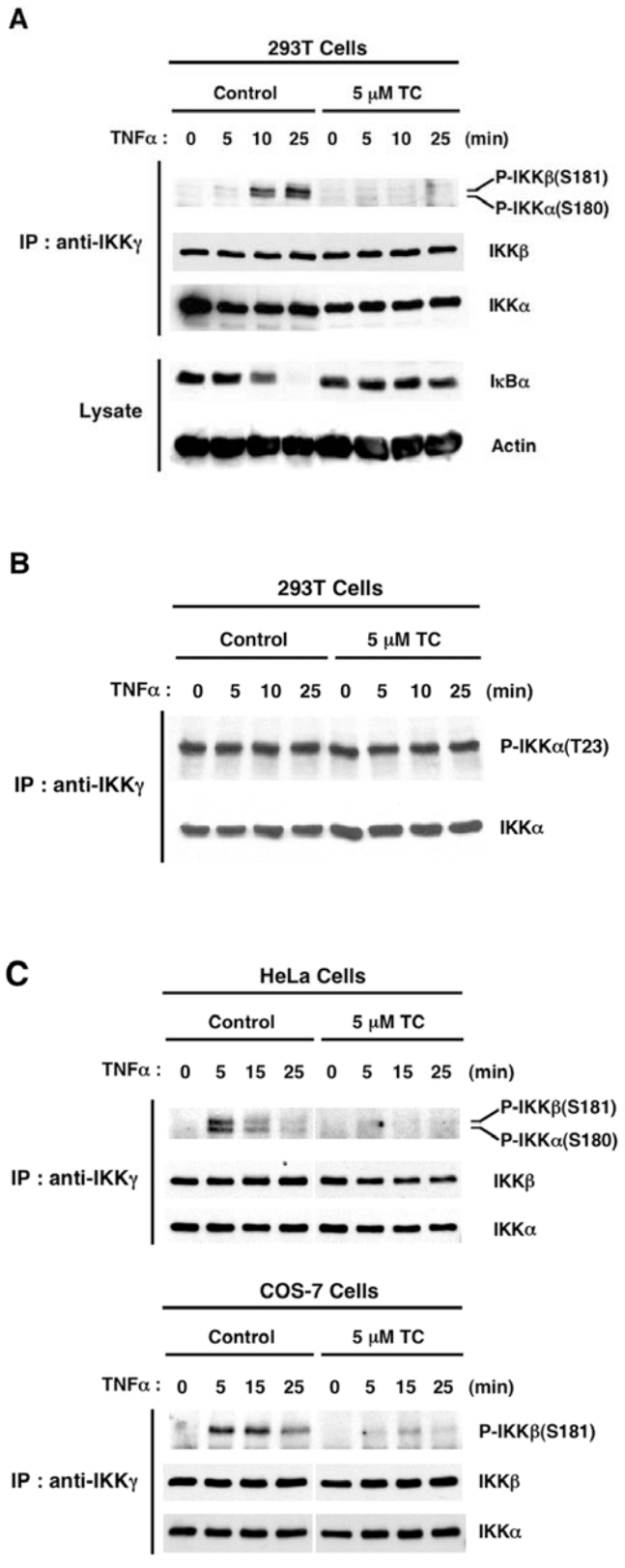

Figure 5. Effects of TC on IKKα and IKKß phosphorylation. 293-T cells (A and B) or HeLa and COS-7 cells (C) were treated with vehicle (control) or 5 μM TC for 5 h and then stimulated for the indicated periods with 10 ng/ml TNFα. Lysates were immunoprecipitated and blotted using the anti-IKKγ antibody. (A) Immunoblots with anti-phospho-IKKα/ß (S180/S181), anti-IKKß, anti-IKKα, anti-IκBα and anti-actin antibodies. Degradation of IκBα in lysates was seen following TNFα treatment. Actin served as an internal standard. (B) Immunoprecipitates were immunoblotted with anti-phospho-IKKα/ß (T23) and anti-IKKα antibodies. (C) Immunoprecipitates were immunoblotted with anti-phospho-IKKα/ß (S180/S181), anti-IKKß and anti-IKKα antibodies.

