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N-Tosyl-L-phenylalanine Chloromethyl Ketone Inhibits NF-κB Activation by Blocking Specific Cysteine Residues of IκB Kinase β and p65/RelA†

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ABSTRACT: *N*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), a serine/cysteine protease inhibitor, has been reported to inhibit expression of inflammatory mediators by blocking nuclear factor-κB (NF-κB) activation. We examined the effect of TPCK on the NF-κB activation pathway in HeLa cells by measuring the activity of IκB kinase (IKK) and p65/RelA−DNA binding. TPCK inhibited tumor necrosis factor-α-induced IKK activation and directly blocked IKK activity in vitro. TPCK-induced inhibition of NF-κB and IKK activation was abrogated by addition of the thiol-reducing agent dithiothreitol, suggesting that the effect of TPCK occurred through modification of a thiol group in IKK. Consistent with this, an IKKβ mutant in which Cys-179 was substituted with alanine was not more susceptible to TPCK. Our result also showed that TPCK inhibits the DNA binding of transiently expressed p65/RelA in HeLa cells. Inhibition of p65/RelA−DNA binding was recovered in the presence of dithiothreitol, and substitution of Cys-38 with Ser in p65/RelA rendered the protein resistant to inhibition by TPCK. Mass spectrometry analysis of IKKβ and p65/RelA isolated from cells treated with TPCK by UPLC-ESI-Q-TOF tandem MS revealed the labeling of Cys-179 of IKKβ and Cys-38 of p65/RelA with a tosylphenylalanylmethyl group. These results suggest that TPCK inhibits NF-κB activation by directly modifying thiol groups on two different targets: Cys-179 of IKKβ and Cys-38 of p65/RelA.

Nuclear factor-κB (NF-κB)[1] is a family of transcription factors that regulate the expression of many genes involved in immune and inflammatory responses, as well as cell proliferation and survival. In mammalian cells, NF-κB family members include five related proteins, p65/RelA, c-Rel, RelB, p50/p105/NF-κB1, and p52/p100/NF-κB2, which form various homo- or heterodimers (*1*, *2*). In unstimulated cells, NF-κB is retained in the cytoplasm by a family of inhibitory proteins known as inhibitors of NF-κB (IκBα, -β, and -ε). In the canonical pathway of NF-κB activation, proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β as well as bacterial lipopolysaccharide induce phosphorylation of IκBα at specific serine residues, leading to its ubiquitination and subsequent degradation. Release of NF-κB, typically composed of p65/RelA and p50, allows it to translocate to the nucleus and induce expression of target genes (*1*, *2*).

Phosphorylation of IκBα occurs with IκB kinase (IKK), which is present as a 700 kDa complex composed of two catalytic subunits, IKKα (or IKK1) and IKKβ (or IKK2), and a regulatory subunit, IKKγ/NEMO/IKKAP1 (*1*−*3*). In studies of mice deficient in IKK subunits, IKKβ was shown to play an essential role in the activation of NF-κB in response to TNF-α and other inflammatory stimuli (*1*, *3*). Activation of IKKβ involves the phosphorylation of Ser-177 and Ser-181, which are located in the "activation loop" within the kinase domain, and conversion of these serines to alanine prevents IKK activation by TNF-α and interleukin-1 (*1*, *3*). Cys-179 of IKKβ, located between the two serine residues in the activation loop, has been shown to play a critical role in enzyme regulation by modulating both the phosphorylation of Ser-177/181 and the catalytic activity of the active enzyme (*4*). It was also reported that various thiol-reactive agents commonly inhibit IKKβ by blocking Cys-179 (*5*−*12*).

N-Tosyl-L-phenylalanine chloromethyl ketone [TPCK (Figure 1A)], a serine and cysteine proteinase inhibitor, was shown to inhibit NF-κB activation by blocking signal-induced phosphorylation and subsequent degradation of IκBα in various cell types (*13*−*20*). In addition, TPCK and other protease inhibitors were shown to block binding of NF-κB to its cognate sequence in vitro (*17*). This effect of TPCK was suppressed by the reducing agent dithiothreitol, implicating the involvement of a

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[1]Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione *S*-transferase; IKK, IκB kinase; MEKK1, MAPK/extracellular signal-regulated kinase kinase 1; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; RT-PCR, reverse transcription polymerase chain reaction; TNF-α, tumor necrosis factor-α; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; UPLC-ESI-q-TOF tandem MS, ultraperformance liquid chromatography electrospray ionization quadrupole time-of-flight tandem mass spectrometry.

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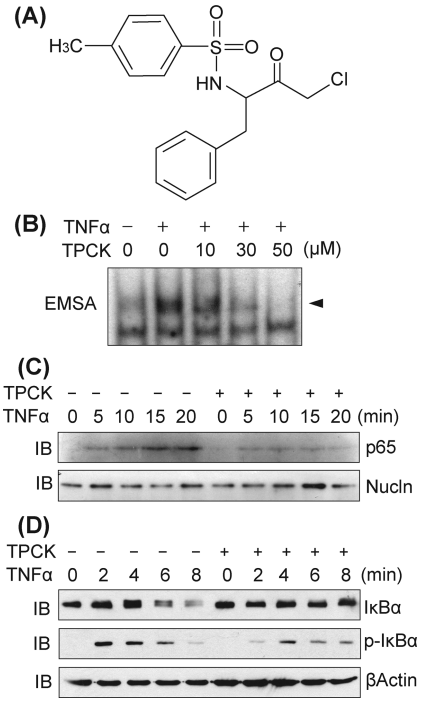


