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N-acetylcysteine blocks apoptosis induced by N-α-tosyl-L-phenylalanine chloromethyl ketone in transformed T-cells

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Abstract

The serine protease inhibitor N-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK) can interfere with cell-cycle progression and has also been shown either to protect cells from apoptosis or to induce apoptosis. We tested the effect of TPCK on two transformed T-cell lines. Both Jurkat T-cells and *Theileria parva*-transformed T-cells were shown to be highly sensitive to TPCK-induced growth arrest and apoptosis. Surprisingly, we found that the thiol antioxidant, N-acetylcysteine (NAC), as well as L- or D-cysteine blocked TPCK-induced growth arrest and apoptosis. TPCK inhibited constitutive NF-κB activation in *T. parva*-transformed T-cells, with phosphorylation of IκBα and IκBβ being inhibited with different kinetics. TPCK-mediated inhibition of IκB phosphorylation, NF-κB DNA binding and transcriptional activity were also prevented by NAC or cysteine. Our observations indicate that apoptosis and NF-κB inhibition induced by TPCK result from modifications of sulphydryl groups on proteins involved in regulating cell survival and the NF-κB activation pathway(s).

Keywords: cell death; IκB; NF-κB; *Theileria parva*; thiol

Abbreviations: CAT, chloramphenicol acetyl transferase; EMSA, electrophoretic mobility shift assay; NAC, N-acetylcysteine; TPCK, N-α-tosyl-L-phenylalanine chloromethyl ketone; Tpi T-cells, *Theileria parva*-infected T-cells

Introduction

The serine protease inhibitor N-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK) has been used in a range of studies to investigate the signal transduction pathways that are involved in gene expression, cell proliferation and apoptosis. The effect of TPCK on apoptotic pathways differs markedly depending on cell type and the particular part of the apoptosis pathway that is being examined. In a number of cases, TPCK appears to protect cells against apoptosis. Thus, TPCK has been shown to prevent endonucleolysis induced by camptothecin in HL60 leukemic cells and also to protect rat thymocytes against endonucleolysis induced by prednisolone.[1] TPCK also blocks 'apoptotic nuclei promoting activity' induced by Fas antigen ligation in T-cells[2] thus preventing anti-Fas-induced apoptosis.[3] Etoposide-induced DNA fragmentation in HL60 cells is also inhibited[4] and TPCK-sensitive proteases have been described, that appear to be specifically involved in DNA fragmentation induced by treatment of human myelogenous leukemia ML-1a cells with TNF.[5] Also LPS-induced cytotoxicity in macrophages could be prevented by TPCK.[6] Interestingly, in myeloid leukemic cells, TPCK, but not other serine protease inhibitors, was found to inhibit apoptosis by wild-type p53.[7] Likewise, activation of calmodulin-dependent protein kinase II which appears to participate in the signalling pathways regulating UV- or TNF-induced apoptosis could be blocked by TPCK, but not by other inhibitors of serine proteases.[8]

In other cases, however, TPCK promotes apoptosis. TPCK has been shown to induce the early morphological and biochemical changes associated with apoptosis in immature rat thymocytes[9] and murine WEHI 231 B-cells undergo apoptosis when treated with TPCK.[10] The effects of TPCK on the cell are not restricted to apoptosis, however, and TPCK also affects pathways that control cellular proliferation. In this context, TPCK has been shown to be a potent inhibitor of $pp70^{S6K}$, a kinase which is particularly important for controlling protein translation and regulating the G1 to S-phase transition of the cell cycle.[11] TPCK has also been demonstrated to inhibit the degradation of PKC,[12] a component of a range of signal transduction pathways that regulates cellular proliferation and differentiation.

NF-κB is a transcription factor which plays a central role in the regulation of cellular differentiation and proliferation.[13,14] NF-κB, however, has also been implicated in the process of apoptosis.[15] NF-κB is normally present as a dimer, consisting of members of the Rel protein family, such as p65 (RelA), p50, p52, c-Rel or RelB. In its inactive form, NF-κB is associated with cytoplasmic inhibitors (IκB) keeping it sequestered in the cytoplasm. IκBα and IκBβ are the most important and best characterised forms of IκB. Upon cellular activation, IκBα is functionally inactivated, resulting in the nuclear translocation of NF-κB where it binds to specific DNA sequences which regulate gene expression.[13,16] The inactivation of IκB involves phosphorylation by activated IκB kinases (IKK), which targets IκB for ubiquitination and proteasomal degradation.[17,18]

