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Differential Activation of Phospholipases During Necrosis or Apoptosis: A Comparative Study Using Tumor Necrosis Factor and Anti-Fas Antibodies

Dirk De Valck, Dominique Vercammen, Walter Fiers, and Rudi Beyaert*

Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology and University of Gent, 9000 Gent, Belgium

Abstract Phospholipases generate important secondary messengers in several cellular processes, including cell death. Tumor necrosis factor (TNF) can induce two distinct modes of cell death, viz. necrosis and apoptosis. Here we demonstrate that phospholipase D (PLD) and cytosolic phospholipase A_2 ($cPLA_2$) are differentially activated during TNF-induced necrosis or apoptosis. Moreover, a comparative study using TNF and anti-Fas antibodies as cell death stimuli showed that PLD and $cPLA_2$ are specifically activated by TNF. These results indicate that both the mode of cell death and the type of death stimulus determine the potential role of phospholipases as generators of secondary messengers. J. Cell. Biochem. 71:392–399, 1998. © 1998 Wiley-Liss, Inc.

Key words: apoptosis; necrosis; phospholipases; tumor necrosis factor; Fas

Tumor necrosis factor (TNF) receptors and Fas are members of a large TNF receptor superfamily and mediate cell death induced by TNF and Fas ligand, respectively [Itoh et al., 1991; Wallach, 1997]. The strong death signal is transduced from a homologous region within the cytoplasmic tail of both receptors, called the death domain [Itoh and Nagata, 1993; Tartaglia et al., 1993]. The death domain serves as a protein-protein interaction domain that recruits cytoplasmic adaptors and effectors involved in cytotoxicity and gene induction [Wallach, 1997]. Despite the high similarity at the receptor and early signal transduction level, the mode of cell death induced by either receptor can be quite different. Fas ligand induces specifically apoptosis [Itoh et al., 1991], the most typical features being chromatin condensation, internucleosomal DNA fragmentation, membrane blebbing, and general disintegration of the cell into apoptotic bodies [Kroemer et al., 1995]. TNF can induce either apoptosis or necrosis [Laster et al., 1988], the latter characterized by swelling of the cell and organelles, followed by collapse, disruption of the cell membrane, and lysis [Grooten et al., 1993].

TNF receptors and Fas have been shown to trigger at least partially different signaling pathways leading to cell death [Schulze-Osthoff et al., 1994; Wong and Goeddel, 1994; Vercammen et al., 1997]. In contrast, recent evidence suggests that in some cell types the decision to die in a necrotic or apoptotic way is determined by the amount of ATP present in the cell, with necrosis occurring when ATP is limiting [Leist et al., 1997]. During the past few years, we and others obtained evidence that TNF cytotoxicity is associated with activation of phospholipase A_2 (PLA_2) [Suffys et al., 1987, 1991; Hayakawa et al., 1993; Thorne et al., 1996; Voelkel-Johnson et al., 1996], phosphatidylinositol/phospholipase C [Beyaert et al., 1993], and phospholipase D (PLD) [De Valck et al., 1993]. The majority of these experimental results was obtained

Abbreviations used: ActD, actinomycin D; $cPLA_2$, cytosolic phospholipase A_2; PLA_2, phospholipase A_2; PLD, phospholipase D; TNF, tumor necrosis factor; TPA, phorbol 12-myristate 13-acetate.

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*Correspondence to: Rudi Beyaert, Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology and University of Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium. E-mail: rudi@dmb.rug.ac.be

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with the L929 fibrosarcoma cell line, which is a model system for TNF-induced necrosis [Grooten et al., 1993; Beyaert and Fiers, 1994]. However, after transfection of L929 cells with human Fas cDNA, these cells can be induced to undergo apoptosis by treatment with agonistic anti-Fas antibodies [Vercammen et al., 1997], providing a useful tool to study necrosis and apoptosis in a single cell line. On the other hand, HeLaH21 cervix carcinoma cells undergo apoptosis after treatment with either TNF or anti-Fas antibodies. In a comparative study on these cell lines, we investigated whether cytosolic phospholipase A_2 ($cPLA_2$) and PLD are similarly or differentially activated during necrosis and apoptosis. We observed that PLD activation is specifically associated with TNF-induced necrosis, whereas $cPLA_2$ activation is associated with early TNF-induced apoptosis. In contrast, apoptosis induced by anti-Fas antibodies was not associated with activation of any of these phospholipases in the same cell lines studied.