FIGURE 1: TPCK inhibits TNFα-induced NF-κB activation, nuclear translocation of p65/RelA, and phosphorylation and degradation of IκBα. (A) Structure of TPCK. (B) HeLa cells were incubated with the indicated concentrations of TPCK for 1 h and stimulated with TNF-α (30 ng/mL) for 10 min. κB binding activity in the nuclear extracts was measured with an EMSA using a probe containing the NF-κB consensus motif. The retarded band is marked with an arrowhead. (C and D) HeLa cells were incubated in the presence or absence of TPCK (30 μM) for 1 h and stimulated with TNF-α (30 ng/mL) for the indicated times. Nuclear extracts were analyzed by immunoblotting using antibodies to p65/RelA and nucleoporin p62 (Nucln) (C). Cytoplasmic extracts were analyzed by immunoblotting (IB) using antibodies to IκBα, Ser-32/36-phosphorylated IκBα, and β-actin (D).

cysteine thiol group in TPCK-induced inhibition of NF-κB−DNA binding. These results suggest that the inhibitory effect of TPCK occurred in at least two different steps during NF-κB activation, i.e., phosphorylation of IκBα and binding of NF-κB to DNA. In addition, inhibition of the latter step is thiol-dependent. However, the target and inhibitory mechanism of TPCK in the NF-κB activation pathway have yet to be determined. Here, we investigated TPCK-mediated inhibition of NF-κB activation in TNF-α-stimulated HeLa cells. The results showed that TPCK blocks both IKK activity and p65/RelA−DNA binding, and mutations of Cys-179 in IKKβ and Cys-38 in p65/RelA prevent inhibition by TPCK.

MATERIALS AND METHODS

Materials. TPCK and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies to IKKα, IKKβ, IκBα, and p65/RelA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against the phosphorylated form of IκBα were obtained from Cell Signaling Technology (Beverly, MA). Nucleoporin p62 and β-actin antibodies were obtained from BD Biosciences (San Jose, CA) and Sigma, respectively. The recombinant glutathione *S*-transferase (GST)−IκBα fusion protein containing the 54 N-terminal residues of IκBα and recombinant human TNF-α were prepared by expression in *Escherichia coli*, as described previously (*21*). The Flag-tagged IKKβ expression vector was kindly provided by F. Mercurio (Signal Pharmaceuticals, San Diego, CA). Substitution of Cys-179 with alanine in IKKβ was accomplished by site-directed mutagenesis, as described previously (*8*). The MAPK/extracellular signal-regulated kinase kinase 1 (MEKK1) expression vector was a kind gift from J. H. Kim (Korea University, Seoul, Republic of Korea).

Site-Directed Mutagenesis. The cDNA of human p65/RelA was generated by reverse transcription polymerase chain reaction (RT-PCR) with specific primer sets (sense, 5′-CGCGATATCATGGACTACAAGGACGACGATGACAAGGCTAGCGACGAACTGTTC-3′; antisense, 5′-CGCGCGGCCGCTTAGGAGCTGATCTGACT-3′) and template RNA obtained from human peripheral blood leukocytes. The amplified cDNA contained the Flag epitope at its N-terminus and was cloned between the *Eco*RV and *Not*I sites of pCR3.1 (Invitrogen, Carlsbad, CA). Mutant p65/RelA C38S was generated by overlapping PCR-based mutagenesis using wild-type p65/RelA as a template. Two mutagenesis oligonucleotide primers containing the underlined point mutation (sense, 3′-CCGCTACAAGTCCGAGGGGC-5′; antisense, 3′-CCCTCGGACTTGTAGCGGAA-5′) and two flanking primers were used for PCR. The final PCR product was digested with *Hin*dIII and *Bst*EII and used to replace the corresponding *Hin*dIII−*Bst*EII fragment in pCR3.1-p65/RelA. The mutation was confirmed by DNA sequencing.

Cell Culture, Transfection, and the IKK Assay. HeLa and HEK-293 cells were obtained from the American type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Cells were transiently transfected with expression vectors for IKKβ, p65/RelA, and their mutants using Fugene 6 (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's protocol, and incubated for 48 h. Preparation of cytoplasmic extracts and immunoprecipitation using anti-IKKα or anti-Flag antibodies were performed as previously described (*21*). The kinase activity of the immune complex was measured in reaction mixtures containing [γ-^{32}P]ATP (2−5 μCi) and GST−IκBα fusion protein (2 μg). Samples were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and electrophoretically transferred to a nitrocellulose membrane. Phosphorylated GST−IκBα fusion protein was visualized by autoradiography and quantitated using a phosphor image analyzer (Fujifilm, Tokyo, Japan). Proteins in the cell extracts were analyzed by immunoblotting using the ECL system (GE Healthcare, Buckinghamshire, U.K.), according to the manufacturer's protocol.

Electrophoretic Mobility Shift Assay (*EMSA*). Nuclear and cytoplasmic extracts were prepared from HeLa cells as described previously (*22*). The oligonucleotide containing the consensus recognition sequence for NF-κB was obtained from Santa Cruz Biotechnology and end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The binding reaction was performed with 15 μg of nuclear protein, and the reaction products were analyzed by electrophoresis on a 6% polyacrylamide gel in 0.5× TBE buffer [22.5 mM Tris-HCl (pH 8.5), 22.5 mM borate, and 0.5 mM EDTA] (*21*).

