**N**α-Tosyl-**L**-phenylalanine Chloromethyl Ketone Induces Caspase-dependent Apoptosis in Transformed Human B Cell Lines with Transcriptional Down-regulation of Anti-apoptotic HS1-associated Protein X-1**§**

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Nα-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) has been widely used to investigate signal transduction pathways that are involved in gene expression and cell survival/cell death. However, contradictory effects of TPCK on apoptosis have been reported, and the underlying signaling events leading to TPCK-induced promotion or prevention of apoptosis are not fully understood. Here, we show that TPCK induces caspase-dependent apoptosis in Epstein-Barr virus (EBV)-transformed human B cell lines with release of pro-apoptotic proteins from mitochondria. TPCK treatment also results in down-regulation of the anti-apoptotic proteins, cIAP1, cIAP2, and HAX-1, and caspase-dependent cleavage of the anti-apoptotic proteins, Bcl-2 and XIAP. Quantitative PCR analysis confirmed that the TPCK-induced down-regulation of HAX-1 occurred at the transcriptional level, and experiments using the specific pharmacological inhibitor, Bay 11-7082, suggested that HAX-1 expression and cell survival/cell death. However, contradictory effects of TPCK on apoptosis have been reported. Hence, TPCK was shown to prevent apoptosis in a cell death stimulus-dependent manner in some model systems (2–4), and a recent study also suggested that TPCK may diminish apoptosis by direct and nonspecific inhibition of active caspases (5). On the other hand, TPCK was also reported to potentiate apoptosis induced by certain apoptotic stimuli, and this was suggested to occur, at least in some cases, through activation of cell cycle checkpoints via inhibition of the proteasome (3, 6).

TPCK alone may also induce apoptosis. TPCK thus caused a reduction in activity of the transcription factor, NF-κB, in a murine B lymphoma cell line resulting in a decrease in c-myc and activation of apoptosis (7, 8). Subsequent studies have shown that TPCK can induce apoptosis in a human acute monocytic leukemia cell line through activation of mitochondria-dependent apoptosis signaling (4, 9). Studies in transformed human T cells also indicated that TPCK can induce apoptosis through inhibition of constitutive NF-κB activation, and it was proposed that TPCK might affect sulfhydryl groups on proteins involved in regulating cell survival and NF-κB acti.

4 The abbreviations used are: TPCK, Nα-tosyl-L-phenylalanine chloromethyl ketone; z, N-benzyloxycarbonyl; fmk, fluoromethyl ketone; AIF, apoptosis-inducing factor; cIAP1, cellular inhibitor of apoptosis protein-1; cIAP2, cellular inhibitor of apoptosis protein-2; AMC, 7-amino-4-methylcoumarin; DHE, dihydroethidium; Δm, mitochondrial transmembrane potential; EBV, Epstein-Barr virus; HAX-1, HS1-associated protein X-1; IκB, inhibitor of NF-κB; NAC, N-acetylcysteine; NF-κB, nuclear factor κB; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; ROS, reactive oxygen species; SCN, severe congenital neutropenia; TMRE, tetramethylrhodamine ethyl ester; XIAP, X chromosome-linked inhibitor of apoptosis protein.
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Additionally, it has been reported that TPCK may inhibit 3-phosphoinositide-dependent kinase 1 signaling thus disabling central kinase cascades governing proliferation and survival (11). More recently, studies in human colorectal carcinoma cell lines pointed to a requirement of dephosphorylation of p53 during TPCK-induced apoptosis (12). Overall, therefore, TPCK appears to exert cell type- and stimulus-dependent effects on cell survival and cell death (apoptosis) and, moreover, these opposing effects may not necessarily be related to a direct inhibition of serine protease activity, as was originally believed to be the case.

To further explore the molecular pathways of TPCK-induced apoptosis in normal (non-malignant) cells, we utilized Epstein-Barr virus (EBV)-transformed human B cell lines. Our studies show that TPCK activates caspase-dependent apoptosis in EBV-transformed B cell lines with generation of reactive oxygen species (ROS) and release of the pro-apoptotic proteins, cytochrome c, and apoptosis-inducing factor (AIF) from mitochondria. TPCK also induced a pronounced down-regulation of cIAP1, cIAP2, and HAX-1 and concomitant caspase-dependent cleavage of Bcl-2 and XIAP. TPCK-induced modulation of HAX-1 occurred at the transcriptional level, and our data suggest that HAX-1 is a novel target gene for NF-κB. The pro-apoptotic effects of TPCK in human B cell lines were abolished in the presence of the thiol-containing anti-oxidant, N-acetylcysteine (NAC).

**EXPERIMENTAL PROCEDURES**

**Generation and in Vitro Culture of Human B Cell Lines**—The EBV-transformed B cell lines were established from peripheral blood lymphocytes separated from a healthy blood donor according to a standard protocol (13). In brief, lymphocytes were isolated by centrifugation at 800 × g on a Ficoll® gradient and incubated in EBV-containing transformation medium supplemented with 20% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. Cyclosporin A (1 mg/ml) was added to inactivate the T lymphocytes. Transformed cell lines were maintained in a humidified 5% CO₂ atmosphere in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mM l-glutamine. The study was approved by the ethical committee at Uppsala University, Uppsala, Sweden.