NF-κB may play a dual, apparently opposite role in apoptosis.[15,19] Compelling evidence has recently been presented, showing that NF-κB can protect cells against apoptosis,[15] most likely by inducing the expression of genes that are required for cell survival.[20] On the other hand, it has also been proposed that NF-κB activation is

required for apoptosis; this appears to apply in particular to apoptotic pathways that depend on *de novo* protein synthesis.[21] TPCK is a potent inhibitor of NF-κB activation[22–28] which blocks IκBα degradation and subsequent nuclear translocation of NF-κB. Inhibition of NF-κB by TPCK appears to involve a block in IκBα phosphorylation,[29,30] a step in the pathway which precedes proteasomal IκB degradation. Whether TPCK also acts directly by blocking the proteolytic processing of IκB, however, is still subject to debate. TPCK has been shown to induce apoptosis in an immature murine B-cell lymphoma line and it was proposed that this occurred through the inhibition of NF-κB.[10] In preliminary investigations, we tested a number of inhibitors of NF-κB, among them TPCK and N-acetylcysteine (NAC). The latter is a thiol antioxidant which has been reported in a number of different systems either to induce apoptosis[31,32] or to protect cells from apoptosis[33–36] by functioning as a synthetic glutathione precursor. We report that TPCK can indeed induce growth arrest and apoptosis in two transformed T-cells. Surprisingly, however, we found that TPCK-mediated growth arrest, induction of apoptosis and also the inhibition of the NF-κB activation pathway were all prevented by treatment of the cells with different forms of cysteine, including the enantiomer D-cysteine which can not be used as a glutathione precursor. Our data strongly indicate that the sulphydryl groups of NAC, D- and L-cysteine form direct targets for the alkylating effects of TPCK, thus protecting cellular proteins with important functions in cell survival from irreversible inactivation by alkylation.

Results

NAC prevents growth arrest and the induction of apoptosis induced by TPCK in transformed T-cell lines

Considering the fact that TPCK can inhibit pp70^{S6K}-dependent pathways which control the entry into the S-phase of the cell cycle,[11] we tested its effect on proliferation of two transformed T-cell lines. The first cell line consisted of Jurkat T-cells; the second cell line is a bovine T-cell which is transformed by infection with the intracellular parasite *Theileria parva*.[37] Both cell lines proliferate in an uncontrolled manner, independent of exogenous growth factors or antigenic stimulation. Cells were cultured for 5 h in the presence of increasing concentrations of TPCK and proliferation was monitored by measuring [^{3}H]thymidine incorporation (Figure 1). The proliferation of both Jurkat and Tpi T-cells was inhibited in a dose-dependent manner. Addition of NAC to the culture medium, however, largely prevented TPCK-mediated growth arrest.

We next examined whether the inhibition of proliferation was also accompanied by apoptosis. When cells undergo apoptosis, phosphatidylserine is translocated to the outer membrane. Surface phosophatidylserine can be demonstrated by the fact that it binds annexin-V.[38] Binding of fluoresceinated annexin-V (annexin-V-FITC) to the surface of Tpi and Jurkat T-cells was monitored by flow cytometry

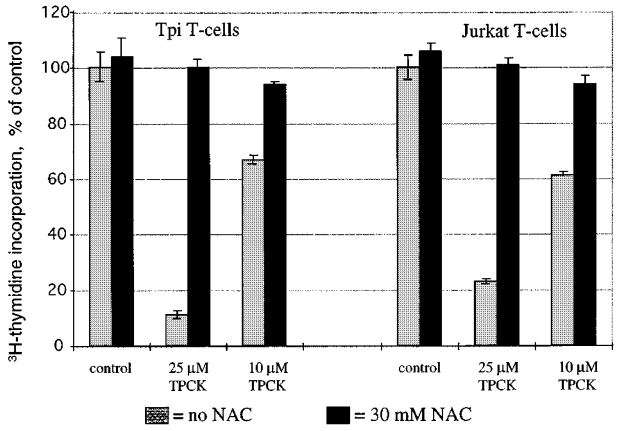

Figure 1 NAC prevents growth arrest induced by TPCK. Tpi and Jurkat T-cells were cultured for 5 h with TPCK at the indicated concentration, in the presence (black bars) or absence (grey bars) of 30 mM NAC. [^{3}H]thymidine was added to the culture medium and incorporation into DNA monitored by liquid scintillation counting

(Figure 2). Upon treatment with 25 μM TPCK for 5 h, the majority of Tpi and Jurkat T-cells were found to bind annexin-V-FITC. Annexin-V binding was strongly reduced, however, when TPCK treatment occurred in the presence of NAC, demonstrating that NAC is capable of blocking TPCK-induced apoptosis.

A number of additional protease inhibitors were also tested (Table 1). The related compound 3,4-dichloroisocoumarin (DCI) functions by acylating, and in some cases alkylating, serine protease active sites. Annexin-V-FITC binding experiments revealed that DCI was also a potent inducer of apoptosis for Tpi T-cells and to a lesser extent for Jurkat T-cells. Interestingly, as was found for TPCK, treatment with NAC prevented DCI-induced apoptosis. Leupeptin, a cell-permeable inhibitor of serine and cysteine proteases, on the other hand, did not induce apoptosis. Calpain inhibitor I (N-aceytl-Leu-Leu-norleucinal), a cysteine endopeptidase inhibitor which blocks cyclin B degradation and arrests cells in G1, induced apoptosis which could not be inhibited by NAC. On the contrary, NAC actually enhanced calpain inhibitor I-induced apoptosis.