Analysis of TPCK Labeling by UPLC-ESI-q-TOF Tandem MS. HEK-293 cells were transfected with expression vectors for Flag-IKKβ or Flag-p65/RelA and incubated for 48 h as described previously (*4*). A group of cells was treated with 30 μM TPCK for 1 h before cell lysis. Cell extract was prepared and passed through a column of anti-Flag M2 affinity

gel (Sigma) twice, and the bound protein was eluted with Flag peptide (Sigma, 0.5 mg/mL). After dialysis against PBS, a 5 μg aliquot of the eluted protein was used for SDS−PAGE and trypsin digestion. Samples for mass spectrometry (MS) analysis were prepared as described previously (*23*). MS data were collected on a nanoAcquity UPLC/ESI/MS system (SYNAPT HDMS, Waters Co., Hertfordshire, U.K.). The operating conditions for MS analysis were as follows: positive ion mode; capillary voltage, 2.8 kV; cone voltage, 35 V; collision energy, 25−40 ramping; N_2 gas. Peptides were separated by using a C18 reversed-phase 75 μm (inside diameter) × 250 mm analytical column (1.7 μm particle size, BEH300 C18, Waters) with a PicoTip integrated electrospray ionization (±10 μm, New Objective, Woburn, MA). Five microliters of peptide mixtures was dissolved in buffer A (H_2O/formic acid, 100:0.1, v/v), injected on a column, and eluted with a linear gradient from 5 to 80% buffer B (acetonitrile/formic acid, 100:0.1, v/v) over 120 min. The mass spectrometer was programmed to record scan cycles composed of one MS scan followed by MS/MS scans of the three most abundant ions in each MS scan. The flow rate was directly 0.3 μL/min. The collected MS spectra were deconvoluted using ProteinLynx global server version 2.3. Modifications were identified using an inclusion list by the database search program Mascot (global search engine) and MOD^i (http://prix.uos.ac.kr/modi/) (*24*). MS/MS spectra were matched against amino acid sequences in SwissProt. All reported assignments were verified by automatic and manual interpretation of spectra from Mascot and MOD^i in a blind mode.

Glutathione Assay. Total glutathione and oxidized glutathione (GSSG) were measured using an assay kit (Bioxytech GSH/GSSG-412, OxisResearch, Foster City, CA) using a procedure recommended by the manufacturer. HeLa cells (3×10^6) resuspended in 60 μL of PBS were lysed by twice being frozen and thawed. For the measurement of total glutathione, the cell lysate was mixed with 9 volumes of 5% metaphosphoric acid (Sigma) and then centrifuged at 8000 rpm for 10 min. The supernatant was diluted in GSH buffer (OxisResearch), and glutathione was measured by spectrophotometry at 412 nm in a reaction mixture containing 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), glutathione reductase, and NADPH. For GSSG measurements, the cell extract was mixed with 1-methyl-2-vinylpyridium trifluoromethane sulfonate (OxisResearch) to block reduced glutathione (GSH). After addition of 5% metaphosphoric acid (Sigma) and dilution in GSSG buffer (OxisResearch), GSSG was measured by spectrophotometry. The level of GSH was calculated by subtracting the level of GSSG from the total level of glutathione.

RESULTS

TPCK Inhibits TNF-α-Induced NF-κB Activation, Phosphorylation, and Degradation of IκBα. To determine the effect of TPCK on NF-κB activation, HeLa cells were incubated with various concentrations of TPCK for 1 h and stimulated with TNF-α. An EMSA of nuclear extracts showed that the TNF-α-induced increase in κB binding activity was suppressed by TPCK in a dose-dependent manner (Figure 1B). Incubation of cells with 30 μM TPCK reduced κB binding activity to a basal level. Immunoblotting analysis demonstrated an increased level of nuclear p65/RelA after TNF-α stimulation for 5 min; however, this increase was suppressed, and a low level of p65/RelA was detected in the nucleus by TPCK treatment (Figure 1C). Because phosphorylation at Ser-32 and -36 residues of IκBα and its subsequent degradation are critical for NF-κB activation by TNF-α, levels of IκBα phosphorylated at Ser-32 and -36 and total IκBα were determined by immunoblotting analysis using specific antibodies (Figure 1D). TNF-α induced IκBα degradation within 6 min of treatment, but this effect was almost completely suppressed by pretreatment of cells with 30 μM TPCK. TNF-α-induced phosphorylation of IκBα was not completely blocked by TPCK, although it was more delayed and reduced compared with control cells treated with TNF-α alone. The levels of nucleoporin p62 and β-actin were not changed by TPCK treatment.

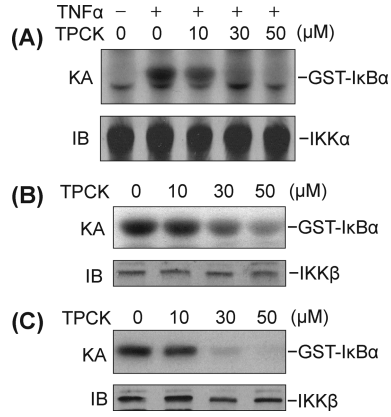


FIGURE 2: TPCK inhibits TNF-α-induced IKK activation and directly blocks IKKβ in vitro. (A) HeLa cells were incubated as described in the legend of Figure 1A. IKK was immunoprecipitated from whole cell extracts with anti-IKKα antibody, and the kinase assay (KA) was performed with [γ-^{32}P]ATP and GST−IκBα fusion protein. The level of IKK in the immunoprecipitate was measured by immunoblotting (IB) analysis using anti-IKKα antibody. (B) HeLa cells grown in six-well plates were transiently transfected with a plasmid (1 μg) encoding Flag-IKKβ. After 48 h, the cells were treated with the indicated concentrations of TPCK for 1 h, IKKβ was immunoprecipitated using anti-Flag antibody, and the kinase assay was performed. IKKβ in the immunoprecipitate was detected using anti-Flag antibody. (C) Cells were transfected with a plasmid encoding Flag-IKKβ as in panel B, and the expressed enzyme was immunoprecipitated with anti-Flag antibody. The isolated Flag-IKKβ was incubated with the indicated concentrations of TPCK for 30 min at 30 °C. An in vitro kinase assay and immunoblotting analysis were performed as described for panel B.