**Generation of B Cell Lines from Kostmann Patients**—EBV-transformed B cell lines were established from two patients with severe congenital neutropenia (SCN) or Kostmann disease according to the procedures described above. The patients both harbor inherited mutations in the HAX1 gene known to be an underlying cause of autosomal recessive SCN (14). Detailed clinical descriptions of these patients were recently reported (15). Patient 1 thus belongs to the original Kostmann family in Stockholm, Sweden, whereas patient 2 harbors an alternative homozygous HAX1 mutation (131G→A, W44X). The patient studies were approved by the ethical committee at Umeå University, Umeå, Sweden.

**Cultivation of the Jurkat T Cell Line**—The human T cell leukemia cell line Jurkat from the European Collection of Cell Cultures (Salisbury, UK) was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Flow Cytometric Determination of Apoptosis**—Cells in logarithmic growth phase were treated with TPCK (Calbiochem) at the indicated concentrations in the presence or absence of N-benzyloxy carbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk, Sigma, 25 μM) or N-acetylcysteine (NAC, Sigma, 5 mM). For some experiments, cells were treated with 50 μM TPCK in the presence or absence of l-cysteine (Sigma, 3 mM) or d-cysteine (Sigma, 3 mM). Apoptosis was determined by flow cytometric quantification of phosphatidylserine (PS) exposure using annexin V-FITC staining and/or DNA content using propidium iodide (PI) staining. Briefly, for detection of PS exposure using recombinant annexin V-FITC (Calbiochem), cells were washed and resuspended in HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1.8 mM CaCl₂) containing annexin V-FITC (2 μg/ml) and PI (125 ng/ml) prior to analysis on a FACSScan (BD Biosciences, San Jose, CA) equipped with a 488 nm argon laser. Ten thousand events were collected for each sample and analyzed using CellQuest software (BD Biosciences). Cell debris was gated out before analysis. For evaluation of hypodiploid DNA content, cells were harvested, washed once in PBS, and resuspended in staining solution containing 50 μg/ml PI, 0.1% Triton X-100, and 0.1% sodium citrate in PBS. Cells were then analyzed on a FACSScan (BD Biosciences) operating with CellQuest software (BD Biosciences).

**Determination of Nuclear Morphology**—For detection of typical features of nuclear apoptosis, cells were harvested and resuspended in 2% paraformaldehyde. Cytospin preparations were then stained with 1 μg/ml Hoechst 33342 (Molecular Probes, Leiden, The Netherlands), and cells were scored for chromatin condensation and fragmentation using a fluorescence microscope (Nikon ECLIPSE TE2000-S, Nikon Corp., Kanagawa, Japan).

**Quantification of Caspase Activation**—Activation of caspase-3-like enzymes was monitored using the specific fluorogenic substrate, Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin (DEVD-AMC). Briefly, cell lysates and fluorogenic peptide substrate (Sigma) were combined in a standard reaction buffer (100 mM HEPES, 10% sucrose, 5 mM dithiothreitol, 1.0 × 10⁻⁶ M Nonidet P-40, and 0.1% CHAPS), and real-time recordings of enzyme-catalyzed AMC release were obtained using a Fluoroscan II plate reader operating with Genesis software (LabSystems, Stockholm, Sweden). Fluorescence values were converted to picomoles of AMC release using a standard curve generated with free AMC and the maximum rate of AMC release (picomoles/min) was calculated.

**ROS Production and Mitochondrial Transmembrane Potential (ΔΨₘ)**—Intracellular production of ROS was measured using dihydroethidium (DHE, Molecular Probes). Oxidation of DHE by superoxide anion (O₂⁻) yields the fluorescent hydroethidium. Briefly, cells were incubated with 5 μM DHE in RPMI 1640 medium for 30 min at 37 °C. After resuspension in PBS, cells were submitted to flow cytometric analysis using a FACScan (BD Biosciences) operating with CellQuest software (BD Biosciences).
Biosciences). To evaluate the dissipation of mitochondrial transmembrane potential (Δψm), cells were incubated for 20 min at 37 °C in cell culture medium with the cationic fluorescent probe, tetramethylrhodamine ethyl ester (TMRE, Molecular Probes, 25 nM). Cells were then spun down, washed once in PBS, and resuspended in HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1.8 mM CaCl₂) containing 25 nM TMRE. As a positive control, cells were incubated for 10 min with the uncoupling agent, carbonyl cyanide 3-chlorophenylhydrazone (Sigma, 100 μM). Flow cytometric analysis was performed on a FACScan (BD Biosciences) using CellQuest software (BD Biosciences). For some experiments, monoclonal anti-Fas antibodies (Medical & Biological Laboratories, Ltd., Nagoya, Japan) were used as a positive control.

Fluorescence-based Assay for Determination of Free Sulphydryl Groups—To assess the potential for direct interactions between NAC and TPCK, cell-free experiments were conducted in which NAC and TPCK were mixed at the indicated concentrations and incubated at 37 °C. To determine free sulphydryl groups in NAC, the maleimide fluorescent sulphydryl reagent ThioGlo™3 (Covalent Associates, Corvallis, OR) was added to each reaction at a final concentration of 1 μM. After incubation for 30 min at 25 °C, the fluorescence content of each sample was determined on a multimode microplate reader (Infinite 200, Tecan, Männedorf, Switzerland) using excitation and emission wavelengths 360 nm and 465 nm, respectively. Results were normalized to control (1 μM ThioGlo™3 solution). All reagents were diluted in PBS, pH 7.2, prior to use.