Experiments were also carried out to exclude the possibility that the inhibitory effect of NAC is mediated solely by neutralising TPCK outside the cell. For this purpose, cells were pre-treated with NAC for 2 h, extensively washed to remove all external NAC and then exposed to TPCK for 5 h. Cells pre-treated with NAC were largely resistant to TPCK-induced apoptosis (not shown), confirming that NAC is capable of neutralising TPCK intracellularly.

Effects of TPCK and NAC on IκB phosphorylation in Tpi T-cells

TPCK has been shown to inhibit the induction of NF-κB activation in T-cells,[30,39] but the effects on constitutive NF-κB activity in T-cells has not yet been monitored. Considering the

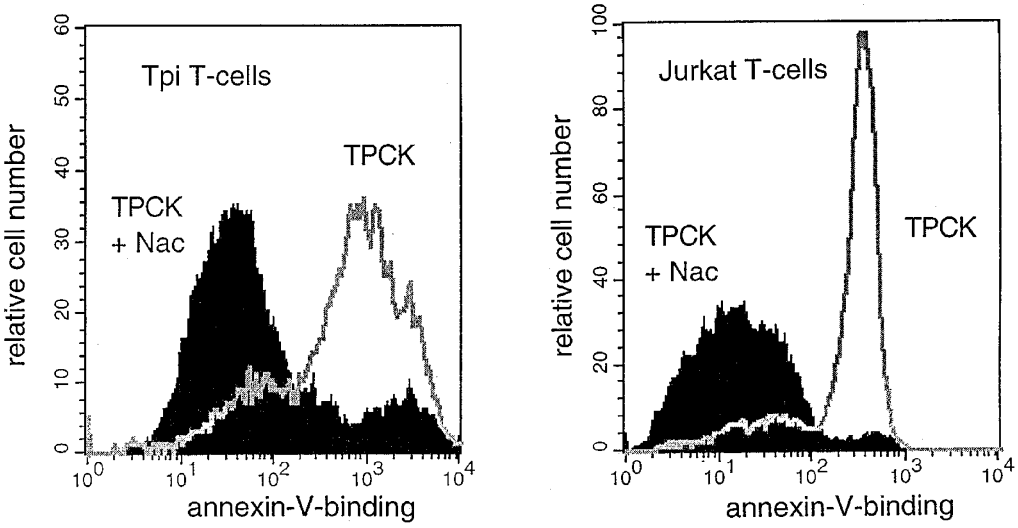

Figure 2 TPCK-induced apoptosis is blocked by NAC. Tpi and Jurkat T-cells were treated for 5 h with 25 μM TPCK in the absence (white profile) or presence (black profile) of NAC, and annexin-V-FITC binding to surface phosphatidylserine was monitored by flow cytometry

Table 1 Effect of different protease inhibitors on the viability of Jurkat and Tpi T-cells

			Apoptotic cells (% of Annexin-V positive cells)			
			Jurkat		Tpi	
Compound	**Type**	**Concentration**	**Control**	**NAC**	**Control**	**NAC**
Control			5	7	14	18
TPCK	Inhibitor of chymotrypsin-like serine proteases	10 μM	78	6	81	19
		25 μM	83	5	85	18
DCI	Serine protease inhibitor	10 μM	12	5	62	21
Leupeptin	Serine and cysteine protease inhibitor	100 μM	45	5	76	35
		10 μg/ml	5	5	13	21
		100 μg/ml	5	6	16	21
Calpain inhibitor I	Cysteine endopeptidase inhibitor	10 μg/ml	12	20	60	71
		100 μg/ml	24	29	62	77

Tpi and Jurkat T-cells were incubated for 16 h with protease inhibitors alone or with NAC. NAC was added at a concentration of 30 mM. The induction of apoptosis was monitored by Annexin-V-FITC binding. TPCK (N-α-tosyl-L-phenylalanine chloromethyl ketone), DCI (3,4-dichloroisocoumarin), Leupeptin (N-acetyl-Leu-Leu-argininal), Calpain inhibitor I (N-acetyl-Leu-Leu-norleucinal)

potential role of NF-κB in the regulation of proliferation and apoptosis, we monitored the effects of both TPCK and NAC on different steps in the NF-κB activation pathway in transformed T-cells. For this purpose we used the Tpi T-cell line in which NF-κB is constitutively activated[40] through a mechanism which involves the continuous degradation of both IκBα and IκBβ.[41] As IκBα itself is transcriptionally regulated by NF-κB,[42,43] increased IκBα levels can be found in Tpi T-cells, despite its constitutive turnover.[41] Phosphorylated IκBα migrates with reduced electrophoretic mobility and is readily detected in untreated Tpi T-cells (Figure 3A, lower panel). Culturing Tpi T-cells in the presence of TPCK prevented IκBα phosphorylation as judged by the disappearance of the slower migrating IκBα form. This effect was visible after 1 h of treatment and lasted throughout the experiment (5 h, data shown for the first 3 h). The same filter was reprobed with antibodies directed against IκBβ. IκBβ is phosphorylated in Tpi T-cells and TPCK treatment also resulted in deficient IκBβ phosphorylation as is evident from the appearance of IκBβ forms with lower molecular weight (Figure 3A, upper panel). Alterations in IκBβ phosphorylation were also obvious within 1 h. As we have demonstrated previously,[41] NAC did not affect IκB. A consistent feature observed for both IκBα and IκBβ, however, was that the inhibition of phosphorylation by TPCK was completely prevented by treatment with NAC. In order to analyse the kinetics with which TPCK affects IκB phosphorylation, a more detailed time-course analysis was also performed (Figure 3B). A reduction in levels of phosphoylated IκBα was first seen after 30 min of TPCK treatment and became more pronounced after 45 min and 1 h. The first changes in IκBβ phosphorylation, on the other hand, could already be detected after 5 min of treatment and became increasingly obvious after 15 and 30 min of treatment.

