Elevated $cPLA_2$ levels as a mechanism by which the p70 TNF and p75 NGF receptors enhance apoptosis

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Abstract The 70-kDa TNF cell surface receptor (p70TNFR) and the related 75-kDa nerve growth factor (NGF) receptor (p75NGFR) can enhance cell death. Expression of p70TNFR or p75NGFR in HeLa cells resulted in enhanced TNF-induced apoptotic cell death with a corresponding elevation of cytosolic phospholipase A_2 ($cPLA_2$) levels. This response was apparent by 24 h, did not occur with NGF treatment or when vector or *Trk*A NGFR were expressed and was reversed by dexamethasone pretreatment. These findings reveal a novel mechanism by which the p70TNFR and p75NGFR achieve enhanced TNFR-mediated programmed cell death by elevating $cPLA_2$ levels.

Key words: Tumour necrosis factor; Nerve growth factor; Receptor; Cytosolic phospholipase A_2; Apoptosis; HeLa cell

1. Introduction

Tumour necrosis factor alpha (TNF), a cytokine secreted primarily by activated macrophages and monocytes, has a wide range of biological involvements, such as in inflammatory responses, antitumour activity, enhancement of cell growth and selective cellular cytotoxicity [1]. Two high-affinity TNF receptor subtypes have been cloned, representing single transmembrane domain glycoproteins of molecular masses 55–60 (p55TNFR) and 70–80 (p70TNFR) kDa. Both these receptors have significant extracellular domain homology, however, their cytoplasmic domains are unrelated, contain no sequences with recognisable catalytic capabilities and give no indication as to the signalling processes by which these receptors may act [2]. In its cytoplasmic domain, the p75 nerve growth factor (NGF) receptor (p75NGFR) has sequence homology to the p70TNFR cytoplasmic domain [3–8]. All of these receptors have been reported to influence cell death [9–13], although the exact cellular signalling mechanisms by which these receptors achieve their effects are poorly understood.

Arachidonic acid is the precursor in the biosynthesis of both prostaglandins and leukotrienes [14,15] and cytosolic phospholipase A_2 ($cPLA_2$) is a 100-kDa enzyme that selectively utilises phospholipid substrates containing arachidonic acid in the *sn*-2 position. Agonists, such as thrombin, platelet-derived growth factor, epidermal growth factor and ATP [16–18], plus the serine kinases protein kinase C (PKC) and mitogen-activated protein (MAP) kinase, were shown to phosphorylate and activate $cPLA_2$ [19,20]. Furthermore, it has been shown that cytokines, macrophage colony-stimulating factor can influence $cPLA_2$ activity [21–23]. Cytokines, such as interleukin-1β and TNF, increase the activity of $cPLA_2$ by phosphorylation and induce the synthesis of new $cPLA_2$ protein. Such profound effects of TNF on $cPLA_2$ suggests a possible role for the it in TNF actions, such as apoptosis. Indeed, it was shown that $cPLA_2$ is critical in the cytotoxic action of TNF [24] and that lipoxygenase metabolites of arachidonic acid were mediating TNF-induced cell death. Furthermore, oxygen radicals which are generated by the peroxidation of arachidonic acid metabolites, are thought to be involved in TNF-induced cytotoxicity [25] and the cytotoxic actions of these radicals can be reversed by the oxygen radical-scavenging enzymes, manganous superoxide dismutase and *bcl-2* [26,27].

We undertook investigations to reveal the influence of $cPLA_2$ in TNF-induced cytotoxicity using the HeLa cell model of TNF-induced cytotoxicity (a cell line reported to contain p55TNFR with little endogenous p70TNFR content [10]) and such cells stably expressing p70TNFR and p75NGFR receptors.

2. Experimental

2.1. Materials

Recombinant human TNF was purchased from R&D Systems. Human NGF and *Trk*A riboprobe vector were kindly provided by E.M. Shooter (Stanford University, Palo Alto, CA). Polyclonal anti-$cPLA_2$ antiserum and $cPLA_2$ cDNA was generated by the Genetics Institute (Boston, MA). [^{3}H]AA ([5,6,8,9,11,12,14,15-^{3}H(N)]arachidonic acid, specific activity = 100 Ci/mmol) was purchased from NEN/DuPont.