Mass Spectrometric Analysis—TPCK (>97% pure) and NAC (99% pure, according to the manufacturer) were mixed together in PBS buffer at 200 and 10 μM, respectively, and placed in an incubator at 37 °C. Samples (100 μL) were taken out and infused with a syringe pump at a flow rate of 20 μL/minute into a Q-TOF Micro (Micromass, Manchester, UK). Samples were analyzed in positive ion mode with the following source settings: capillary voltage, 3500 V; cone voltage, 20 V; source temperature, 200 °C; desolvation gas flow rate, 200 L/h; and desolvation temperature, 200 °C. Mass spectra were acquired over mass-to-charge ratios of 100 to 2000.

Preparation of Mitochondrial and Cytosolic Fractions and Total Cell Lysates—For total cell lysates, cells were lysed for 30 min at 4°C in radioimmune precipitation assay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.5% deoxycholate). To obtain mitochondrial and cytosolic fractions, cells were resuspended in buffer A (50 mM PIPES-KOH, pH 7.4, 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, and 1 mM dithiothreitol) supplemented with a mixture of protease inhibitors (Roche Diagnostics GmbH, Basel, Switzerland). Cell suspensions were then incubated with 100 μg/ml digitonin, and homogenates were centrifuged at 12,000 × g for 10 min at 4 °C, and supernatant (cytosolic fraction) as well as pellet (enriched for nuclei and mitochondria) were recovered. Protein concentration was measured using the Bradford reagent (Pierce), and samples were denatured in standard Laemmli buffer. Purity of the mitochondrial and cytosolic fractions was determined by probing with antibodies to cytochrome c oxidase subunit IV (COXIV, Molecular Probes) and β-actin (Sigma), respectively.

Western Blot Analysis—Mitochondrial and cytosolic fractions or total cell extracts (20–60 μg) were resolved by electrophoresis on a 10–12% SDS-polyacrylamide gel and transferred to Immun-Blot™ polyvinylidene difluoride membranes (0.2 μM, Bio-Rad). Membranes were blocked with 5% nonfat milk in 0.1% TBS-T and subsequently probed with antibodies directed against Bcl-2 (DakoCytomation, Glostrup, Denmark), cytochrome oxidase subunit VIIa (OxPhos complex IV subunit VIIa, COXIV, Molecular Probes), poly(ADP-ribose) polymerase (PARP, Biomol International, Plymouth Meeting, PA), X-linked inhibitor of apoptosis protein (XIAP, Nordic Biosite, Täby, Sweden), HAX-1 (BD Biosciences), cytochrome c (BD Biosciences), cellular inhibitor of apoptosis protein-1 (cIAP1, Santa Cruz Biotechnology, Santa Cruz, CA), AIF (Santa Cruz Biotechnology), or cellular inhibitor of apoptosis protein-2 (cIAP2, R&D Systems, Minneapolis, MN). After washing, membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (DakoCytomation). Proteins were visualized with the enhanced chemiluminescence (ECL) detection reagents (Amersham Biosciences). Membranes were reprobed with antibodies to β-actin (Sigma) to control for equal loading of protein.

Quantitative Real-time PCR—Total RNA from cells treated with TPCK or Bay 11-7082 (Calbiochem) was isolated using NucleoSpin RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Additional incubation with DNase I (Roche Applied Science) was carried out to ensure the elimination of genomic DNA from the samples. cDNA was synthesized from 400 ng of RNA using SuperScript II reverse transcriptase (Invitrogen), according to the manufacturer’s instructions. Quantitative real-time PCR was performed using 20 ng of cDNA template, Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), and 100 nM of the appropriate forward and reverse primers. The following primers were used to amplify 126 bp of the human HAX-1 transcript: 5’-GACCTCGGAGGCAAGCAGAT-3’ (forward primer sequence), 5’-GGTGCTGAGACTTGGAAC-3’ (reverse primer sequence). The β-actin gene was used as a reference (forward primer sequence 5’-AGCCTGCGCTTTGGCA-3’, reverse primer sequence: 5’-GGCGGCGCATTCATC-3’). The amplification was carried out using an ABI PRISM 7000 (Applied Biosystems), and the cycling conditions were as follows: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 60 s (for β-actin: 40 cycles of amplification at 95 °C for 30 s, 55 °C for 60 s, and 72 °C for 30 s). Each sample was run in triplicate from three independent experiments. To assess specificity, amplification products were subjected to melting curve analysis. Relative mRNA levels were calculated using the comparative cycle threshold method, and values were normalized against β-actin.

NF-κB Binding Site Prediction—The 5’-flanking region of the human HAX-1 genomic sequence was analyzed using the GenBank™ record containing a partial sequence of human chromosome 1 (GenBank™ accession number AL354980). Putative binding sites of the human NF-κB transcription factor family proteins, p50, p52, c-Rel, RelA/p65, and RelB, were investigated in silico using the AliBaba2 software (16), which uses the TRANSFAC 4.0 transcription factor data base (17).
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The genomic sequence 5 kb upstream of the first ATG codon was analyzed.