TPCK Inhibits TNFα-Induced IKK Activation and in Vitro IKKβ Activity. Since TPCK inhibited phosphorylation of IκBα, we tested whether TPCK inhibits TNF-α-induced IKK activation in HeLa cells. Cells incubated with various concentrations of TPCK were stimulated with TNF-α for 10 min, and IKK was immunoprecipitated from the cell extracts with anti-IKKα antibody to measure the kinase activity (Figure 2A). TPCK suppressed the TNF-α-induced IKK activation in a dose-dependent manner. Because the IKKβ subunit of the IKK complex is essential for activation of NF-κB in response to TNF-α, we examined whether TPCK suppresses the IKKβ expressed in HeLa cells. Cells transfected with IKKβ expression vectors were treated with various concentrations of TPCK for 1 h, followed by measurement of enzyme activity. The overexpressed IKKβ, which was active in the absence of any stimulation, was suppressed by TPCK (Figure 2B). To determine whether TPCK directly inhibits IKKβ, it was isolated from transfected cells and kinase activity was determined after addition of TPCK in vitro. The results show that TPCK blocked IKKβ activity in vitro at a range of concentrations similar to that effective for cultured cells (Figure 2C).

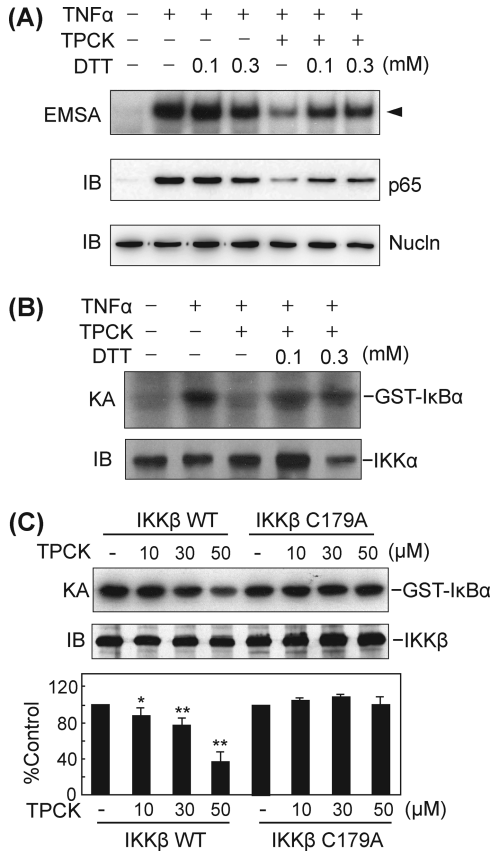


FIGURE 3: TPCK inhibits IKK by modifying Cys-179 of IKKβ. (A) Dithiothreitol (DTT) abrogates TPCK-mediated inhibition of NF-κB activation. HeLa cells were incubated with 30 μM TPCK and indicated concentrations of dithiothreitol for 1 h and stimulated with TNF-α (30 ng/mL) for 10 min. Nuclear extracts were prepared, and κB binding activity was measured by an EMSA. The retarded band is marked with an arrowhead. The amounts of p65/RelA and nucleoporin p62 (Nucln) in the nuclear extract were measured by immunoblotting analysis (IB) using specific antibodies. (B) Cells were treated as described for panel A and lysed, and the IKK complex was isolated by immunoprecipitation using an anti-IKKα antibody. The immune complex was analyzed by the kinase assay (KA), and the level of IKKα in the immune complex was determined by immunoblotting analysis. (C) HeLa cells were transiently transfected with the expression vectors for Flag-IKKβ or Flag-IKKβ C179A (1 μg each) together with an expression vector for MEKK1 (0.1 μg). Cells were treated with various concentrations of TPCK, and the activity of IKKβ and its expression level were determined by the kinase assay and immunoblotting analysis using anti-Flag antibody, respectively. The radioactivity of phosphorylated GST-IκBα was measured by phosphor image analysis, and the results are presented as the percent of untreated control cells (bottom). The experiment was repeated three times, and the results are presented as means ± the standard deviation. One asterisk indicates $P < 0.05$, and two asterisks indicate $P < 0.01$ (by the Student's unpaired t-test).

Inhibition of IKKβ by TPCK Occurs through Modification of Cys-179. The inhibitory effect of TPCK on NF-κB−DNA binding has been shown to be prevented by the thiol reducing agent dithiothreitol (*17*). When dithiothreitol together with TPCK was added to cells, TPCK-induced inhibition of NF-κB activation was suppressed (Figure 3A). The recovery of κB binding activity by dithiothreitol was accompanied by restoration of the nuclear p65/RelA level, as determined by immunoblotting analysis. To test whether dithiothreitol also prevents TPCK-induced IKK inactivation, the IKK complex was isolated from cells treated with TPCK and dithiothreitol and assessed for kinase activity (Figure 3B). Dithiothreitol prevented TPCK-induced IKK inactivation, and recovery of IKK activity was detected after addition of 0.1 mM dithiothreitol.