2.2. HeLa cell transfections

Stable transfections of HeLa cells with receptor cDNAs were performed in a Bio-Rad Gene Pulser electroporator (960 μFD, 0.25 V). 50 μg of the full-length receptor cDNAs in the pCDM8 mammalian expression vector (Invitrogen) were cotransfected with the pSV2 plasmid carrying the hygromycin phosphotransferase gene, mixed with 350 μg of yeast tRNA acting as carrier in 20 mM HEPES (pH 7.4). HeLa clones were cultured as described [10]. Hygromycin-resistant colonies were selected in growth medium supplemented with 400 μg/ml hygromycin (Boehringer Mannheim) and maintained at 200 μg/ml hygromycin. Positive clones were identified by a combination of Western analysis and [^{125}I]TNF or -NGF ligand-binding studies [28].

2.3. Northern analysis

Total RNA was isolated by extraction with guanidine thiocyanate then centrifugation on a CsCl gradient as described [7]. Glyoxal-denatured RNA was electrophoresed and transferred to positively charged Nytran (Schleicher and Schuell). Full-length cDNA probes were [^{32}P]dCTP-labelled by random prime reaction (Gibco BRL kit) and hybridized to the blots overnight at 42°C in hybridization solution (50% formamide, 5 × Denhardt's, 0.5% sodium dodecylsulphate (SDS), 100 μg/ml tRNA, 200 μg/ml salmon sperm DNA and 5 × SSPE), then washed (3 × 20 min in 0.1 × SSPE, 0.2% SDS, 50°C) before autoradiographic exposure at −80°C with intensifying screens.

2.4. Western analysis

Cell monolayers were directly lysed with 2% SDS, 70 mM Tris-HCl

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(pH 6.8), then boiled for 15 min to denature proteins and nucleic acids. Whole cell sample protein concentrations were determined (Pierce assay kit) then supplemented with 6 × loading buffer (60% glycerol, 12.5% β-mercaptoethanol, 1% bromophenyl blue). Samples were boiled for a further 3 min before loading equal amounts of total protein followed by SDS/PAGE. Electrophoresed proteins were electrically transferred to a Millipore Imobilon-P membrane and blocked with Ca^{2+}/Mg^{+}-free PBS + 5% non-fat dried milk, 0.5% Tween-20. Antibody-binding to the blot (1–2 h, 25°C, in blocking solution) was followed by washing (3 × 5 min) then a 30 min incubation with protein A-horseradish peroxidase (1:10,000) (Calbiochem). After further washing (3 × 5 min), specific protein–antibody interactions were detected with ECL detection agents (Amersham).

2.5. Cytotoxicity assay

Freshly dispersed cells were aliquoted into 96-well culture plates (Costar) at a cell density of 1×10^5 cells/ml (100 μl of cell suspension/well). After 24 h growth, the plates were treated with the required combination of agents and incubated for a further 24 h. Colourimetric determination of the attached cell number in each well was performed as described [10].

2.6. Arachidonic acid release studies

Confluent HeLa cells in Costar 12-well culture plates were isotopically labelled for 2–4 h in growth medium with 0.5 μCi/ml [^{3}H]arachidonic acid. After labelling, wells were washed once with 1 ml of Opti-MEM (Gibco-BRL) and incubated with Opti-MEM with or without the desired concentration of cytokine. At the required time, the medium from the well was centrifuged (16,000 × *g*, 3 min, 4°C) to pellet any detached cells, then its radioactivity determined which measures the [^{3}H]AA (or its radioactive metabolites) liberation from labelled phospholipid pools.