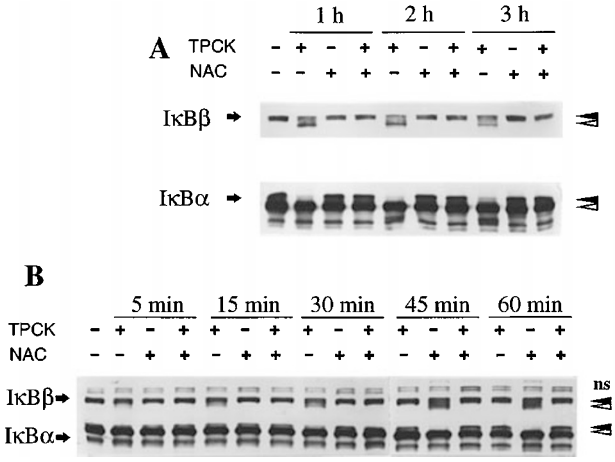

Figure 3 Effects of NAC and TPCK on constitutive IκB degradation in Tpi T-cells. (**A**) Tpi T-cells were treated for 1, 2 or 3 h with 25 μM TPCK and/or 30 mM NAC as indicated. Whole cell extracts were subjected to immunoblot analysis using antibodies directed against IκBα (lower panels) and IκBβ (upper panel). The closed arrowheads represent the phosphorylated IκB forms, the open arrrowheads the hypophosphorylated IκB forms. (**B**) Time-course analysis. Cells were treated with TPCK and/or NAC as indicated and whole cell extracts subjected to immunoblot analysis using anitbodies directed against IκBα and IκBβ together. The band labelled 'ns' corresponds to a protein carrying IκBβ-related epitopes[41] recognised by anti-IκBβ antibodies

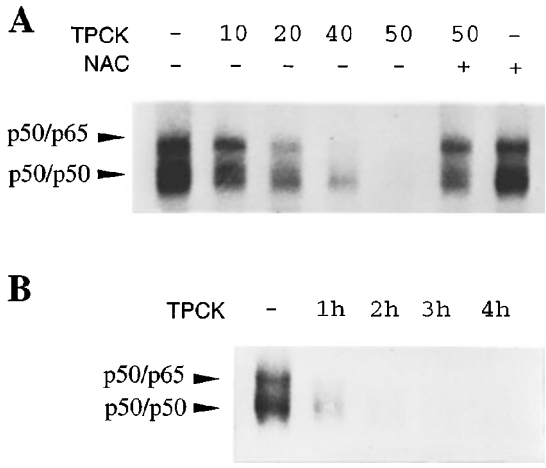

Figure 4 TPCK-mediated inhibition of NF-κB-binding is partially blocked by NAC. (**A**) Nuclear extracts were prepared from Tpi T-cells that were treated for 5 h with different concentrations of TPCK (μM) and DNA binding analyzed by EMSA using oligonucleotides corresponding to the HIV-LTR κB site. (**B**) Time-course experiment showing the TPCK-mediated disappearance of NF-κB DNA binding complexes. Nuclear extracts of cell treated with 25 μM TPCK for 1, 2, 3 or 4 h were analyzed by EMSA

The TPCK-mediated inhibition of constitutive NF-κB DNA binding and transcriptional activity is partly prevented by NAC

NF-κB translocation to the nucleus and DNA binding are constitutive in Tpi T-cells.[40,41] Electrophoretic mobility shift assays (EMSA) were performed, using oligonucleotides corresponding to the HIV-LTR κB motif which bind complexes consisting of p65 and p50 in Tpi T-cells (J.M. unpublished observation). As has also been shown in other systems, TPCK potently inhibited NF-κB DNA binding activity in a dose dependent manner (Figure 4A). In agreement with earlier observations,[41] NAC did not inhibit NF-κB DNA binding. In TPCK-treated cells, however, NF-κB DNA binding was restored to a large extent, albeit not completely, in the presence of NAC. A time course analysis was also performed in which cells were treated with TPCK for 1–5 h. Within 1 h of TPCK treatment, strong reduction in NF-κB DNA binding was already obvious (Figure 4B), reflecting the rapid inhibition of IκB phosphorylation (Figure 3A and B).