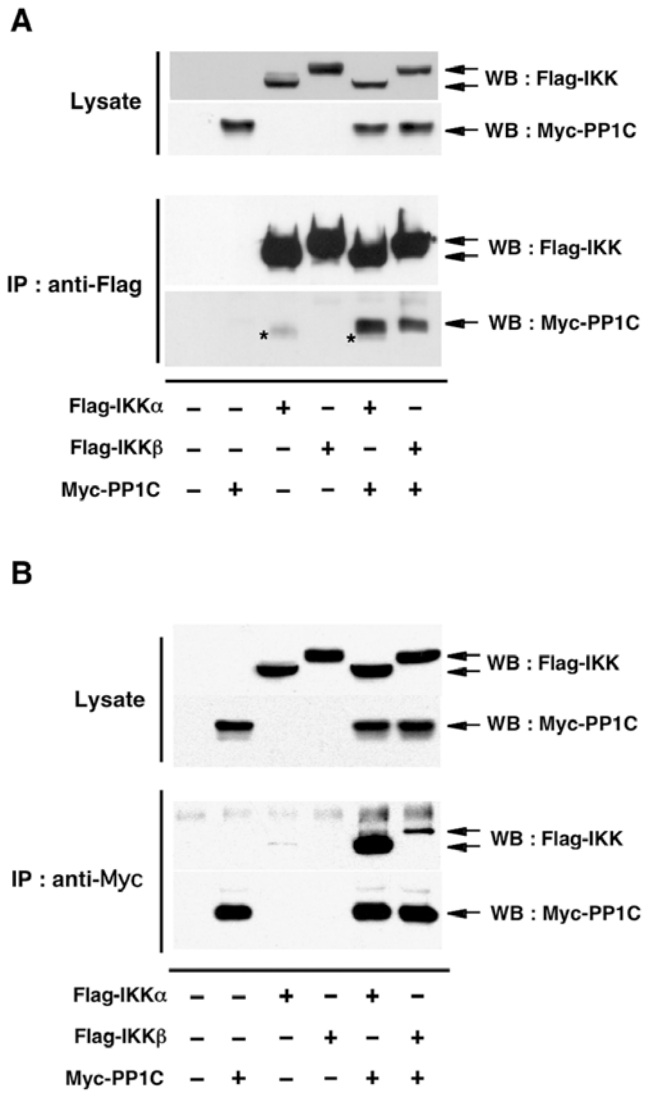

Figure 6. PP1Cα interacts with IKKα and IKKß in 293-T cells. 293-T cells were transfected with either pRK7-N-Flag-IKKα, pRK5-C-Flag-IKKß or empty vector and either pcDNA3-Myc-PP1Cα or empty vector. Expression levels of Flag-IKKs and Myc-PP1Cα in lysates were analyzed by immunoblotting with anti-Flag M2 or anti-Myc 9E10 monoclonal antibodies. (A) Samples were immunoprecipitated (IP) with anti-Flag M2 antibody and subjected to immunoblotting using anti-Flag (upper) or anti-Myc (lower) antibody. (A) Asterisks indicate non-specific bands. (B) Samples were immunoprecipitated (IP) with anti-Myc antibody and subjected to immunoblotting using anti-Flag (upper) or anti-Myc (lower) antibody.

TNFα treatment led to the recruitment of TAK1, RIP, IKKα and IKKß to TNFR1. PP1C was also detected together with these signaling molecules. Interestingly, the recruited amount of the signaling molecules including PP1C increased to a top at 10 min and then decreased to the lowest level at 60 min after stimulation of TNFα. Here, the change of the amount of PP1C recruited to TNFR1 corresponded with that of IKK activity (Fig. 4A). These results suggested that PP1C was recruited to TNFR1 via binding to IKK complex and plays a role as a positive regulator in the TNFα/NF-κB signaling pathway.

Discussion

Engagement of TNFR1 by TNFα activates three important signaling pathways: NF-κB, JNK and caspases. The extent to