2.7. Apoptosis assay

Cells were incubated in growth medium supplemented with various agents for 15 h before confluent cells were detached by incubation with Ca^{2+}/Mg^{+}-free PBS+0.5 mM EDTA (37°C). Both adherent and non-adherent cells were pooled, washed once in growth medium (4°C) and DNA was stained by inclusion of 5 μg/ml Hoechst 33342 stain. A 5-μl aliquot of the stained cell sample was examined by epifluorescence using a Leitz Aristoplan microscope equipped with an A cube and photographed at 320× magnification. Fields with large numbers of cells were use to ascertain the % of the cells which were intensely fluorescing under ultraviolet excitation due to chromosomal compaction and nuclear karyorrhexis (an indication of apoptotic mechanisms of cell death). The existence of non-fluorescent cells was confirmed by visible light microscopy.

3. Results

As has been previously reported [23] and can be seen from Fig. 1, TNF (50 ng/ml for 4 and 8 h) induced $cPLA_2$ mRNA levels in HeLa cells. Treatment with 100 ng/ml NGF had no effect on $cPLA_2$ mRNA levels (Fig. 1b). Interestingly, the basal levels of $cPLA_2$ mRNA were higher in both the p75NGFR- and p70TNFR-stably overexpressing HeLa clones. In spite of raised basal levels of $cPLA_2$ mRNA in p75NGFR and p70TNFR clones, TNF still increased the message levels in a time-dependent manner (Fig. 1a), again confirmed by $cPLA_2$ Western analysis (Fig. 1c). TNF treatment resulted in a time-dependent (0–18 h) increase in $cPLA_2$ protein levels in wild-type and p70TNFR-and p75NGFR-expressing cells. Moreover,

Table 1
Enhanced TNF-induced HeLa cell death occurs through apoptotic mechanisms

HeLa clone	TNFα-induced apoptotic cells (% of total cells, mean ± S.D.)
Wild-type	26.1 ± 10.0
p75NGFR	66.1 ± 10.4
p70TNFR (#1)	59.2 ± 10.5
p70TNFR (#2)	78.1 ± 9.4
*Trk*A	31.3 ± 6.9
Hygromycin-resistant	17.8 ± 8.5

Cells were incubated in growth medium supplemented with 50 ng/ml TNF for 15 h before confluent cells were detached by incubation with Ca^{2+}/Mg^{+}-free PBS plus 0.5 mM EDTA (37°C). Both adherent and non-adherent cells were pooled, washed once by resuspension-centrifugation in growth medium (4°C), DNA was stained and cells were analysed as described in section 2. Approximately 4% of unstimulated cells appeared apoptotic.