**Statistical Analysis**—The results are expressed as mean values ± S.D. Student’s t test was used to evaluate differences between samples, and differences between mean values were considered significant when \( p < 0.05 \).

**RESULTS**

**TPCK Induces Prominent, Caspase-dependent Apoptosis in EBV-transformed Human B Cells**—To investigate whether TPCK can trigger apoptosis in EBV-transformed B cells, we incubated cells with TPCK at various concentrations ranging from 10 to 75 μM. Apoptosis was determined by quantification of PS externalization and measurement of hypodiploid DNA content. In addition, activation of caspase-3-like enzymes as well as cleavage of the nuclear caspase substrate, PARP, was also determined. TPCK triggered PS exposure and caspase activation in a concentration-dependent manner (Fig. 1, A and B). Cleavage of PARP to its characteristic 85-kDa fragment was also observed in TPCK-treated cells (Fig. 1C). Next, we performed studies of the time course of TPCK-induced apoptosis. TPCK (50 μM) triggered time-dependent PS externalization, and the population of annexin V-positive/PI-negative cells displayed a maximum at 6 h of treatment, followed by increasing numbers of annexin V-positive/PI-positive cells, indicative of secondary necrosis (sometimes referred to as “late” apoptosis) (Fig. 1D). TPCK also induced a time-dependent induction of apoptosis as determined by the measurement of hypodiploid DNA content (Fig. 1E). Apoptosis induction was confirmed by the detection of typical nuclear chromatin condensation and fragmentation (data not shown).

To further study whether TPCK induces caspase activation in human EBV-transformed B cells, a time course of TPCK-triggered DEVD-AMC cleavage was performed. Caspase-3-like enzyme activity was induced by TPCK (50 μM), and the activity peaked at 4 h (Fig. 2A). To confirm that specific caspase-3 activation occurred in these cells, a similar time course (1–12 h) was performed, and samples were harvested for detection of pro-caspase-3 processing using specific anti-caspase-3 antibodies. As seen in Fig. 2B, TPCK induced the processing of pro-caspase-3 (32 kDa) into its active form (17 kDa). TPCK-induced activation of caspase-3 coincided with the cleavage of PARP (Fig. 2B). Additionally, to assess whether TPCK-induced apoptosis was dependent upon caspase activation, cells were pre-treated in the presence or absence of the pan-caspase inhibitor, z-VAD-fmk (25 μM), and then stimulated with TPCK. TPCK-induced caspase activation was completely blocked by z-VAD-fmk (Fig. 2C). Moreover, TPCK-induced apoptosis, as determined by detection of hypodiploid DNA content and PS externalization, was also suppressed by the pan-caspase inhibitor (Fig. 2, d and e). Taken together, these results demonstrate that TPCK-induced apoptosis in EBV-transformed human B cells is caspase-dependent.

**TPCK Induces Mitochondrial Depolarization with Release of Pro-apoptotic Factors into the Cytosol**—Previous studies have shown that TPCK-induced apoptosis may occur through a mitochondria-dependent pathway (4, 9). We therefore asked whether TPCK induces mitochondria-dependent apoptosis in EBV-transformed B cells. Reduction of mitochondrial transmembrane potential (Δψm) was detected at 1 h and Δψm progressively decreased in a time-dependent manner (Fig. 3A). Further studies showed that the effect of TPCK was also concentration-dependent (Fig. 3B). Next, we studied the release of the pro-apoptotic factors, cytochrome c and AIF, from mito-
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TPCK triggers caspase-dependent apoptosis in EBV-transformed B cells. A, B cells were incubated with TPCK (50 μM) for the indicated time points, and caspase activation was determined using the fluorogenic caspase substrate, DEVD-AMC. Data are shown as mean values ± S.D. of at least three independent experiments. B, Western blotting of B cells treated with TPCK. Total cell lysates were probed with antibodies to pro-caspase-3 (32 kDa) and its active subunit (17 kDa), and membranes were then stripped and reprobed with specific antibodies to PARP, a well known nuclear caspase substrate and to β-actin to control for equal loading of protein. C–E, B cells were pre-treated with the pan-caspase inhibitor z-VAD-fmk (25 μM) for 30 min and then subjected to treatment with TPCK (50 μM) for 6 or 12 h. Apoptosis was determined by measurement of caspase activation (C), hypodiploid DNA content (D), and PS exposure (E). Data are shown as mean values ± S.D. of at least three independent experiments. *, p < 0.001 TPCK versus TPCK plus z-VAD-fmk (C and E); #, p < 0.01 TPCK versus TPCK plus z-VAD-fmk (D).

The release of mitochondrial factors and drop in membrane potential during apoptosis are associated with the production of ROS. Indeed, TPCK induced a time-dependent increase in the generation of ROS as detected by the DHE assay (Fig. 4A). The dissipation of Δψm and the production of ROS were both inhibited to some extent by z-VAD-fmk in B cells treated with TPCK (Fig. 4, B and C). However, z-VAD-fmk failed to block these mitochondrial events completely, suggesting that caspase-independent events are involved. Furthermore, TPCK-induced release of cytochrome c was unaffected in the presence of the pan-caspase inhibitor (see Fig. 8B). In contrast, TPCK-induced release of AIF was almost completely inhibited by z-VAD-fmk, indicating that the release of AIF occurred downstream of cytochrome c release and caspase activation.