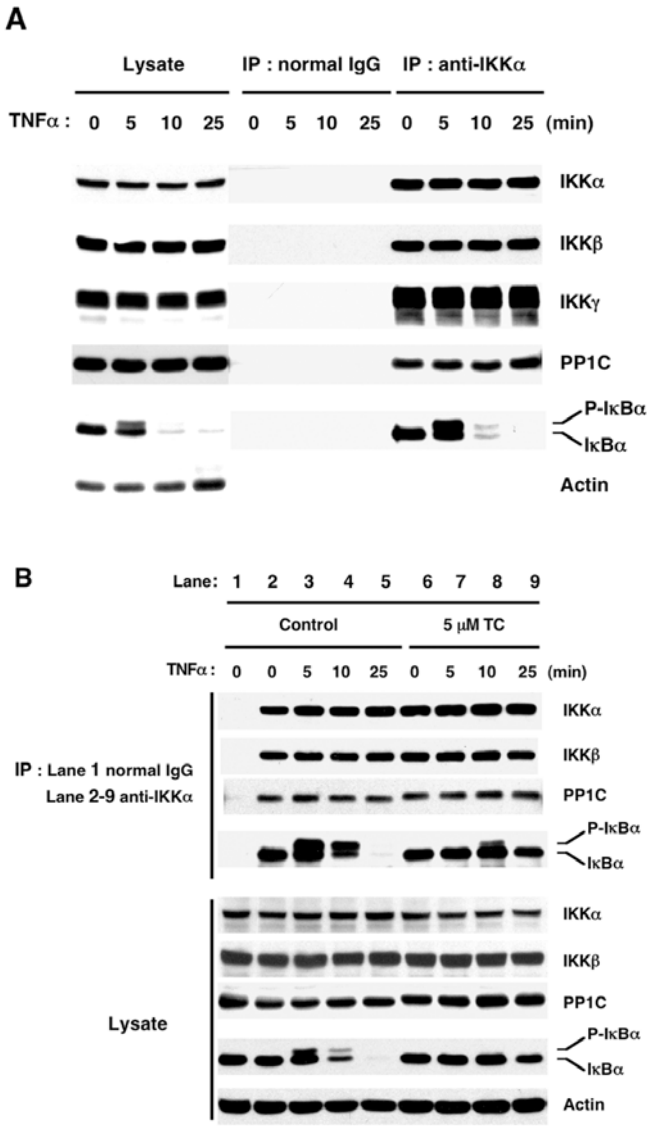

Figure 7. PP1C associates with the IKK complex in 293-T cells. (A) 293-T cells were stimulated for the indicated periods with 10 ng/ml TNFα. Immunoblots of cell lysates and immunoprecipitates with either normal IgG or anti-IKKα were analyzed using anti-IKKα, anti-IKKß, anti-IKKγ, anti-PP1C, anti-IκBα and anti-actin antibodies. (B) 293-T cells were treated with vehicle or 5 μM TC for 5.5 h and then stimulated for the indicated periods with 10 ng/ml TNFα. Following immunoprecipitation with either normal IgG or anti-IKKα immunoblots were analyzed using anti-IKKα, anti-IKKß, anti-PP1C, anti-IκBα and anti-actin antibodies. In a separate experiment, we confirmed that the upper band of IκBα is a phosphorylated form using phospho antibody (data not shown).

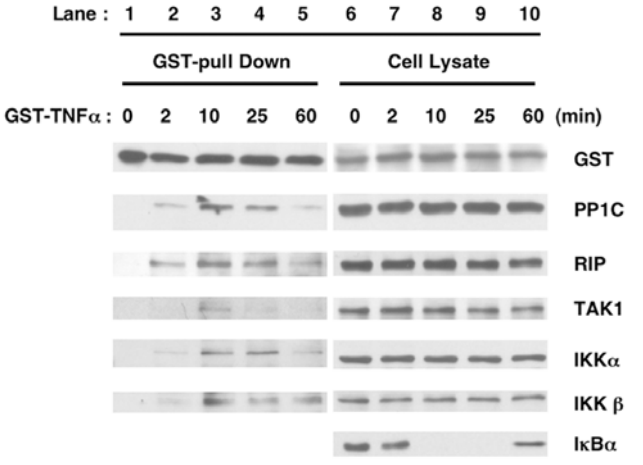

Figure 8. PP1C is recruited to TNFR1 with TNFα stimulus in 293-T cells. 293-T cells were stimulated with 1 μg/ml GST-TNFα for the indicated time and then the GST-TNFα and its binding proteins were pulled-down using glutathione sepharose. The bound proteins and whole cell lysate were immunoblotted with the indicated primary antibodies.