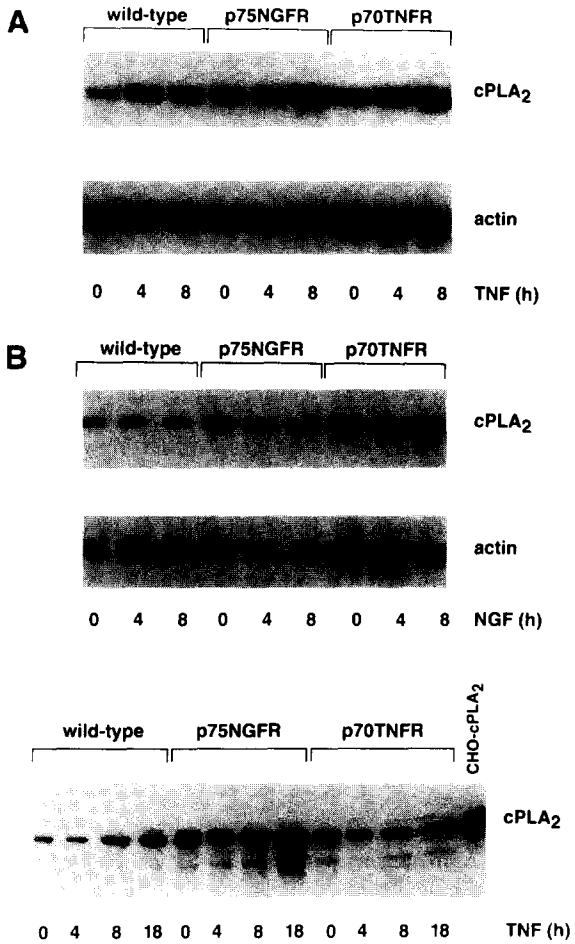

Fig. 1. Basal and TNF-induced levels of $cPLA_2$ mRNA and protein are greater in p75NGFR- and p70TNFR-expressing HeLa cells. Panel A: autoradiograms of $cPLA_2$ and β-actin mRNAs from wild-type, p75NGFR-overexpressing and p70TNFR-overexpressing HeLa cells treated with 50 ng/ml recombinant human TNF for the indicated times. Total RNA (15 μg/lane) was analysed for expression of the 3.4 kb $cPLA_2$ and 1.5 kb β-actin mRNAs using specific ^{32}P-labelled cDNA probes. Autoradiographic exposure was at −80°C with intensifying screens for 24 and 4 h, respectively. Panel B: same as in panel A, except cells were treated with 100 ng/ml human NGF. Panel C: Western analysis of $cPLA_2$ protein levels in wild-type, p75NGFR-overexpressing and p70TNFR-overexpressing HeLa cells treated with 50 ng/ml TNF for the indicated times. 50 μg protein/lane was electrophoresed on an 8% SDS gels. By comparison, the extreme most right lane contains 10 μg of total protein from a Chinese hamster ovary (CHO) cell line which is stably overexpressing $cPLA_2$ as a standard. Specific polyclonal $cPLA_2$ antibody (1:1000 dilution) binding was visualized by enhanced chemiluminescence by a 15-s exposure. The results in Fig. 1 are typical data from at least 4 independent experiments.

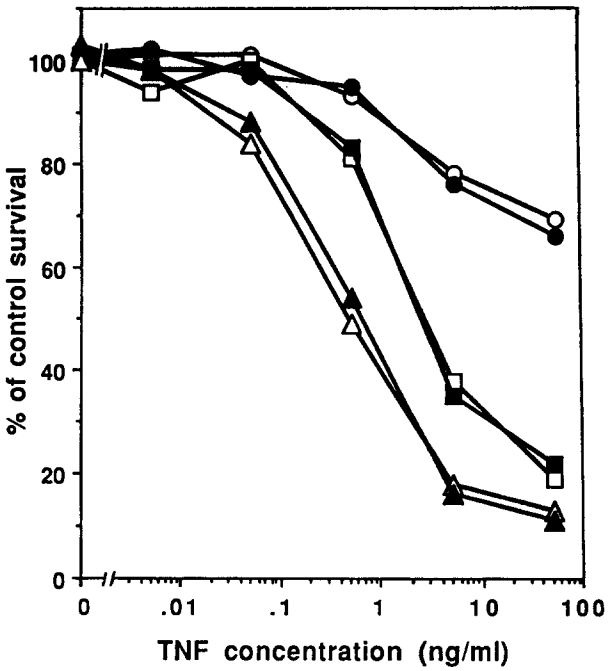

Fig. 2. Enhanced TNF-induced cytotoxicity in p75NGFR- and p70TNFR-expressing HeLa cells. Concentration–response curves for TNF were determined in the absence (open symbols) or additional presence (closed symbols) of 100 ng/ml NGF for wild-type (circles), p75NGFR-overexpressing (squares) and p70TNFR-overexpressing (triangles) HeLa cells. Levels of TNF-induced cell death were determined as described in section 2. The data represents the mean of at least 8 treatment wells, from at least 3 independent experiments. S.D. values were < 5%.

basal $cPLA_2$ levels were higher in the p75NGFR- and p70TNFR-overexpressing HeLa cells, resulting (by 8–18 h TNF treatment, times at which cell death is occurring) in markedly greater levels of $cPLA_2$ in the overexpressing cells, as compared with the wild-type HeLa. These finding are of interest such that both the p70TNFR and p75NGFR cells were found to be much more susceptible to TNF-induced cytotoxicity as compared with wild-type HeLa cells.