To monitor the effect of TPCK on NF-κB transcriptional activity, Tpi T-cells were transfected with the plasmid HIV-CAT, in which the chloramphenicol aceytyl transferase (CAT) gene expression is dependent on transcriptional activation through the two HIV-LTR κB motifs. At a concentration of 10 μM TPCK, CAT activity was reduced to 5% of that observed in untreated cells (Figure 5). In agreement with the observations made for NF-κB DNA binding (Figure 4A), full transcriptional activity could not be restored by NAC. Nevertheless, CAT activity amounting to almost 60% of the control values could be measured, demonstrating that the antagonistic effect of NAC on TPCK-mediated inhibition of NF-κB can also be observed at the transcriptional level.

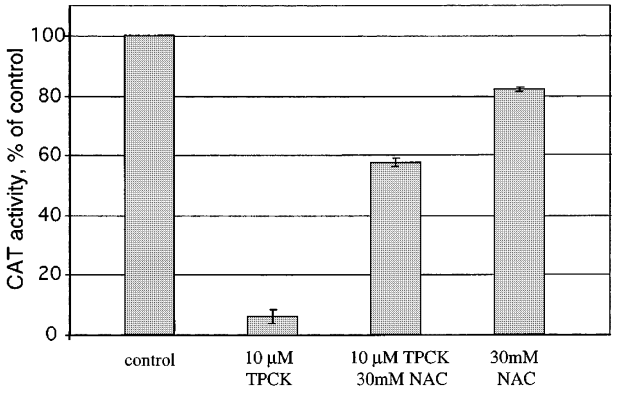

Figure 5 TPCK-mediated inhibition of NF-κB transcriptional activity is partially restored by treatment of Tpi T-cells with NAC. Tpi T-cells were transfected with the HIV-CAT plasmid and cultured for 16 h in the presence of TPCK (10 μM) or NAC (30 mM) as indicated. Cell extracts were prepared and CAT activity measured. Results are presented as the percentage of CAT activity observed in untreated Tpi T-cells and are the means ± S.D. from three independent transfections and CAT assays

TPCK-mediated growth arrest and apoptosis can also be inhibited by the amino acid cysteine

TPCK is an alkylating agent which also shows reactivity to thiol groups.[44] The possibility therefore exists that the sulphydryl group of NAC functions as a target for TPCK, thus potentially protecting thiol groups of proteins with a function in cellular signalling from alkylation. Alternatively,

NAC, in its capacity as a glutathione precursor, could protect the cell by increasing the levels of intracellular glutathione. Since NAC is a cysteine derivative, we tested whether the amino acid cysteine on its own was also capable of protecting cells against TPCK-induced apoptosis. Tpi T-cells and Jurkat T-cells were treated for 5 h with