TPCK Induces Down-regulation of HAX-1, cIAP1, and cIAP2 and Cleavage of Bcl-2 and XIAP—The transcription factor, NF-κB, regulates the expression of numerous genes, including anti-apoptotic factors belonging to the cellular inhibitor of apoptosis protein (IAP) family. Furthermore, it has been suggested that TPCK induces apoptosis by inhibiting IkB phosphorylation resulting in inactivation of NF-κB (7, 8, 10). We therefore evaluated the expression of cIAP1, cIAP2, and XIAP in EBV-transformed B cells following treatment with TPCK. In addition, the expression of two mitochondria-targeted anti-apoptotic proteins, Bcl-2 and HAX-1, was also investigated; both proteins have been shown to prevent loss of mitochondrial membrane potential and/or release of pro-apoptotic factors from mitochondria during apoptosis (14, 19).

B cells were treated with different concentrations of TPCK in the presence or absence of z-VAD-fmk (Fig. 5A) and at different time points (Fig. 5B). Bcl-2 and XIAP were both cleaved in a caspase-dependent manner in TPCK-treated cells; the cleavage of both proteins was evident at doses of 25 μM or more (Fig. 5A) and occurred in a time-dependent manner starting at 2–4 h of treatment (Fig. 5B). The processing of Bcl-2 and XIAP in TPCK-treated cells thus occurs after caspase-3 activation and PARP cleavage (Fig. 2, A and B), and, furthermore, this event is completely prevented by z-VAD-fmk (Fig. 5A). This is in line with previous studies in other model systems showing that the cleavage of Bcl-2 and XIAP during apoptosis is caspase-dependent (20, 21). Moreover, TPCK treatment caused prominent down-regulation of cIAP1, as well as a detectable and time-dependent decrease in the expression of cIAP2 and HAX-1 (Fig. 5, A and B). TPCK-induced down-regulation of cIAP1, cIAP2, and HAX-1 protein expression was also prevented by z-VAD-fmk.

The role of HAX-1 in apoptosis has not been fully elucidated to date, although a number of previous studies have indicated that HAX-1 can prevent apoptosis in various model systems (22–24). Moreover, SCN patients harboring homozygous HAX1 mutations display increased apoptosis of myeloid progenitor cells with excessive release of cytochrome c from mitochondria (25). To test the putative role of HAX-1 in the protection against TPCK-induced apoptosis, EBV-transformed B cell

FIGURE 2. TPCK triggers caspase-dependent apoptosis in EBV-transformed B cells. A, B cells were incubated with TPCK (50 μM) for the indicated time points, and caspase activation was determined using the fluorogenic caspase substrate, DEVD-AMC. Data are shown as mean values ± S.D. of at least three independent experiments. B, Western blotting of B cells treated with TPCK. Total cell lysates were probed with antibodies to pro-caspase-3 (32 kDa) and its active subunit (17 kDa), and membranes were then stripped and reprobed with specific antibodies to PARP, a well known nuclear caspase substrate and to β-actin to control for equal loading of protein. C–E, B cells were pre-treated with the pan-caspase inhibitor z-VAD-fmk (25 μM) for 30 min and then subjected to treatment with TPCK (50 μM) for 6 or 12 h. Apoptosis was determined by measurement of caspase activation (C), hypodiploid DNA content (D), and PS exposure (E). Data are shown as mean values ± S.D. of at least three independent experiments. *, p < 0.001 TPCK versus TPCK plus z-VAD-fmk (C and E); #, p < 0.01 TPCK versus TPCK plus z-VAD-fmk (D).

chondria. As seen in Fig. 3C, TPCK triggered early release (evident from 1 h) of cytochrome c and a subsequent, time-dependent release of AIF into the cytosol. The release of cytochrome c coincided with the processing and activation of pro-caspase-9 (Fig. 3D), an event that is known to occur in a cytosolic complex termed the apoptosome, which consists of cytochrome c, pro-caspase-9, and the adaptor protein, apoptotic protease-activating factor-1 (18).

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FIGURE 3. Loss of mitochondrial membrane potential ($\Delta \psi_m$) and release of mitochondrial proteins in TPCK-treated human B cells. EBV-transformed B cells were incubated with 50 $\mu$M TPCK for 1, 2, 4, 6, 8, and 12 h (A) or with 10, 25, 50, and 75 $\mu$M TPCK for 6 h (B). Cells were then harvested for flow cytometric detection of $\Delta \psi_m$ using the fluorescent cationic probe, TMRE. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 100 $\mu$M) was included as a positive control. Data shown are mean values ± S.D. of at least three independent experiments. C, EBV-transformed B cells were incubated with TPCK (50 $\mu$M) for the indicated time points. Cytosolic (supernatant) and mitochondrial (pellet) fractions were prepared and subjected to Western blot analysis using specific antibodies against cytochrome c and AIF. Expression of $\beta$-actin and COXIV were also monitored to determine the purity of cytosolic and mitochondrial fractions, respectively. D, total cell lysates were probed with antibodies directed toward pro-caspase-9 and its cleavage products (active caspase-9). The membrane was stripped and reprobed with anti-$\beta$-actin antibodies to control for equal loading of protein.

lines were derived from two non-related SCN patients. Western blotting confirmed the absence of HAX-1 protein expression in the patient-derived cell lines (Fig. 5C). The cell lines were then incubated with TPCK (50 $\mu$M) for the indicated time points, and caspase activation was monitored using the DEVD-AMC assay (Fig. 5D). These experiments indicated that B cell lines from the SCN patients were more sensitive to TPCK-induced caspase activation when compared with transformed B cells from a normal donor.