Fig. 2 displays the TNF cytotoxicity concentration–response curves for wild-type and p75NGFR- or p70TNFR-overexpressing HeLa cells. TNF caused a concentration-dependent cytotoxic response in all three clones, however, TNF far more potently killed the p70TNFR- and p75NGFR-overexpressing cells (maximal typical cytotoxic response of 75–95% death) than wild-type HeLa cells (typically, only 20–30% death). It is noteworthy that the cytotoxic responses seen here are: for a period of only 24 h in the presence of cytokine (times of up to 72 h can be routinely used by others); and also in the absence of any cyclohexamide, an agent used by others (in addition to TNF) permissively to 'reveal' any cytotoxic action of TNF [29]. Thus, our system demonstrates acute cytotoxic responses to TNF.

To assess whether the enhanced cytotoxic actions of TNF are affected by the raised levels of $cPLA_2$ in the p70TNFR- and p75NGFR-overexpressing cells, we generated several more stably expressing HeLa clones for our investigations. The p75NGFR and *Trk*A receptors both bind NGF, however, they are both sequentially and functionally distinct in that the *Trk*A receptor contains an intrinsic tyrosine kinase [30]. *Trk*A and hygromycin-resistant HeLa cells were similar to wild-type HeLa cells in that they displayed both low basal $cPLA_2$ levels and low susceptibility to TNF-induced cytotoxicity (Fig. 3). Thus, the raised $cPLA_2$ levels and enhanced death is specific to HeLa cells expressing either the p70TNFR or the p75NGFR and not due purely to expression of a cell surface NGF receptor, or of exerting hygromycin resistance upon the cells.

Using the Hoechst staining method of determining condensation of nuclear chromatin (an indication of apoptotic mechanisms of death), we determined that the HeLa cells were indeed dying from apoptotic, rather than necrotic mechanisms. Apoptotic mechanisms of cell death were also confirmed by the detection of nuclear DNA 'laddering' in TNF-treated HeLa cells (data not shown). Table 1 illustrates that the greater TNF-induced apoptosis in p70TNFR- or p75NGFR-expressing HeLa cells is clone-specific. This enhanced apoptosis was not observed in wild-type, *Trk*A-expressing or hygromycin-resistant cells treated with TNF. Furthermore, another independently derived p70TNFR-overexpressing HeLa clone (p70TNFR (#2)) showed similar results in these studies (see Table 1) as the original p70TNFR-overexpressing clone.

The [^{3}H]arachidonic acid release assay was used as a functional display of $cPLA_2$ activity in HeLa clones. As seen in Fig. 4, once again the HeLa clones which displayed significantly greater TNF-induced apoptosis (the p70TNFR and p75NGFR-expressing clones) than clones which displayed only minimal TNF-induced death (wild-type, *Trk*A and hygromycin-resistant clones), had markedly greater TNF-induced $cPLA_2$ activity. This clonal difference was manifested as greater induction of cellular $cPLA_2$ activity by TNF treatments of 6 h or longer (similar to the $cPLA_2$ mRNA and protein induction time course in Fig. 1). Although it appears that the initial transient liberation of [^{3}H]arachidonic acid is of a similar magnitude in all the clones tested, it is the secondary, longer-term increase in [^{3}H]arachidonic acid liberation (corresponding to $cPLA_2$ mRNA induction) that separates the clones into those which are susceptible or are relatively resistant to TNF-induced death. As cell death occurs at times of 8 h TNF treatment or greater, it is probable that it is the TNF-stimulated $cPLA_2$ gene expression which is critical for mediating apoptosis in HeLa cells.

Pretreatment of p70TNFR- and p75NGFR-overexpressing and wild-type HeLa cells for periods of up to 24 h with 1 μM dexamethasone, reversed TNF-induced cytotoxicity. This reduction of TNF-induced cell death was matched by a reduction in $cPLA_2$ protein levels in all three clones (data not shown). As was reported previously [23], dexamethasone could specifically block the induced synthesis of $cPLA_2$ by TNF treatment and it appears here that it can reduce the elevated $cPLA_2$ levels in p70TNFR- and p75NGFR-expressing cells to those levels seen in wild-type HeLa cells. This consistent observation of elevated $cPLA_2$ levels is apparently a prerequisite for observing enhanced TNF-induced death in HeLa cells. Evidence in support of a role for PLA_2 in TNF-induced death was reported in WEHI-S cells, where expression of a 70-kDa major heat-shock protein inhibits TNF-induced cytotoxicity. This heat-shock protein acts to inhibit TNF-induced PLA_2 activity [31]. Previously, Hayakawa et al. [24] observed that TNF-induced cytotoxicity in L929 cells was utterly dependent on the presence of $cPLA_2$ in those cells.