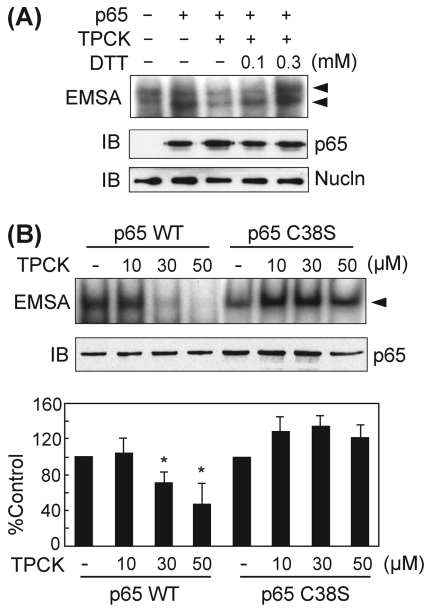
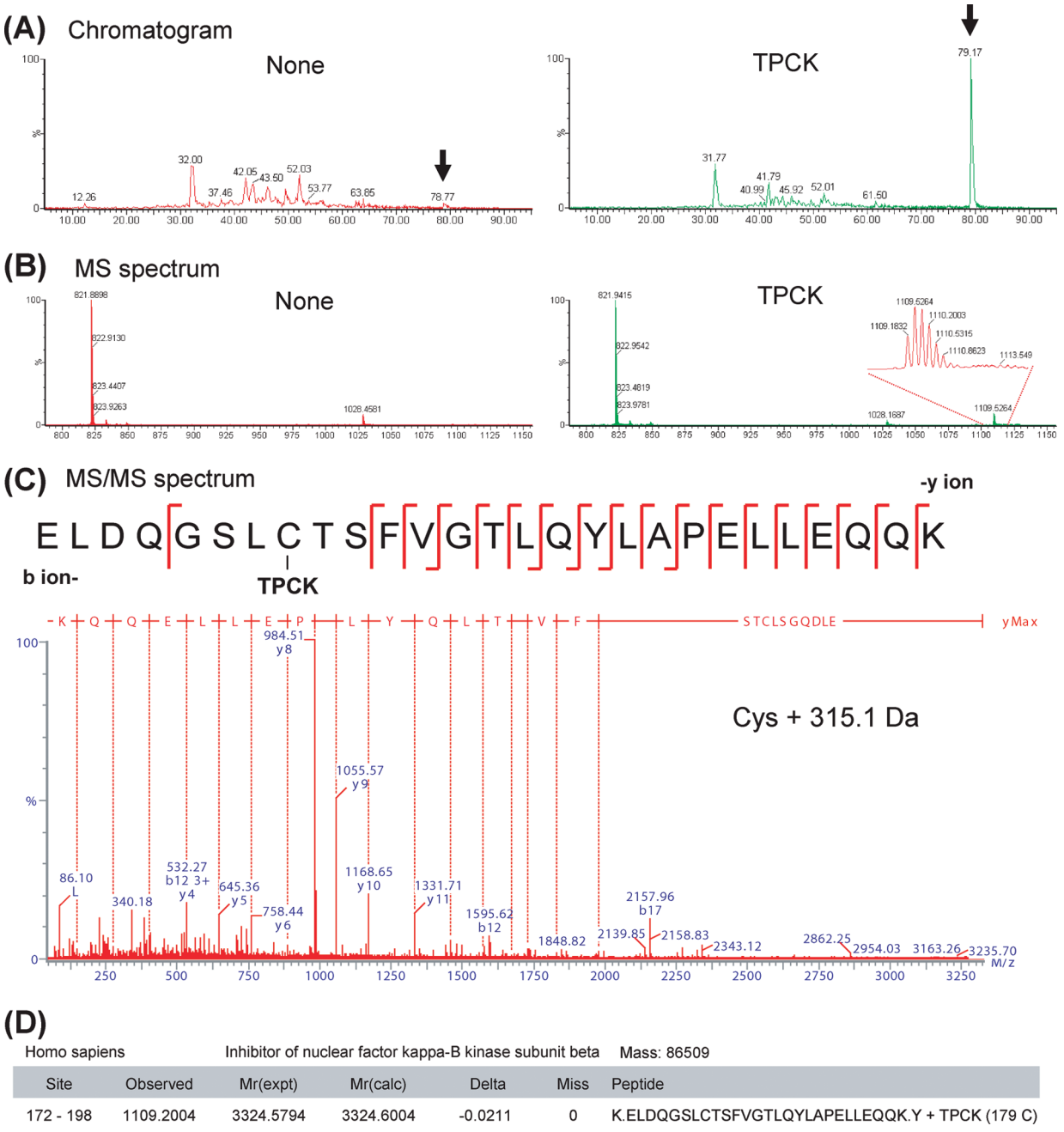


FIGURE 4: TPCK inhibits DNA binding of p65/RelA by blocking Cys-38 of p65/RelA. (A) Cells were transiently transfected with expression vectors for p65/RelA. After 48 h, the cells were incubated in the presence or absence of 30 μM TPCK and/or 0.1 and 0.3 mM dithiothreitol (DTT) for 1 h. Nuclear extracts were prepared, and κB binding activity and p65/RelA and nucleoporin p62 (Nucln) protein levels were measured by an EMSA and immunoblotting analysis (IB), respectively. (B) Cells were transiently transfected with expression vectors for wild-type p65/RelA or the p65/RelA C38S mutant. After 48 h, the cells were incubated with the indicated concentrations of TPCK for 1 h. Nuclear extracts were prepared, and κB binding activity and p65/RelA levels were measured by an EMSA and immunoblotting analysis, respectively (top panel). The radioactivity of the retarded bands was measured by phosphor image analysis. The results are presented as means ± the standard deviation ($n = 3$) (bottom panel). An asterisk indicates $P < 0.01$.