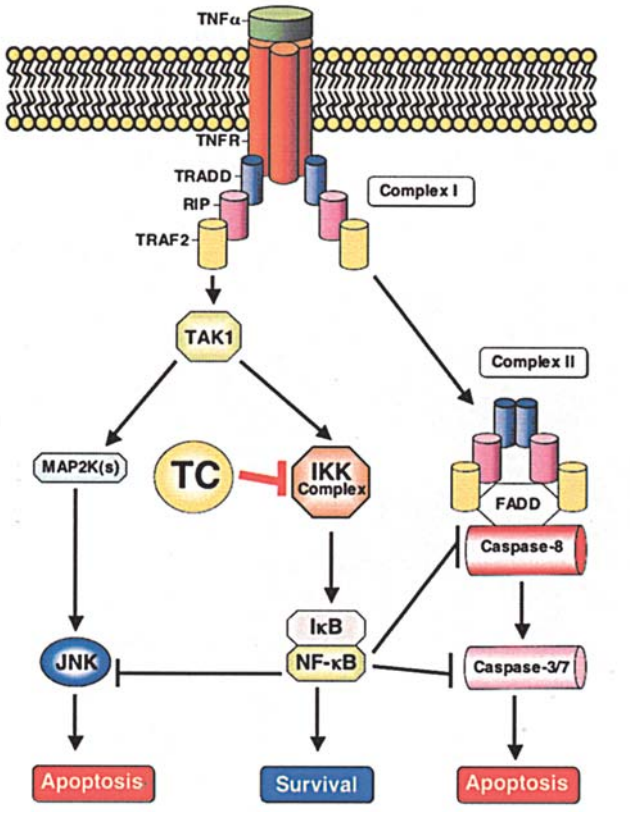

Figure 9. Tautomycetin suppresses IKK activation downstream of TNFα without affecting JNK or caspase activation. PP1 is involved in the IKK complex functions as a positive regulator of IKK activation following TNFα stimulation. This conceptual scheme is based on data shown in ref. 3. Red arrows indicate results obtained in this study.

which each pathway is activated determines the overall effect of TNFα on cell function (2-4). Numerous studies strongly indicate that the transcription factor NF-κB is involved in carcinogenesis. In addition, compelling experiment indicates an important role of NF-κB in modulating cancer therapy efficacy. Therefore, it is important to dissect how molecular mechanism is regulated. Here, using specific inhibitors of PP1 and PP2A, TC and OA, respectively, we dissected differential roles of these phosphatases in TNFα-induced signal pathways.

We found that 5 μM TC, which causes complete inhibition of PP1 activity without affecting PP2A activity (25,26), inhibits TNFα-induced NF-κB activation in 293-T cells (Fig. 1). We also found that TC inhibited TNFα-induced IκBα phosphorylation/degradation in 293-T (Fig. 3), HeLa, COS-7, HepG2 and Jurkat cells (data not shown), suggesting that a target of TC is present upstream of IκB. TC did not affect TNFα-induced caspase (Fig. 2) or JNK activation (Fig. 3), indicating that a TC target resides downstream of

the complex I bifurcation pathway towards NF-κB activation. We focused on the IKK complex, because it is an upstream kinase of IκBα and an effector of TAK1, at a point where the TNFα signal bifurcates towards NF-κB and JNK signaling. We found that treatment of cells with TC resulted in inhibition of T-loops phosphorylation of IKKs (Fig. 5) and that PP1C physically associates with the IKK complex (Figs. 6 and 7) and was recruited to TNFR1 with TNFα stimulus (Fig. 8). These data showed that TC, possibly by inhibiting PP1, blocks the TNFα/NF-κB pathway via prevention of IKK activation (Fig. 9).

We have shown that PP1 is present in a complex with IKK, that it positively regulates IKK activity. Recently, a novel inhibitory phosphorylation site, the NEMO/γ binding domain (NBD/γBD), in IKKß was identified (15). In this study, IKKß-γBDAA, a nonphosphorylatable mutant, had significantly higher basal level IKK activity compared to the wild-type protein. By contrast, IKKß-γBDEE, a phosphorylation mimicking mutant, had basal level activity similar to the wild-type, but its activity was not activated by IL-1ß. These results suggest that phosphorylation of Ser-740 and -750 in NBD/γBD negatively regulates IKK activity. If PP1 recognizes the C-terminal serine-rich region as a substrate, TC might induce an upward mobility shift of IKKß, but this was not the case (Fig. 5A). Thus it is possible that the NBD/γBD in IKKß is a PP1 substrate. However, several studies show that there are multiple phosphorylation sites in IKKγ (35) and that the IKK complex contains other associated proteins, such as cdc37 and Hsp90 (36). Further analysis of these proteins in relation to PP1 activity is required.

NF-κB and IKK are strongly implicated in a variety of tumor malignancies (8-11). Activated NF-κB regulates the expression of genes involved in the cancer such as c-myc, cyclin D1, matrix metalloproteinases, Bcl-xL, c-FLIP and VEGF. Inhibition of NF-κB alone or in combination with cancer therapies leads to tumor cell death or growth inhibition (8). For example, thalidomide and immunomodulatory thalidomide analogues have shown activity against relapsed or refractory multiple myeloma. Importantly, thalidomide blocks NF-κB activation via suppression of IKK activity and was shown to inhibit NF-κB in multiple myeloma. A small molecule inhibitor of IKK (PS-1145) was found to be selectively toxic for subtypes of diffuse large B-cell lymphoma cells that are associated with NF-κB activation. Present data suggest that TC might be another member of IKK inhibitor and a potential candidate for prevention or treatment of certain types of cancer.

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