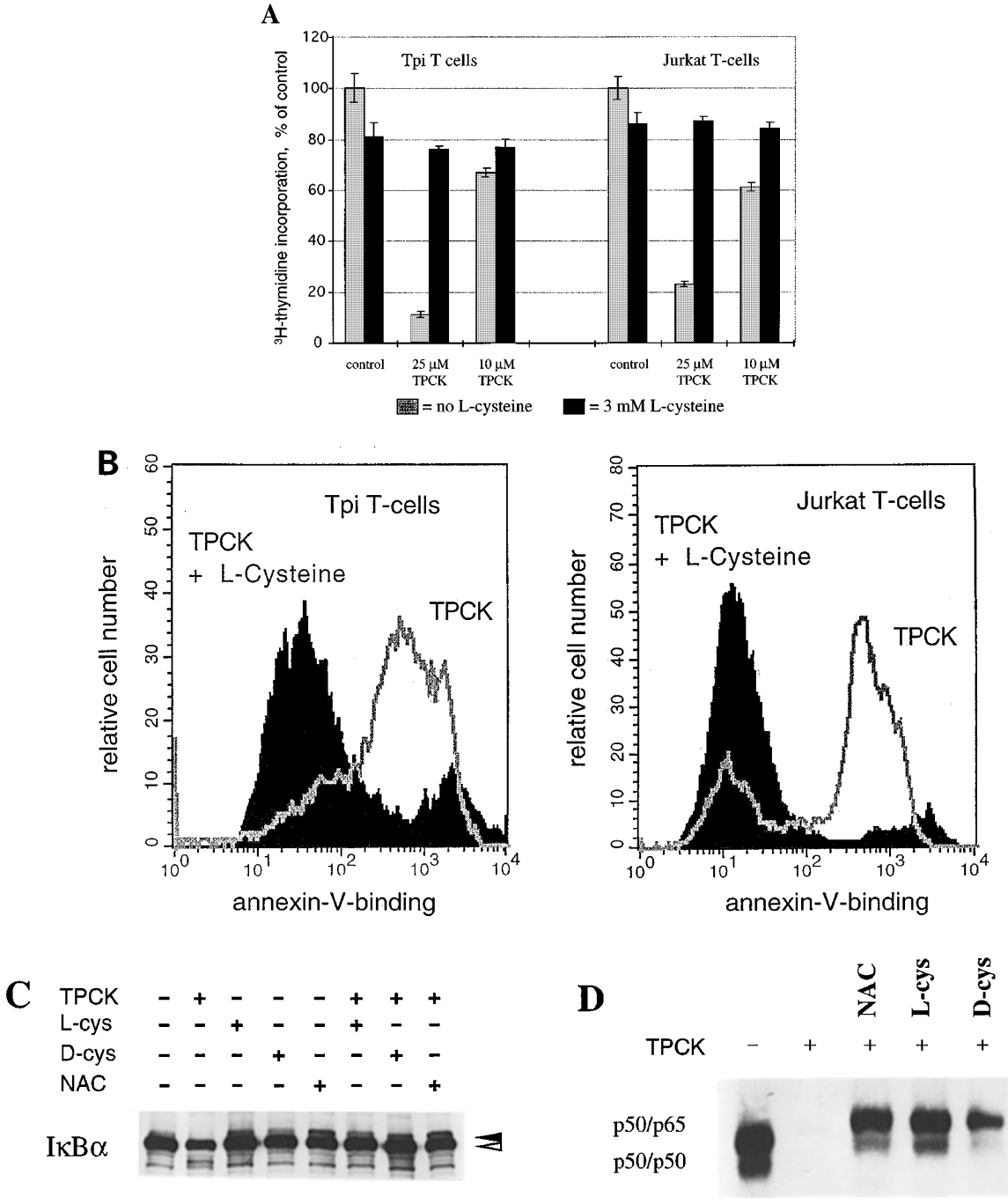

Figure 6 The amino acid cysteine blocks TPCK-induced growth arrest and apoptosis and also counteracts the effects of TPCK on the NF-κB activation pathway. (**A**) Tpi and Jurkat T-cells were treated for 5 h with TPCK at the doses indicated, in the absence (grey bars) or presence (black bars) of 3 mM L-cysteine. DNA synthesis was monitored by measuring [^{3}H]thymidine incorporation. (**B**) L-cysteine prevents TPCK-induced apoptosis. Tpi and Jurkat T-cells were treated with 25 µM TPCK for 5 h in the presence (black profile) or absence (white profile) of 3 mM L-cysteine. Annexin-V-FITC binding to surface phosphatidylserine was monitored by flow cytometry. (**C**) L- and D-cysteine prevent the TPCK-mediated inhibition of IκBα phosphorylation. Tpi-T-cells were treated for 2 h with TPCK (25 µM), L-cysteine (3 mM) D-cysteine (3 mM) or NAC (30 mM) as indicated. Whole cell extracts were subjected to immunoblot analysis using anti-IκBα antibodies. The open arrowhead indicates hypophosphorylated IκBα, the closed arrowhead phosphorylated IκBα. (**D**) L- and D-cysteine counteract TPCK-mediated inhibition of NF-κB DNA binding. Cells were treated for 5 h with TPCK (25 µM), L-cysteine (3 mM) D-cysteine (3 mM) or NAC (30 mM) as indicated and nuclear extracts were analyzed by EMSA

TPCK in the presence or absence of L-cysteine. Figure 6A shows that TPCK-mediated inhibition of [^{3}H]thymidine incorporation was prevented when 3 mM L-cysteine was added to the medium. This effect could be observed for both Tpi and Jurkat T-cells. The effect of L-cysteine on TPCK-induced apoptosis was also examined by monitoring the number of annexin-V-FITC positive cells (Figure 6B). Upon treatment for 5 h with 25 μM TPCK, the majority of cells bound annexin-V-FITC. This strong increase in the number of annexin-V-FITC-binding cells could not be observed, however, when cells were treated with TPCK in the presence of 3 mM L-cysteine. Finally, we also tested whether the cysteine enantiomer D-cysteine, which can not be incorporated into glutathione, could also block TPCK. D-cysteine did not differ from L-cysteine in its capacity to prevent TPCK-induced growth arrest or apoptosis. As was the case for NAC, pre-treatment for 2 h with both cysteine enantiomers, followed by washes to remove extracellular cysteine, also prevented apoptosis (data not shown).

The inhibition of IκBα phosphorylation by TPCK was prevented by both D- and L-cysteine (Figure 6C). The block in NF-κB DNA binding was prevented by D-cysteine as well as by L-cysteine (Figure 6D), with L-cysteine, however, being more efficient than D-cysteine. Together, these experiments indicate that the amino acid cysteine, on its own and like its derivative NAC, is capable of countering the effects of TPCK.

Discussion

We demonstrate that TPCK induces growth arrest and apoptosis in two transformed T-cell lines and also inhibits the constitutive NF-κB activation observed in Tpi T-cells. Unexpectedly, we found the induction of growth arrest, apoptosis and the inhibition of NF-κB to be prevented by the thiol NAC and also by L- and D-cysteine.