HAX-1 Down-regulation Occurs at the Transcriptional Level and Is Associated with NF-κB Inhibition—Cilenti et al. (23) have reported that HAX-1 is cleaved by the mitochondrial serine protease, Omi/HtrA2, during cisplatin-induced apoptosis. However, we could not detect any HAX-1 cleavage product(s) in the current study (the same source of anti-HAX-1 antibody was used). The expression of HAX-1 was recently shown to be regulated by cytokines, suggesting a level of transcriptional control of HAX-1 (26). Moreover, as mentioned in the preceding section, TPCK is known to suppress the transcription factor, NF-κB. We therefore hypothesized that TPCK induces down-regulation of HAX-1 expression through direct or indirect inhibition of NF-κB. HAX-1 mRNA expression was thus monitored in EBV-transformed B cells treated with TPCK (50 $\mu$M) by quantitative reverse transcription-PCR. As shown in Fig. 6A, TPCK induced a significant and time-dependent decrease in HAX-1 mRNA expression. To further investigate whether a decrease in HAX-1 mRNA level is associated with inhibition of NF-κB, we utilized a specific NF-κB inhibitor, Bay 11-7082. Cells were incubated with different concentrations of Bay 11-7082 or TPCK, and HAX-1 mRNA and protein expression was then determined by quantitative reverse transcription-PCR and Western blot analysis, respectively. Bay 11-7082 (50 $\mu$M) as well as TPCK induced a significant decrease in HAX-1 mRNA expression (Fig. 6B). These results are consistent with the decrease in HAX-1 protein levels (Fig. 6C). For comparison, Bay 11-7082 also triggered a decrease in protein expression of the well known NF-κB target genes, XIAP, cIAP1, and cIAP2, whereas TPCK treatment resulted in a down-regulation of cIAP1 and cIAP2 and cleavage of XIAP (Fig. 6C). To assess whether down-regulation of HAX-1 is restricted to EBV-transformed B cell lines, HAX-1 protein expression was also determined in the Jurkat leukemic T cell line. Caspase-dependent apoptosis induction with down-regulation of HAX-1 expression was also seen in this cell line following TPCK treatment (Fig. 6, D and E). Collectively, our results demonstrate that HAX-1 is down-regulated at the transcriptional level during apoptosis leading to a substantial decrease in its protein expression. Moreover, we identified four putative binding sites for NF-κB approximately 1 kb upstream of the first ATG codon in the genomic sequence of HAX1 (supplemental Fig. S1). The current data suggest that HAX-1 is a novel target for the transcription factor, NF-κB.

The Thiol-containing Anti-oxidant, NAC, Prevents TPCK-induced Apoptosis in Human B Cells—It has previously been reported that a thiol anti-oxidant, NAC, prevented growth arrest and apoptosis in TPCK-treated T cells (10). Therefore, we determined whether TPCK-induced apoptosis in EBV-transformed B cells could be inhibited by NAC. Cells were incubated with TPCK (50 $\mu$M) in the presence or absence of NAC (5
Apoptosis was then determined following TPCK treatment for 12 h. NAC completely inhibited TPCK-induced apoptosis as assessed based on PS externalization and hypodiploid DNA content (Fig. 7, A and B). Additionally, caspase activation was significantly, although not completely inhibited in the presence of NAC (Fig. 7C). On the other hand, NAC completely prevented PARP cleavage in TPCK-treated cells (Fig. 7D). Next, we determined the effect of NAC on ROS production and reduction in $\Delta\psi_m$ in TPCK-treated cells. NAC significantly prevented TPCK-induced ROS production as well as dissipation of $\Delta\psi_m$ (Fig. 8A). Moreover, TPCK-induced translocation of cytochrome c and AIF from mitochondria to cytosol was also abrogated in the presence of NAC (Fig. 8B). Finally, the cleavage of Bcl-2 and XIAP observed in TPCK-treated cells was also inhibited in the presence of NAC (Fig. 8C). Moreover, the down-regulation of cIAP1, cIAP2, and HAX-1 in TPCK-treated cells was prevented in the presence of NAC (Fig. 8C). In sum, TPCK-induced mitochondrial events and subsequent apoptosis induction in normal B cells are effectively blocked by NAC.