Since Cys-179 of IKKβ is known to play a critical role in regulating enzyme activity (*4*) and acts as a target site for various IKK inhibitors (*5*−*12*), we examined the role of Cys-179 in TPCK-induced IKKβ inhibition. To test this, the cells were transfected with a vector encoding wild-type IKKβ or the IKKβ mutant in which Cys-179 is substituted with alanine (IKKβ C179A). Because IKKβ C179A demonstrates a greatly reduced activity in the absence of the activating kinase compared with wild-type IKKβ (*4*), an expression vector for MEKK1 was coexpressed with IKKβ and IKKβ C179A. In these cells, TPCK inhibited the kinase activity of wild-type IKKβ; the kinase activity was decreased by more than 60% after addition of 50 μM TPCK (Figure 3C). In contrast, the activity of IKKβ C179A remained unchanged in the presence of the same concentration of TPCK.

TPCK Inhibits DNA Binding of p65/RelA by Modifying Cys-38. Our results suggested that TPCK inhibits IKK through modification of the thiol group of IKKβ Cys-179. It has been shown that, like Cys-179 of IKKβ, Cys-38 of the NF-κB subunit p65/RelA plays an important role in DNA binding and is susceptible to inhibition by various thiol-reactive agents (*11*, *25*−*28*). To determine whether TPCK inhibits DNA binding of p65/RelA, its effect was monitored in HeLa cells transfected with the p65/RelA expression vector. An EMSA of nuclear extracts demonstrated a significant increase in κB binding activity in cells transfected with the



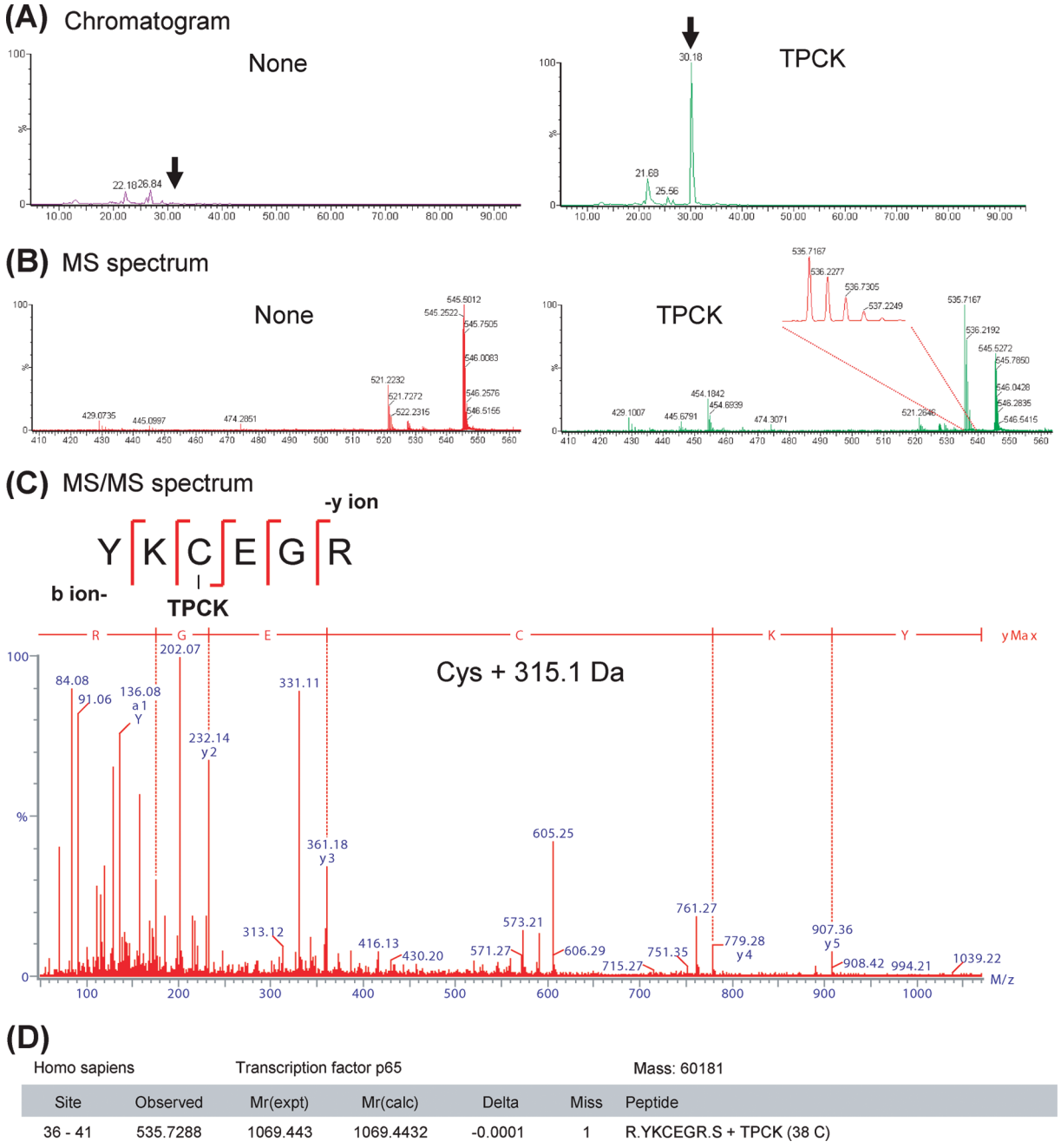
Site	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Peptide
172 - 198	1109.2004	3324.5794	3324.6004	-0.0211	0	K.ELDQGSLCTSFVGTLQYLAPELLEQQK.Y + TPCK (179 C)