Work from several laboratories indicates that the anti-serine protease activity of TPCK can protect cells from cytotoxicity in general[6] and can also interfere with defined steps in the apoptosis process such as the formation of apoptotic bodies, chromatin degradation, DNA fragmentation and proteolysis.[1,2,5,45] TPCK and DCI, but not other cell-permeable protease inhibitors, have been shown to inhibit the generation of ceramide and apoptosis, by blocking daunorubicin-induced sphingomyelinase activation.[46] Also internucleosomal DNA fragmentation induced by the topoisomerase I inhibitor camptothecin was suppressed by TPCK in B lymphoma Namalwa cells[47] and HL60 cells,[1,48] but caspase-3-like activity and high molecular weight DNA fragmentation were not affected.[47] On the other hand, our work and that of others[9,10,49] shows that TPCK can clearly also induce apoptosis. This is in apparent conflict with the anti-apoptotic activity ascribed to the serine protease-inhibiting activity of the compound. There are cell type differences in the proteases involved in apoptosis and multiple pathways leading to apoptosis that can be selectively induced and suppressed by different agents.[7] The likelihood is therefore great that these differences relate to cell type, nature of the stimulus and state of differentiation of the cells that were examined. It is worth noting that in several studies, serine protease inhibitors other than TPCK did not interfere with apoptotic mechanism.[57] In our hands, TPCK and DCI induced apoptosis, but leupeptin, which inhibits both serine and cysteine proteases did not, indicating that apoptosis, induced by the former compounds, may not solely depend on their protease blocking activity.

TPCK inactivates chymotrypsin by specifically alkylating the histidine at postition 57. Several observations, however, show that the activity of TPCK is not restricted to blocking serine proteases. As a chloromethyl ketone with alkylating activity, TPCK also inhibits other enzymes, an activity which can be attributed to its reactivity to nucleophiles such as thiol groups.[44,50,51] The irreversible inactivation of the catalytic subunit of cAMP-dependent protein kinase[44] and the inactivation of the transcription factor TFIIIC by TPCK occur solely through thiol group modification.[52] Importantly, TPCK shows considerable reactivity to the thiol group of glutathione[51] and can impair the glutathione redox system which regulates several cellular processes including apoptosis.[53] TPCK also diminishes glutathione reductase activity and intracellular levels of reduced glutathione.[54] Furthermore, TPCK also prevents increased transcription of the gene encoding γ-glutamylcysteine synthetase, the rate-limiting enzyme in the biosynthesis of glutathione.[55] Reduced glutathione appears to play a role in the rescue of cells from apoptosis.[56–58] Since NAC and cysteine are precursors of glutathione, it could be argued that the anti-apoptotic effect of NAC is effectuated by restoring the glutathione balance in TPCK-treated cells. However, the fact that D- as well as L-cysteine could both block the effects of TPCK, strongly suggests that the inhibition of TPCK is based on the cysteine thiol groups. This way, the reactivity of TPCK to the sulphydryl groups of NAC and D- or L-cysteine may help to prevent thiol modification and irreversible inactivation of proteins that contribute to maintaining cell survival, including the tripeptide glutathione. Also, the fact that NAC was capable of blocking the early TPCK-mediated inhibition of IκB phosphorylation, without pre-treatment of the cells, also argues in favour of a direct effect by NAC, rather than an indirect effect based on increased glutathione levels. This would be in agreement with earlier observations, that NAC-mediated protection against apoptosis in a murine T-cell hybridoma did not correlate with increased glutathione levels.[59] Likewise, NAC-promoted survival of PC12 cells has been shown to be glutathione-independent.[60] It can not of course be excluded that both precursor (NAC) and glutathione synergise to counter the alkylating effects of TPCK. In this regard, it has been shown that the TPCK-mediated inhibition of superoxide production in neutrophils could also be prevented by reduced glutathione[50] and, in this case, it was also suggested that inhibition was largely due to the inhibition of sulphydryl groups. Finally, the hypothesis that NAC-mediated inhibition of TPCK may not be limited to blocking its anti-serine-protease activity is further supported, albeit indirectly, by the fact that glutathione has been shown to prevent the inhibition of transcription in transformed cells by the closely related N-tosyl-L-lysine-chloromethyl ketone, a trypsin inhibitor.[61]

Thioredoxin has recently been identified as a direct inhibitor of apoptosis signal-regulating kinase-1 (ASK-1) in a redox-dependent manner, with two cysteines in the redox-active site controlling its activity.[62] Oxidation of these residues neutralises the anti-apoptotic activity of thioredoxin. It could be imagined that TPCK-mediated alkylation of the cysteine sulphydryl groups could have a similar effect. It will therefore be of interest to determine if TPCK also inhibits the anti-apoptotic function of thioredoxin.

Apart from inducing growth arrest and apoptosis, TPCK was also a potent blocker of the constitutive NF-κB activation observed in Tpi T-cells. Also in this case, inhibition of the NF-κB pathway by TPCK was prevented by NAC or cysteine. This could be observed at the level of IκB phosphorylation along with NF-κB DNA binding and transcriptional activity. The antagonistic effects of NAC and cysteine against TPCK-mediated inhibition of NF-κB at the level of DNA binding and κB-dependent CAT activity was not complete (see Figures 4, 5 and 6). This suggests that TPCK may inhibit these processes in part through other pathways, which are not affected by NAC or cysteine, conceivably by influencing factors that are not required for optimal DNA binding and transcriptional activity.