MATERIALS AND METHODS

Cell Lines and Materials

L929 murine fibrosarcoma cells, a L929hFas.Cl4 subclone selected for stable expression of human Fas [Vercammen et al., 1997], and HeLaH21 human cervix carcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum and 5% newborn calf serum, L-glutamine, penicillin, and streptomycin. Recombinant TNF was produced in *E. coli* and purified to at least 99% homogeneity. Agonistic monoclonal anti-Fas antibody of the IgM type (clone CH-11) was purchased from Immunotech (Alston, MA). Actinomycin D (ActD), the calcium ionophore A23187, and phorbol 12-myristate 13-acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, MO). Antirabbit horseradish peroxidase–linked antibodies and an ECL Western blotting detection kit were purchased from Amersham International (Amersham, UK).

Detection of Apoptosis by Flow Cytometry Analysis

DNA profiles showing hypoploidy in cells undergoing apoptosis were obtained after addition of propidium iodide (Sigma Chemical Co.) to a final concentration of 50 μg/ml and application of one freeze/thaw cycle to permeabilize cells and cell fragments. Propidium iodide fluorescence was measured by FACS analysis.

PLD-Catalyzed Transphosphatidylation Reaction

Cells were grown in six-well plates up to near confluency. To isotopically label the phospholipid pool, we incubated cells for 24 h in 1 ml fresh complete medium, containing 0.5 μCi [1-^{14}C]palmitic acid (specific activity 55.6 mCi/mmol) (Amersham International). At the end of the labeling period, cells were washed free from nonincorporated label with phosphate-buffered saline, followed by addition of fresh complete medium. PLD-catalysed transphosphatidylation of ethanol was performed as previously described [De Valck et al., 1993].

Detection of $cPLA_2$ Phosphorylation by Gel Shift Using Western Blotting

Cells were grown in six-well plates up to confluency and were serum-starved for 24 h by incubation in DMEM supplemented with 5 mg bovine serum albumin/milliliter. After addition of various stimuli, cells were lysed in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% NP-40, 1 mM EDTA, 50 mM NaF, 30 mM Na pyrophosphate, 0.2 mM $NaVO_3$, 25 mM β-glycerophosphate, supplemented with protease inhibitors. After removal of nuclei and residual cell debris, 50 μg of protein was separated by 8% SDS-PAGE and blotted onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Detection of $cPLA_2$ protein was performed using rabbit polyclonal antihuman $cPLA_2$ antiserum (a kind gift of Dr. J. Clark and Dr. J. Knopf, Genetics Institute, Cambridge, MA). Antirabbit horseradish peroxidase–linked antibodies were used as secondary antibodies; detection was performed by the ECL method. Recombinant human $cPLA_2$ (a kind gift of Dr. R. Kramer, Lilly Research Laboratories, Indianapolis, IN) was used as a positive control for detection of both unphosphorylated $cPLA_2$ and retarded phosphorylated $cPLA_2$.

Arachidonic Acid Release

Cells were grown in six-well plates up to near confluency. Medium was removed, and 1 ml of fresh complete medium containing 0.5 μCi [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid (12 Ci/mmol) (Amersham International) was added.

Cells were further incubated for 24 h up to confluency. At the end of the labeling period, cells were washed free from nonincorporated label with phosphate-buffered saline, followed by addition of DMEM containing 5 mg fatty acid–free bovine serum albumin/milliliter. After an additional hour of incubation, various stimuli were added; supernatant was cleared from cell material by centrifugation. Radioactivity in the medium was measured by scintillation counting after addition of 10 volumes of scintillation liquid.

RESULTS

Induction of Necrosis and Apoptosis by TNF and Anti-Fas Antibodies in Susceptible Cell Lines

Initial definitions of necrosis and apoptosis are mainly based on morphological features. Many different biochemical methods to specifically quantitate apoptosis have been described, including the measurement of DNA hypoploidy as a result of internucleosomal DNA fragmentation [Laster et al., 1988; Grooten et al., 1993; Kroemer et al., 1995]. In contrast to apoptotic cells, living cells or cells undergoing cell death by necrosis do not show hypoploidy but have discrete DNA populations in G_0/G_1, S, and $G_2 + M$ phases of the cell cycle. We used the DNA pattern as a parameter for apoptotic cell death. Since TNF-induced and anti-Fas–induced cell death is often synergistic with inhibitors of transcription [Vercammen et al., 1997], all experiments were performed in the presence of ActD (1 μg/ml), which on its own had no cytotoxic effect.