FIGURE 5: Analysis of TPCK labeling at Cys-179 in IKKβ by UPLC-ESI-q-TOF tandem MS. (A) IKKβ peptides prepared by trypsin digestion were separated on a BEH C18 column. The TPCK-labeled peptide was eluted at 79.17 min, whereas the peak was not detected at the same position in chromatography with the control IKKβ peptide sample. Arrows indicate the retention time of the TPCK-labeled peptide. (B) MS spectra of a precursor ion of the TPCK-labeled peptide that eluted at 79.17 min. The inset shows detailed m/z values of the precursor ion derived from the TPCK-labeled peptide. (C) MS/MS spectrum of decayed products of precursor ion indicating binding of the tosylphenylalanylmethyl group (315.1 Da) at Cys-179. (D) Result obtained from the earch for modification of Cys-179 using global search engine MASCOT and MOD[1].

p65/RelA expression vector compared with control cells transfected with the empty vector (Figure 4A). Treatment of cells with TPCK suppressed p65/RelA−DNA binding to basal levels, whereas concomitant treatment of cells with TPCK and dithiothreitol recovered binding.

To examine the role of Cys-38 of p65/RelA during inhibition by TPCK, a mutant p65/RelA, in which Cys-38 was substituted with serine (p65/RelA C38S), was expressed in HeLa cells. The effect of TPCK was then monitored. The results show that DNA binding of the p65/RelA C38S mutant was resistant to the inhibitory effect of TPCK, suggesting that modification of Cys-38 by TPCK is involved in TPCK-mediated inhibition of p65/RelA−DNA binding (Figure 4B).

Binding of TPCK to Cys-179 of IKKβ and Cys-38 of p65/RelA. To verify whether Cys-179 of IKKβ and Cys-38 of p65/RelA are modified by TPCK treatment, the modifications of proteins isolated from TPCK-treated cells were analyzed by tandem mass spectrometry (MS/MS). HEK-293 cells transfected with expression vectors of IKKβ and p65/RelA were incubated in the presence or absence of TPCK. After purification by immunoaffinity chromatography and gel electrophoresis, the proteins were digested with trypsin, and the resulting peptides were analyzed by UPLC-ESI-q-TOF tandem MS. The result of liquid chromatography (LC)/MS showed that TPCK-labeled peptides of IKKβ and p65/RelA were eluted at 79.17 and 30.18 min, respectively, whereas no peaks were detected at those positions in



Site	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Peptide
36 - 41	535.7288	1069.443	1069.4432	-0.0001	1	R.YKCEGR.S + TPCK (38 C)

FIGURE 6: Analysis of TPCK labeling at Cys-38 in p65/RelA by UPLC-ESI-q-TOF tandem MS. Analysis was conducted as described in the legend of Figure 5. (A) p65/RelA peptides were separated on a BEH C18 column, and the TPCK-labeled peptide eluted at 30.18 min (arrows). (B) MS spectra of a precursor ion. The inset shows detailed m/z values of the precursor ion. (C) MS/MS spectrum of decay products of the precursor ion indicating binding of the tosylphenylalanylmethyl group (315.1 Da) at Cys-38. (D) Result obtained from the search for modification using global search engine MASCOT and MOD[i].

peptides obtained from control cells not treated with TPCK (Figures 5A,B and 6A,B). The precursor ions that eluted at these retention times were selected in the MS scan and fragmented for sequencing by collision-induced dissociation (CID), and the fragments were searched for modification by Mascot and MOD[i]. Among dissociated fragments detected in the MS/MS scan, those containing Cys-179 of IKKβ and Cys-38 of p65/Rel modified with the tosylphenylalanylmethyl group (315.1 Da) were identified (Figures 5C,D and 6C,D).

TPCK-Induced Change in the Cellular Glutathione Level. Glutathione, a tripeptide containing a cysteine thiol group, is a major antioxidant in cells that provides reducing power to maintain cellular redox potential. To determine whether TPCK treatment induces a change in the cellular glutathione level by blocking the thiol group of GSH, we measured the levels of GSH and GSSG and the GSH:GSSG ratio in HeLa cells (Figure 7). After incubation with TPCK for 1 h, cellular levels of GSH and GSSG and the GSH:GSSG ratio were not changed by TPCK at concentrations up to 30 μM. However, incubation of cells with 50 μM TPCK induced a 13% decrease in the level of GSH and 5% increase in the level of GSSG, resulting in a significant decrease in the GSH:GSSG ratio compared with that of untreated control cells.

DISCUSSION

In this study, TPCK was shown to inhibit TNF-α-induced nuclear κB binding activity and increase the nuclear p65/RelA

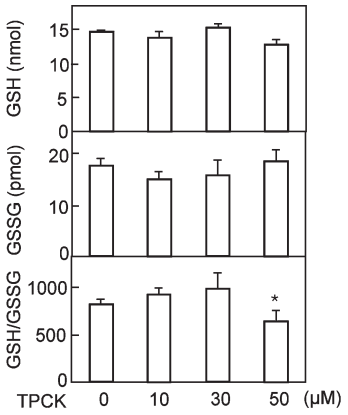


FIGURE 7: TPCK-induced change in cellular glutathione levels. HeLa cells (3×10^6) were incubated in medium containing the indicated concentrations of TPCK for 1 h and lysed. Total levels of glutathione and oxidized glutathione (GSSG) in the cell lysate were measured by spectrophotometry. The amounts of reduced glutathione (GSH) and the GSH:GSSG ratio were calculated. Results are presented as means ± the standard deviation ($n = 3$ per group). An asterisk indicates $p < 0.05$ vs untreated cells, by the Student's unpaired t-test.

level as well as the levels of phosphorylation and degradation of cytosolic IκBα. These results suggest that the effect of TPCK appeared during or before TNF-α-induced phosphorylation of IκBα, and IKK or another upstream signal molecule in the NF-κB activation pathway is a target of TPCK. Determination of IKK activity showed that TPCK indeed blocks activation of IKK in TNF-α-stimulated cells, suppresses enzyme activity in cells expressing IKKβ, and directly inhibits IKKβ in vitro, indicating that blocking of IKKβ is responsible for the inhibition of NF-κB activation by TPCK.