It has been proposed that the serine protease inhibitory activity of TPCK is responsible for the block of IκBα degradation.[6,10,22,27,39,63,64] Guesdon *et al*.,[29] however, reported that TPCK blocked phosphorylation mechanisms, but found no evidence for protease involvement in IκBα degradation. Our finding that NAC, D- and L-cysteine block the effects of TPCK at the level of IκB phosphorylation is in line with these observations and suggests that TPCK interferes with NF-κB activation by modifying sulphydryl groups of proteins that regulate the IκB phosphorylation pathways. It is worth noting that inhibition of IκBα phosphorylation by TPCK could first be observed after 30 min of treatment, whereas IκBβ was affected within 5 min of treatment. These differences indicate that the pathway(s) regulating IκBβ degradation differs from that of IκBα in its sensitivity to TPCK inhibition.

It has been reported that the immunosuppressive effects of TPCK observed in T-cells can be countered by NAC.[39] NF-κB is a pivotal component of T-cell activation[14,16] and our finding that NAC can prevent NF-κB inhibition by TPCK therefore helps to explain this event. As mentioned earlier, however, TPCK can inhibit cellular signalling pathways at different levels and the immunosuppressive activity of TPCK is unlikely to be restricted solely to the NF-κB pathway. For example, the proteolytic processing of protein kinase C, a key kinase in T-cell activation, and pp70^{S6K}, which regulates progression through the cell cycle, are both inhibited by TPCK.[11,12] In this regard we observed in time course studies that the arrest of DNA synthesis clearly precedes the onset of apoptosis (V.H., unpublished). It is therefore conceivable that the inhibition of pp70^{S6K} by TPCK also contributed to the arrest of proliferation observed for Jurkat and Tpi T-cells.

In summary, our findings strongly indicate that the effects on proliferation, NF-κB activation and apoptosis in particular, that have been observed for TPCK may be attributed to its interaction with the sulphydryl groups of proteins that regulate cellular signalling, growth and survival, rather than direct serine-protease inhibitory activity.

Materials and Methods

Cell lines and proliferation

Jurkat and *T. parva*-infected (Tpi) T-cells[37] were cultured at 37°C in Leibovitz L15 medium containing 10% (v/v) heat-inactivated foetal bovine serum, 20 mM Hepes (pH 7.1), L-glutamine (2 mM) benzylpenicillin (100 units/ml) and streptomycin sulphate (100 μg/ml) (cL15). Proliferation assays were carried out in triplicate in 96 flat bottom microtiter plates. Cells (2×10^5 cells/ml) were cultured for 5 h in medium containing 2.5 μCi/ml [^{3}H]thymidine in the presence or absence of drugs. After harvesting on Packard UniFilter 96 microplates, radioactivity was monitored in a beta counter.

Inhibitors

NAC, TPCK, DCI, calpain inhibitor I and Leupeptin were all purchased from Sigma-Aldrich-Fluka (Buchs, Switzerland), TPCK, DCI and calpain inhibitor I were dissolved in DMSO; leupeptin was dissolved in water; NAC in phosphate buffered saline.

Apoptosis assay

Tpi and Jurkat T-cells were cultured at 2×10^5 cells/ml in cL15 for 5 h in the presence or absence of drugs. Cells were harvested by centrifugation and washed in cold PBS. After centrifugation cells were resuspended in cold staining buffer (Hepes 10 mM, NaCl 140 mM, $CaCl_2$ 5 mM). Staining with recombinant Annexin-V-FITC and propidiumiodide was done according to instructions of the manufacturer and cells were analyzed by flow cytometry using standard protocols and a Becton-Dickinson FACScan.

Immunoblot analysis

Cells were collected and washed twice in ice-cold PBS, pH 7.4, and then processed for immunoblot analysis as described before.[41] Rabbit anti-peptide polyclonal antibodies with the following specificities were used: IκBα (C-terminal 21 amino acids; sc-371, Santa Cruz, Basel, Switzerland); IκBβ (C-terminal 20 amino acids; sc-945). Bound primary antibody was detected using horseradish peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

EMSA and CAT assays

The preparation of nuclear extracts and electrophoretic mobility shift assays (EMSA) were performed using standard protocols,[40] using the oligonucleotide (5′-GAGGGGACTTTCCG-3′ and its complement). Prior to preparation of nuclear extracts, cells were treated for 5 h with different drugs at the indicated concentrations. The use of the HIV-CAT plasmids and transfection of Tpi T-cells have been described before.[40] Briefly, Tpi T-cells were washed twice in ice-cold PBS and finally resuspended in ice-cold PBS at 2×10^7 cells/ml. Cells were distributed in 0.4 cm cuvettes in a volume of 600 μl. 60 μg DNA (2 mg/ml) was added, the suspension was mixed and cells were electroporated at 400 V and 250 μF. After 5 min incubation on ice, cells were pooled and transfered to antibiotic free cL15 and left for

30 min to recover. Cells were then centrifuged and resuspended in cL15 containing antibiotics and incubated with the drugs as indicated for 16 h. CAT activity was determined according to standard protocols.

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