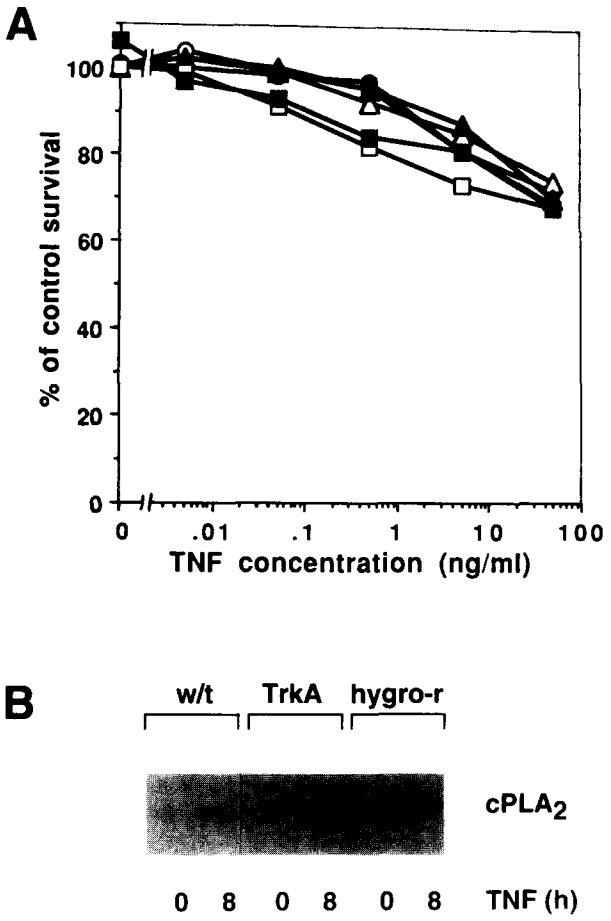

Fig. 3. Compared with wild-type cells, TNF-induced cytotoxicity and $cPLA_2$ levels are similar in *Trk*A-expressing and hygromycin-resistant HeLa cells. Panel A: concentration–response curves for TNF-induced cell death were determined in the absence (open symbols) or additional presence (closed symbols) of 100 ng/ml NGF for wild-type (circles), *Trk*A-overexpressing (squares) and hygromycin-resistant (triangles) HeLa cells. The data represents the mean of at least 8 treatment wells, from at least 2 independent experiments. S.D. values were < 5%. Panel B: autoradiogram of $cPLA_2$ mRNA levels from wild-type, TrkA-overexpressing and hygromycin-resistant HeLa cells treated for 0 or 8 h with 50 ng/ml TNF. Total RNA (15 μg/lane) was probed with ^{32}P-labelled $cPLA_2$ cDNA probe and then exposed at −80°C for 4 days with intensifying screens.

4. Discussion

The ability of the two TNF receptors to signal independently for cytotoxicity is presently a controversial topic. It has been claimed that the p55TNFR exclusively signals to induce cell death [29]. Further work by Tartaglia et al. demonstrated a region within the intracellular domain of p55TNFR (termed the 'death domain') which is crucial for cytotoxicity [32]. Thus, the ability of the p55TNFR to signal for cell death remains convincing. However, the influence that the p70TNFR has on cell death is presently unresolved.