TPCK-mediated suppression of NF-κB and IKK activation was significantly prevented by addition of dithiothreitol, suggesting that TPCK inhibits NF-κB activation by modifying a cysteine thiol in IKK or other signaling molecules in the NF-κB pathway. Other studies also showed that inhibition of NF-κB-dependent gene expression in TPCK-treated cells could be recovered by reducing agents, such as dithiothreitol, *N*-acetylcysteine, and 2-mercaptoethanol (*28*, *29*). In previous studies, various thiol-reactive compounds, including cyclopentenone prostaglandins (*5*), arsenite (*6*), parthenolide (*7*), the gold compound auranofin (*8*), nitric oxide (*9*), cobrotoxin (*10*), epoxyquinone A monomer (*11*), and butein (*12*), were shown to commonly inhibit IKKβ through modification of Cys-179. Our previous study showed that Cys-179 of IKKβ plays a crucial role in signal-induced activation of IKKβ by promoting phosphorylation of adjacent Ser-177 and Ser-181 residues as well as by affecting the catalytic function of the active enzyme (*4*). In this study, an IKKβ mutant, in which Cys-179 had been substituted with alanine (IKKβ C179A), was shown to be resistant to inactivation by TPCK. Moreover, analysis of the IKKβ protein isolated from TPCK-treated cells by MS/MS revealed that the tosylphenylalanylmethyl group was bound to Cys-179 of IKKβ. Taken together, these results indicate that TPCK inhibits IKKβ through modification of Cys-179.

Previous reports have shown that various thiol-reactive compounds that inhibit IKK also inhibit NF-κB−DNA binding through modification of Cys-38 in the NF-κB p65/RelA subunit (*7*, *11*, *25*−*27*). In a previous study, inhibition of NF-κB−DNA binding by TPCK in vitro was shown to be prevented by the reducing agent dithiothreitol, suggesting that modification of a cysteine thiol group is involved in TPCK-mediated inhibition of NF-κB−DNA binding (*17*). In these results, TPCK blocked DNA binding of p65/RelA in transiently expressing cells, and addition of dithiothreitol along with TPCK prevented this inhibition, confirming the modification of a cysteine thiol in p65/RelA by TPCK. The p65/RelA mutant, in which Cys-38 was substituted with serine (p65/RelA C38S), was resistant to inhibition by TPCK, and MS/MS sequencing analysis of p65/RelA obtained from TPCK-treated cells showed attachment of the tosylphenylalanylmethyl group to Cys-38. These results suggest that, in addition to inhibition of IKKβ, direct blocking of NF-κB−DNA binding through modification of p65/Rel Cys-38 is another mechanism for inhibition of NF-κB activity by TPCK.

GSH containing a free thiol group is involved in removal of toxic oxygen radicals and is required for the maintenance of the normal reduced state in the cell. Our result showed that incubation of cells with TPCK up to 30 μM did not change the level of GSH or GSSG or the GSH:GSSG ratio, whereas 50 μM TPCK induced a significant decrease in the GSH:GSSG ratio caused by a decreased level of GSH and an increased level of GSSG compared to those of nontreated control cells. A previous study showed that a near-complete depletion of cellular GSH via treatment of cells with a thiol oxidizing agent diethylmaleate prevented NF-κB induction in endotoxin-treated rat hepatocytes (*30*). However, it was shown that a mild oxidative shift of the glutathione pool via treatment of cells with H_2O_2 or L-lactate rather greatly enhanced NF-κB activation in T cells stimulated with anti-CD3 and anti-CD28 (*31*). These results suggest that a mild decrease in the GSH:GSSG ratio observed in TPCK-treated cells was not a cause of TPCK-induced NF-κB inhibition, but a result of GSH thiol modification by TPCK. Our result also suggested that TPCK reacts more easily with cysteine thiol groups of IKKβ and p65/RelA than that of GSH, because inhibition of IKKβ and p65/RelA occurred at lower concentrations of TPCK than the change in the cellular GSH level.

In conclusion, our results show that the protease inhibitor TPCK inhibited two steps in the NF-κB activation pathway by modifying specific cysteine residues in IKKβ and p65/RelA. Homologous cysteine residues are present in IKKα (*5*), and other NF-κB family proteins such as RelB, c-Rel, p105/p50, and p100/p52 (*32*), suggesting that TPCK also inhibits these proteins in addition to IKKβ and p65/RelA. Although TPCK-induced blocking of these multiple proteins in NF-κB signaling may cause unwanted side effects, it also suggests that TPCK is more effective in inhibiting various canonical and noncanonical pathways of NF-κB activation than inhibitors more specific to IKK or NF-κB subunits. TPCK has been shown to have therapeutic effects in hypoxic-ischemic brain injury and collagen-induced arthritis in experimental animals, without any obvious adverse side effects (*33*, *34*). Understanding the mechanism of compounds like TPCK, which blocks multiple steps in the NF-κB signaling pathway, may lead to the development of more effective therapeutics for ischemic and inflammatory diseases.

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