L-Cysteine and D-Cysteine Block TPCK-induced Apoptosis in Human B Cells—Previous studies have suggested that the sulfhydryl group of NAC may function as a target for TPCK, thus potentially protecting thiol groups of proteins with a critical role in cellular survival signaling from alkylation (10). Alternatively, NAC, in its capacity as a glutathione precursor, could protect cells by increasing the levels of intracellular glutathione. To begin to address these possibilities, we incubated human B cells with TPCK (50 $\mu$M) in the presence or absence of L-cysteine (3 mM) or the cysteine enantiomer, D-cysteine (3 mM), which cannot be incorporated into glutathione. Notably, both L-cysteine and D-cysteine blocked TPCK-induced apoptosis, as compared to TPCK alone (Fig. 8D).
determined by annexin V-FITC labeling (Fig. 9A). Next, cell-
free experiments were conducted using ThioGlo™3, a male-
imide reagent that produces a highly fluorescent adduct on its
reaction with sulfhydryl groups (27). In these experiments, the
ability of TPCK to quench the formation of fluorescent NAC-
ThioGlo™3 adducts was assessed. As seen in Fig. 9B, a time-
and concentration-dependent reduction of the fluorescence
signal was observed upon addition of TCPK to the reaction
mixture. Finally, in an attempt to identify putative reaction
products formed upon interaction between TPCK and NAC,
the two compounds were mixed and incubated at 37 °C, and
reaction mixtures were submitted for mass spectrometric anal-
ysis. Monitoring of the resulting mass spectra over the mass-to-
charge (m/z) range 100 to 2000 revealed a reduction in the
TPCK peak intensity (sodiated TPCK at m/z 375) over the
course of the incubation (supplemental Fig. S2). However, no
major product was detected under these experimental condi-
tions. Notwithstanding, the current set of data demonstrates
that the amino acid cysteine or its derivate, NAC, is capable of
countering the pro-apoptotic effects of TPCK in human B cells,
most likely through a direct interaction with TPCK.

DISCUSSION

The current studies show that TPCK induces apoptosis in
transformed human B cells with multiple effects on cell death
signaling pathways. Indeed, TPCK treatment induces both
transcriptional and post-translational alterations in members
of the IAP family of proteins as well as the anti-apoptotic Bcl-2
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the mitochondria-targeted anti-apoptotic factor, HAX-1, pro-
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TPCK-induced Apoptosis in B Cells

FIGURE 8. Effect of NAC on TPCK-induced mitochondrial events in EBV-transformed B cells. A, B cells were pretreated or not with NAC (5 mM) for 30 min followed by TPCK (50 μM) for 12 h, and ROS production and drop of Δψm were determined using the DHE assay and TMRE staining assay, respectively. Data shown are mean values ± S.D. of at least three independent experiments. *, p < 0.001 TPCK versus TPCK plus NAC (DHE assay); #, p < 0.01 TPCK versus TPCK plus NAC (TMRE assay). B, cytosolic (supernatant) and mitochondrial (pellet) fractions were prepared from B cells treated with TPCK (50 μM) for 6 h in the presence or absence of z-VAD-fmk (25 μM) or NAC (5 mM) and subjected to Western blotting using antibodies to cytochrome c and AIF. Membranes were reprobed with antibodies to β-actin and COXIV to determine the purity of cytosolic and mitochondrial fractions, respectively. C, B cells were pretreated or not with NAC (5 mM) for 30 min followed by TPCK (50 μM) for 6 h, and total cell extracts were subjected to Western blot analysis using antibodies against Bcl-2, XIAP, cIAP1, cIAP2, and HAX-1. Antibodies directed to β-actin were used to control for equal loading of protein.

lacking HAX-1 expression were found to be more susceptible to treatment.

TPCK was shown in the present study to induce caspase activation and we also observed that the subsequent induction of apoptosis was blocked by a pan-caspase inhibitor, z-VAD-fmk. However, the release of cytochrome c from mitochondria was unaffected by pre-treatment of cells with z-VAD-fmk, indicating that a caspase-independent step also exists in this pathway of apoptosis induction. Indeed, the dissipation of Δψm and the production of ROS were only partially inhibited by z-VAD-fmk. On the other hand, AIF translocation from mitochondria to cytosol was completely blocked by z-VAD-fmk, and this event is therefore likely to serve as an amplification of the initial apoptosis signal, downstream of caspase activation. Our studies also showed that Bcl-2, a prominent anti-apoptotic factor that serves to block mitochondrial events during apoptosis, was cleaved in a caspase-dependent manner. Caspase-mediated cleavage of Bcl-2 has been reported previously in other model systems (20, 28), and this event has been suggested to function as a positive feedback loop for the apoptotic execution of the cell (29). We also observed caspase-dependent cleavage of XIAP, an important endogenous caspase-inhibitory molecule upon TPCK treatment of B cell lines. As is the case with Bcl-2 cleavage, one could speculate that this post-translational modification of XIAP also could serve to propagate the initial apoptosis signal. Of note, previous studies have shown that XIAP is cleaved upon death receptor-mediated apoptosis (21).