L929 cells have previously been shown to die by necrosis after TNF treatment [Grooten et al., 1993; Beyaert and Fiers, 1994]. Indeed, treatment of these cells with TNF resulted in cell death without any evidence for DNA hypoploidy (Fig. 1A). Anti-Fas treatment had no detectable effect on these cells. In contrast, L929hFas.Cl4 cells, a subclone selected for stable expression of human Fas [Vercammen et al., 1997], is susceptible to anti-Fas–induced cell death with the appearance of DNA hypoploidy as a hallmark of apoptosis (Fig. 1B). Interestingly, these cells still died in a necrotic way after TNF treatment. In order to study TNF-induced apoptosis, we used HeLaH21 cells, which die in an apoptotic way and show DNA hypoploidy after treatment with either TNF or anti-Fas antibodies (Fig. 1C).

PLD Activation Is Specifically Associated With TNF-Induced Necrosis

We have previously described that TNF cytotoxicity is associated with activation of PLD [De Valck et al., 1993]. That study was mainly performed in L929 cells using transphosphatidylation of phospholipids to the acceptor molecule ethanol as a unique feature of PLD activity [Yang et al., 1967]. The recent availability of L929hFas.Cl4 cells provides an elegant model to study necrosis and apoptosis in the same cell line after TNF or anti-Fas treatment, respectively. PLD activity was determined in parental L929 cells and L929hFas.Cl4 cells after TNF or anti-Fas treatment in the presence of ActD (Fig. 2). Formation of phosphatidylethanol as the product of PLD activity could be determined in both L929 and L929hFas.Cl4 cells after induction of TNF-mediated necrosis. However, no phosphatidylethanol formation was observed in L929hFas.Cl4 cells after induction of apoptosis by anti-Fas antibodies. Similarly, neither TNF-induced nor anti-Fas–induced apoptosis of HeLaH21 cells was associated with PLD activation. Activation of PLD by treatment of cells with a noncytotoxic concentration of TPA was used as a positive control. These data suggest that PLD activation is specifically associated with TNF-induced necrosis. In addition, the observation that TPA activates PLD without inducing cell death demonstrates that activation of PLD as such is not sufficient to induce necrosis.

$cPLA_2$ Activation Is Specifically Associated With TNF-Induced Apoptosis

PLA_2 releases fatty acids from the sn-2 position of phospholipids. Possible involvement of PLA_2 in TNF cytotoxicity is supported by release of arachidonic acid into the medium of TNF-sensitive cells [Suffys et al., 1991]. Additional evidence is provided by the use of various PLA_2 inhibitors which block both TNF-induced arachidonic acid release and cytotoxicity [Suffys et al., 1987]. Although PLA_2 comprises a family of closely related proteins [Dennis, 1994], the 85 kDa $cPLA_2$ has been demonstrated to be the main PLA_2 species activated early during TNF cytotoxicity [Hayakawa et al., 1993; Thorne et al., 1996; Voelkel-Johnson et al., 1996]. Full activation of $cPLA_2$ proceeds by a two-step mechanism. First, $cPLA_2$ becomes phosphorylated on a serine residue by a mito-