Previous reports provided evidence for a role for the p70TNFR in enhancing TNF-induced cytotoxicity [9,33,34]. Other reports suggested that p70TNFR could cause apoptosis independently of p55TNFR activation [35,36]. It may be that the function of the p70TNFR in apoptosis is to enhance p55TNFR-mediated actions. Such a functional role has already been suggested for the p70TNFR by Tartaglia et al. [11]. In this model, the receptor serves to present ligand to another receptor (p55TNFR), which in turn signals for cell death. The enhancements of cytotoxicity by the p70TNFR would be a result of increased local TNF concentration around the p55TNFR. Such a 'ligand passing' model of the p70TNFR and p75NGFR could be consistent with the data presented here. However, the presence of the p75NGFR receptor (which does not bind TNF) was sufficient to enhance TNF-induced apoptosis in HeLa cells, but expression of any of the other receptors used in this study did not result in enhanced apoptosis. These data with the permissive role of either the p70TNFR or p75NGFR suggests that these receptors specifically introduce into the cell, a mechanism by which TNF-mediated cell death can be enhanced. This mechanism may be the increase in basal levels of $cPLA_2$, an enzyme clearly involved in TNF-induced apoptosis. Thus, we propose a model by which the p70TNFR (and p75NGFR) enhance programmed cell death: the presence of the receptor (not necessarily its ligand-mediated signal transducing activities) induces production of an intracellular mediator(s) (including $cPLA_2$ for the production of arachidonic acid metabolites) which is utilised by the TNF-stimulated signalling pathways of the p55TNFR.

No direct signalling function has yet been attributed to the p70TNFR and p75NGFR cytoplasmic domains. These receptors may acquire other cytoplasmic entities to form fully active signalling complexes. Indeed, it is known that p55TNFRs must dimerize in order to activate its signalling pathways [1]. Indeed, the cytoplasmic domain of the p70TNFR was recently found to directly bind two TNF receptor-associated factors (TRAFs 1 and 2) which were necessary for the receptor's signal

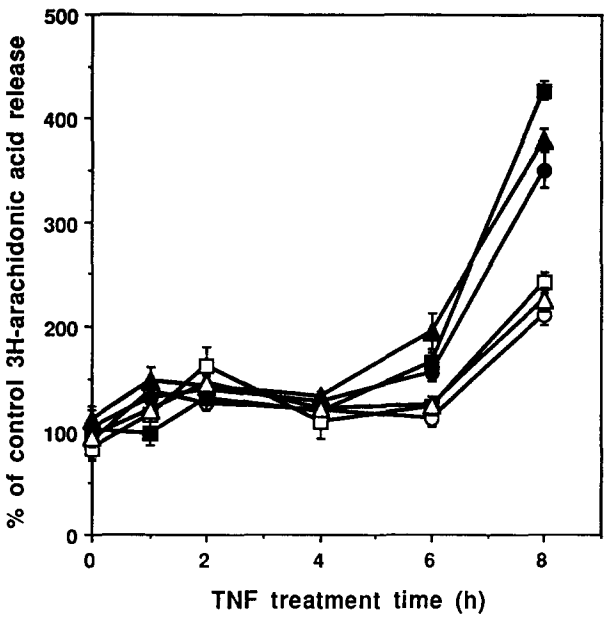

Fig. 4. TNF-induced arachidonic acid release is greater in HeLa cells that display enhanced TNF-induced death. Time courses of 50 ng/ml TNF induced [^3H]arachidonic acid release from wild-type HeLa cells (open circles), TrkA-expressing (open squares), hygromycin-resistant (open triangles), p75NGFR-expressing (closed triangles) and two independently derived clones of HeLa cells expressing p70TNFR (closed circles and closed squares). The data represent the mean ± S.E. of 3 separate experiments.

transduction [37]. The signalling mechanisms utilised by these TRAFs was not determined, however, the p75NGFR was also recently found to couple to sphingomyelin turnover [38]. The sphingomyelin pathway was implicated in apoptosis [39] and it may be that this pathway is involved not only in p75NGFR but also p70TNFR enhancement of cell death. It is uncertain what precise role the enhancement of $cPLA_2$ levels by the p70TNFR and p75NGFR has, but uncovers further pathway mechanisms by which these novel receptors may operate.

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