Several studies have shown that TPCK-induced apoptosis is associated with suppression of NF-κB signaling. Our data obtained in human B cell lines support this notion. Hence, we observed down-regulation of the known NF-κB target genes, cIAP1, cIAP2, as well as down-regulation of HAX-1. Quantitative PCR and Western blot analysis confirmed that HAX-1 mRNA and protein expression are suppressed by TPCK as well as by the specific NF-κB inhibitor, Bay 11-7082. HAX-1 was initially identified by yeast two-hybrid screening as a novel HS1-interacting protein and is ubiquitously expressed in rat, mouse, and human tissues (15, 30, 31). HS1 and HAX-1 (HS1-associated protein X-1) are both expressed in B cells, and Suzuki et al. (32) speculated that HAX-1 could participate in transduction of the signal from the B cell receptor to intracellular organelles, including mitochondria and endoplasmic reticulum. Furthermore, overexpression of HAX-1 has been reported to block apoptosis in various cell types (14, 22–24), and homozygous HAX1 deficiency in mice results in postnatal lethality with extensive apoptosis of neurons in the striatum and cerebellum (26). HAX-1 is sometimes classified as a Bcl-2 family protein, based on its structural similarity to Bcl-2 and its mitochondrial localization (32). However, the fact that HAX-1 appears to be under the transcriptional regulation of NF-κB (the present study) taken together with recent investigations suggesting that HAX-1 interacts with caspase-9 (24) indicates that the function and regulation of HAX-1 also are reminiscent to those of the IAP family members. Further studies are warranted to elucidate the role of HAX-1 in apoptosis as well as in other cellular processes.

Numerous previous reports based on the inhibitory effects of the anti-oxidant, NAC, have assumed that generation of ROS is a primary event in mitochondria-dependent cell death. We observed an inhibitory effect of NAC on TPCK-induced ROS production as well as on cell death (apoptosis) in normal B cell lines. However, previous studies by Heussler et al. (10) suggested that the effects that have been observed for TPCK may be attributed to its interaction with the sulphydryl groups of proteins that regulate cellular signaling, growth, and survival rather than to a direct inhibition of serine protease activity. Our time-course experiments also indicate that ROS production occurs downstream of cytochrome c release and caspase activation, and this leads us to conclude that TPCK-triggered ROS production is probably downstream of apoptosis induction. Therefore, the complete protection against TPCK-induced apoptosis by NAC that we have observed in the present study could potentially be explained by direct actions of NAC on TPCK, rather than its anti-oxidant function and regulation of glutathione levels in the cell. Indeed, we observed that d-cysteine, which cannot be incorporated into glutathione, did not differ from l-cysteine in its capacity to prevent TPCK-induced apoptosis in human B cells. TPCK was also shown to quench the formation of adducts between NAC and ThioGlo™3, a...
FIGURE 9. L-Cysteine and D-cysteine block TPCK-induced apoptosis in EBV-transformed B cells. A, B cells were pre-treated with L-cysteine (3 mM) or D-cysteine (3 mM) for 30 min followed by incubation with TPCK (50 μM) for 6 h. Apoptosis was determined based on flow cytometric detection of annexin V-FITC labeling. Representative histograms are shown. B, TPCK blocks the formation of fluorescent NAC-ThioGlo3 adducts. TPCK at concentrations 0, 10, 25, 50, 100, and 200 μM was incubated with 10 μM NAC at 37 °C for 1, 3 or 6 h in PBS, pH 7.2. ThioGlo3 at a final concentration of 1 μM was added to each reaction mixture. Samples were incubated at 25 °C for 30 min and fluorescence of ThioGlo3-NAC adducts was measured at 465 nm. TPCK alone at the indicated concentrations was used as a negative control. Results are expressed as mean ± S.D. of three independent measurements.
sulfhydryl-reactive maleimide compound. Taken together, these data support the view that the reactivity of TPCK to the sulfhydryl groups of NAC and L- or D-cysteine may help to prevent thiol modification and irreversible inactivation of proteins that contribute to cell survival (10). We were unable to identify the putative TPCK conjugates, but it remains possible that such conjugates are formed in a cellular environment but not in vitro or that our method for detection of such conjugates needs to be refined. For a schematic representation of our findings, and a tentative molecular ordering of TPCK-triggered events in normal human B cells, see Fig. 10.

In conclusion, we have demonstrated that TPCK induces caspase-mediated apoptosis in normal human B cell lines with multiple effects on pro- and anti-apoptotic proteins both at the transcriptional and post-translational level. The TPCK-induced effects were inhibited by NAC. We also report that the expression of the anti-apoptotic factor, HAX-1, is decreased during TPCK-induced apoptosis, and we provide first evidence that HAX-1 expression can be modulated in an NF-κB-dependent manner. Taken together, these studies serve to increase our understanding of the complexity of apoptosis signaling in normal human B cells and further implicate NF-κB in the control of apoptosis of B cells.

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FIGURE 10. TPCK-induced apoptosis in transformed human B cells. A schematic representation of the experimental findings in the current study. Mitochondrial generation of ROS, and the dissipation of mitochondrial transmembrane potential is partially caspase-dependent in this model, suggesting that effector caspses that are activated downstream of mitochondria may act on these organelles in a feedback loop to potentiate the initial apoptotic insult. In addition, the down-regulation and/or inactivation of endogenous caspase inhibitors may unleash further caspase activation in the cell. However, our experimental data suggest that the protective effect of NAC in this model is likely due to a direct interaction between NAC and TPCK. Consult text for abbreviations and a more detailed discussion.