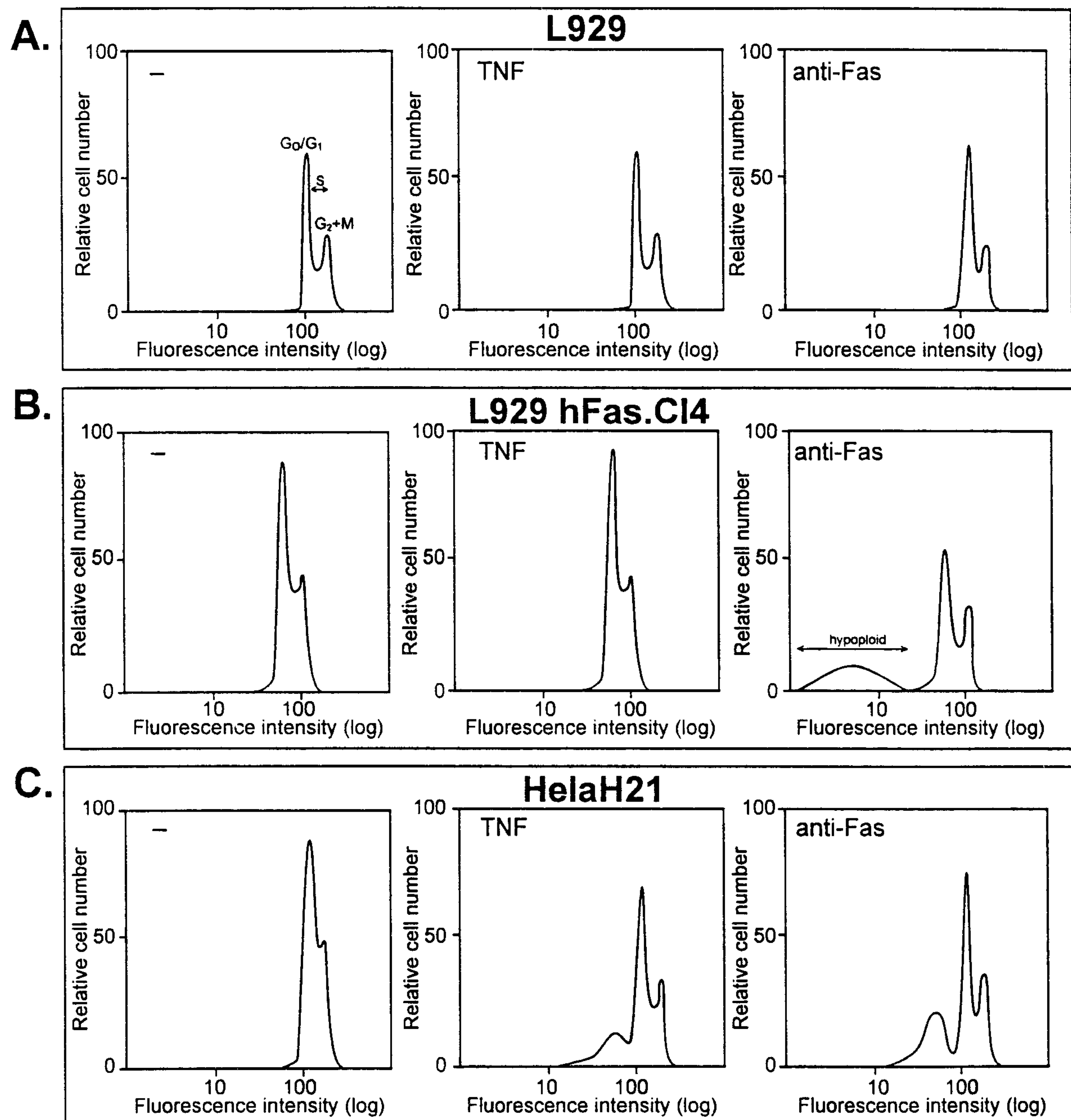

Fig. 1. Detection of DNA hypoploidy by flow cytometry analysis. L929, L929hFas.Cl4, and HeLaH21 cells were either untreated or treated for 6 h with 1,000 IU/ml TNF or 500 ng/ml anti-Fas antibodies as described. ActD (1 µg/ml) was included in all samples in order to sensitize the cells to the cytotoxic effect of TNF and anti-Fas. The DNA was stained with propidium iodide for analysis.

gen-activated protein kinase [Lin et al., 1993], resulting in a slower migration during gel electrophoresis. Second, phosphorylated $cPLA_2$ is translocated from the cytosol to membranes by an increase in intracellular calcium [Clark et al., 1991].

To analyze whether TNF and anti-Fas can both stimulate $cPLA_2$ at early time points, we determined $cPLA_2$ phosphorylation in serum-starved HeLaH21 cells treated with TNF or anti-Fas in the presence of ActD (Fig. 3). Western blotting and detection with an anti-$cPLA_2$ antiserum demonstrated that $cPLA_2$ is present in untreated cells, both as a nonphosphorylated form (Fig. 3, lower band) and a retarded phosphorylated form (Fig. 3, upper band), as de-

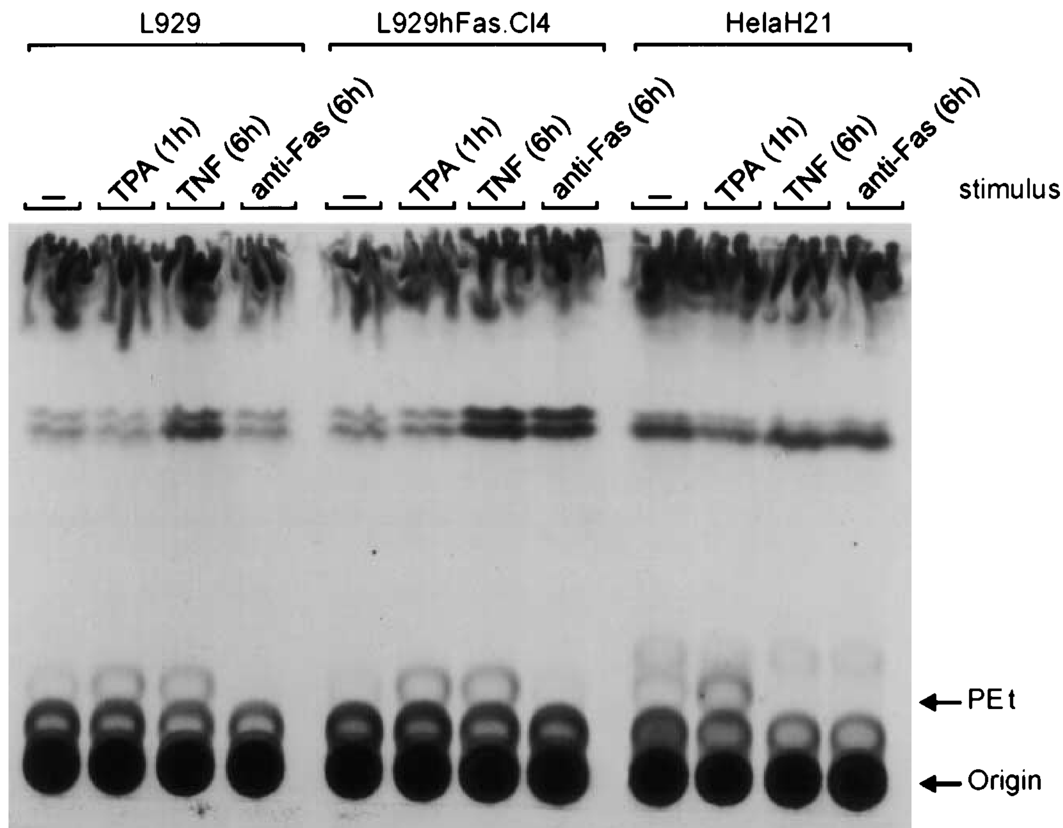

Fig. 2. Effect of TNF and anti-Fas on phosphatidylethanol (PEt) formation by PLD-catalyzed transphosphatidylation. L929, L929hFas.Cl4, and HeLaH21 cells prelabeled with [^{14}C]-palmitic acid were either untreated or treated with 200 ng/ml TPA, 1,000 IU/ml TNF, or 500 ng/ml anti-Fas antibodies. Incubation with TPA lasted for 1 h, and incubations with TNF or anti-Fas lasted for 6 h. ActD (1 μg/ml) was included in all treatments. Samples were analyzed by thin-layer chromatography.

scribed previously [Lin et al., 1993]. Treatment of cells with TPA served as a positive control and resulted in complete $cPLA_2$ phosphorylation, since the lower, nonphosphorylated band shifted completely to the upper, phosphorylated band. TNF stimulation of the cells resulted in a moderate but significant and reproducible $cPLA_2$ phosphorylation. In contrast, stimulation with anti-Fas antibodies had no detectable effect on $cPLA_2$ phosphorylation. We were unable to study $cPLA_2$ activation during TNF-induced necrosis in L929 or L929hFas.Cl4 cells, since these cells contain $cPLA_2$ levels below the detection limit and show no early arachidonic acid release after TPA or TNF treatment. $cPLA_2$ activation as described above was confirmed by measuring arachidonic acid release from HeLaH21 cells stimulated with TNF or anti-Fas. Calcium ionophore A23187 (5 μM) was used as an additional calcium-mobilizing stimulus which should result in translocation of $cPLA_2$ from the cytosol to membranes [Clark et al.,

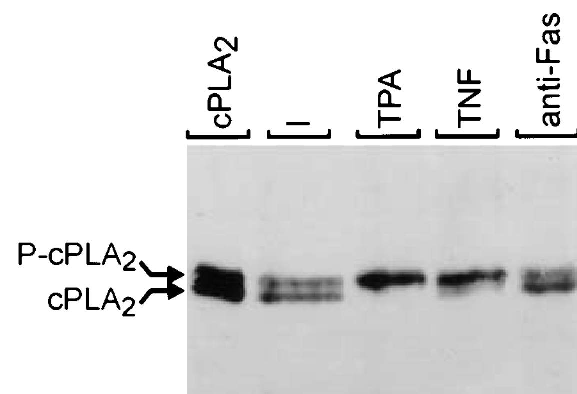

Fig. 3. Effect of TNF and anti-Fas on $cPLA_2$ phosphorylation as detected by gel shift analysis. Serum-starved HeLaH21 cells were either untreated or treated for 1 h with 200 ng/ml TPA, 1,000 IU/ml TNF, or 500 ng/ml anti-Fas antibodies in the presence of 1 μg/ml ActD. Cell lysates were analyzed by PAGE. After Western blotting, $cPLA_2$ and phosphorylated $cPLA_2$ (P-$cPLA_2$) were revealed with anti-$cPLA_2$ antibody. Recombinant human $cPLA_2$ (10 ng) served as a positive control for detection of both unphosphorylated and phosphorylated $cPLA_2$.

1991; Sharp et al., 1991]. Treatment of cells with TPA resulted in a sixfold increase in arachidonic acid release as compared to control cells. TNF stimulation of the cells resulted in a twofold increase in arachidonic acid release, whereas stimulation with anti-Fas antibodies did not induce arachidonic acid release (Fig. 4). These data indicate that early $cPLA_2$ activation is specifically associated with apoptosis induced by TNF but not by anti-Fas antibodies.

DISCUSSION

Activation of phospholipases and of the production of phospholipid metabolites is recognized as a key step in signal transduction after stimulation of different cell types with various hormones and growth factors [Divecha and Irvine, 1995]. Although most of these stimuli induce cell growth and differentiation, we and others previously reported that phospholipase activation can also be associated with cytotoxicity [Suffys et al., 1987, 1991; Beyaert et al., 1993; De Valck et al., 1993; Hayakawa et al., 1993; Thorne et al., 1996; Voelkel-Johnson et al., 1996]. In the present study we provide evidence that PLD and $cPLA_2$ are differentially activated during both forms of cell death, viz. necrosis and apoptosis.

PLD activation is specifically associated with TNF-induced necrosis and not with TNF-induced apoptosis. The underlying molecular mechanism is at present still unclear. There is considerable evidence that the basic mechanism of TNF-induced necrosis involves formation of reactive oxygen species, presumably by the mitochondria [Schulze-Osthoff et al., 1992]. Reactive oxygen species may cause membrane damage by lipid peroxidation, and PLD activation may be a cellular defense mechanism to specifically hydrolyze such lipids in an attempt to repair cell damage.

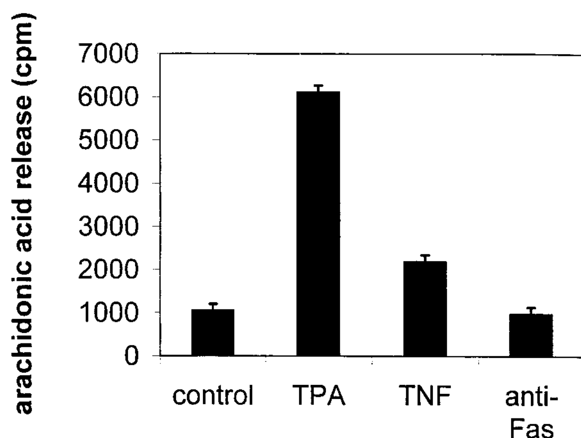

Fig. 4. Effect of TNF and anti-Fas on arachidonic acid release. HeLaH21 cells prelabeled with [^{3}H]-arachidonic acid were either untreated or treated for 1 h with 200 ng/ml TPA, 1,000 IU/ml TNF, or 500 ng/ml anti-Fas antibodies in the presence of 1 µg/ml ActD and 5 µM A23187. [^{3}H]-arachidonic acid released in the medium was quantified by liquid-scintillation counting.

In contrast to PLD, $cPLA_2$ activation was observed in cells susceptible to TNF-induced apoptosis, which is in agreement with reports on TNF-induced $cPLA_2$ activation [Hayakawa et al., 1993; Thorne et al., 1996; Voelkel-Johnson et al., 1996]. Activation of $cPLA_2$ results from its phosphorylation by mitogen-activated protein kinases [Lin et al., 1993], leading to a slower migration after SDS-PAGE. Indeed, we and others have previously shown that TNF activates extracellular signal-regulated kinase [Van Lint et al., 1992] and p38 mitogen-activated protein kinase [Beyaert et al., 1996; Waterman et al., 1996], which can both phosphorylate $cPLA_2$. It has recently also been proposed that caspase-mediated processing of $cPLA_2$ plays a role in $cPLA_2$ activation, although this is still controversial [Voelkel-Johnson et al., 1995; Wissing et al., 1997]. We were unable to study early $cPLA_2$ phosphorylation in cells dying from necrosis since $cPLA_2$ levels are below the detection limit in L929 cells. In addition, an early increase in arachidonic acid release could not be observed in these cells, suggesting that $cPLA_2$ is not activated during the early stages of TNF-induced necrosis. The latter results differ from those previously published on L929 cells with high concentrations of active $cPLA_2$ [Voelkel-Johnson et al., 1996]. However, biochemical discrepancies with L929 cells have been observed before by several laboratories, which might be the result of different culturing conditions or subcloning of the parental cell line [Enari et al., 1996]. Moreover, L929 cells that die by apoptosis after TNF treatment instead of by necrosis as described here have also been reported [Trent et al., 1996]. The way of dying of the L929 cells used by Voelkel-Johnson et al. [1996] is not clear. We previously reported a TNF-induced release of arachidonic acid in L929 cells during TNF-induced necrosis [Suffys et al., 1987, 1991]. However, in the latter studies this could be detected only at late times ($>$5 h) after TNF stimulation, which is in sharp contrast with the early $cPLA_2$ activation studied in this paper. The inability to detect substan-

tial $cPLA_2$ levels in L929 cells makes an involvement of $cPLA_2$ in the late TNF-induced release of arachidonic acid rather unlikely. The PLA_2 species involved in this stage of the necrotic process still needs to be identified.

In contrast to TNF, we were unable to detect any effect of anti-Fas on the activity of PLD or $cPLA_2$ in susceptible cells. This is in agreement with previous data demonstrating that $cPLA_2$ is dispensable for Fas-mediated apoptosis in L929 cells [Enari et al., 1996] but contradicts the previous demonstration of early arachidonic acid release after anti-Fas stimulation of HuT78 lymphoma cells [Cifone et al., 1995]. However, this discrepancy might reflect a cell line–specific effect. Moreover, in the latter study, PLA_2 activity was not demonstrated to be associated with apoptosis, nor was the critical PLA_2 involved characterized as $cPLA_2$. Our results suggest that the TNF receptor p55 and Fas activate at least partially different signaling pathways. Both have a closely related stretch of 90 amino acids in their intracellular domain, which is crucial and sufficient to induce the death signal [Itoh and Nagata, 1993; Tartaglia et al., 1993]. After clustering of the p55 TNF receptor or Fas, similar signaling molecules are believed to be recruited to the death domain of both receptors, suggesting that differences in TNF-induced or Fas-induced signaling pathways should originate at another level. The latter might involve the membrane-proximal part of the receptors, which is unrelated. Indeed, a role for the membrane-proximal part of the p55 TNF receptor has recently been shown to be involved in $cPLA_2$ activation by the recruitment of FAN and a neutral sphingomyelinase [Adam-Klages et al., 1996]. Nevertheless, phospholipase activation by Fas has already been reported in the case of a phosphatidylcholine-specific phospholipase C. Activation of the latter results in activation of an acidic sphingomyelinase, which generates ceramide [Cifone et al., 1993, 1995]. This lipid metabolite can have potent proapoptotic activities [Hannun and Obeid, 1995]. The role of Fas-induced changes in cellular levels of ceramides in apoptosis is nowadays a matter of debate, since Fas-induced apoptosis of T-cells occurs independently of ceramide generation [Watts et al., 1997]. Therefore, the role of sphingomyelinases and ceramide production in apoptosis might have been overstated. One should, however, also be cautious regarding the role of PLD activation in necrosis and $cPLA_2$ activation in TNF-mediated apoptosis since both phospholipases can also be activated by a noncytotoxic TPA treatment. However, the latter seems to activate phospholipases by a protein kinase C–dependent mechanism which is not involved in TNF signaling [Exton, 1997; Leslie, 1997]. In conclusion, the present demonstration of a differential activation of specific phospholipases in TNF and Fas signaling might be important in view of the determination of specific targets for the development of new therapies.

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