Distinct regulation of cytosolic phospholipase A₂ phosphorylation, translocation, proteolysis and activation by tumour necrosis factor-receptor subtypes

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The hormonally regulated Ca²⁺-dependent enzyme, cytosolic phospholipase A₂ (cPLA₂) is activated by a range of inflammatory stimuli. Tumour necrosis factor- α (TNF) is one of the first known stimuli for cPLA₂ but it is not known whether both TNF receptor subtypes are involved in activating the lipase. In the present study, we show for the first time that both type I 55 kDa TNFR (TNFR1) and type II 75 kDa TNFR (TNFR2) stimulate cPLA₂ enzyme, but with distinct signalling mechanisms. TNFR1 activates mitogen-activated protein kinase (MAPK) and p38MAPK. TNFR1 then phosphorylates and activates cPLA₂ in a MAPK-dependent fashion. Furthermore, TNFR1 causes the translocation and caspase-dependent proteolysis of cPLA₂ as

part of its activation profile. TNFR2, on the other hand, does not cause the phosphorylation of cPLA₂ as it does not activate MAPK or p38MAPK, but instead activates cPLA₂ by causing its translocation to plasma membrane and perinuclear subcellular regions. TNFR2 activation causes a delayed, slight increase in [Ca²⁺]_i of < 50 nM that may contribute towards the translocation and activation of cPLA₂. Therefore both TNF receptor subtypes play a role in cPLA₂ activation, but by means of separate signal-transduction pathways.

Key words: apoptosis, cytokine receptor, phosphatase, protein kinase, signal transduction.

INTRODUCTION

Cytosolic phospholipase A₂ (cPLA₂) is a Ca²⁺-dependent lipase that cleaves arachidonic acid-containing phospholipids (mainly phosphocholine) at their *sn*-2 position, liberating arachidonic acid that is a precursor to lipoxins, thromboxanes, leukotrienes and prostaglandin eicosanoids as well as precursor to the platelet-activating factor [1–4]. cPLA₂ is hormonally regulated by a range of receptor stimuli including hormones, neurotransmitters, antigens and mitogens [5]. Activation of cPLA₂ can be through phosphorylations at specific serine residues (notably Ser⁵⁰⁵ and Ser⁷²⁷) by mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and another kinase that may be p38MAPK [5]. One of the first stimuli found to activate cPLA₂ was tumour necrosis factor- α (TNF) [6]. TNF's actions in stimulating cPLA₂ consist of activation by phosphorylation, as well as causing the translocation to plasma membrane but mostly perinuclear subcellular regions [5], and the possible caspase-1- and caspase-8-dependent cleavage of cPLA₂ which may lead to the regulation of its activity [7–12]. Long-term effects of TNF include the stimulation of cPLA₂ gene transcription that is sensitive to anti-inflammatory glucocorticoids [6] that may be involved in the TNF receptor (TNFR) sensitivity of apoptosis [13]. The importance of cPLA₂ in inflammatory processes is clear due to pharmacological evidence (e.g. non-steroidal anti-inflammatory agents or glucocorticoids) as well as genetic evidence in transgenic knock-out animals which display reduced inflammatory and allergic responses as well as decreased cardiovascular infarction and neurological deficits or damage [14].

TNF is a multifunctional cytokine produced mainly by monocytes and macrophages, and is a principal regulator of inflammation and immunity, eliciting a wide variety of biological effects, the nature of which depends on the type and growth state of the target cell [15]. TNF has also been recognized as an important mediator of several autoimmune diseases as well as other chronic and acute inflammatory diseases, e.g. endotoxic shock, graft-versus-host disease, cachexia, rheumatoid arthritis and Crohn's disease. TNF exists in two biologically active forms, a 17 kDa homotrimer in solution and a 26 kDa membrane-bound precursor protein.

TNF activity is transduced via two distinct high-affinity membrane receptors designated as type I 55 kDa TNFR (TNFR1; also known as p55TNFR, TNFR60, p60, CD120a and TNFRSF1a) and type II 75 kDa TNFR (TNFR2; also known as p75TNFR, p80, CD120b and TNFRSF1b), which are constitutively co-expressed in most tissues [16]. An important question to be asked is: which signal pathways are mediated by TNFR1 and TNFR2? The role of each receptor in mediating the signal for cell death is widely debated. It is probable that both TNFRs participate in cell death, with TNFR1 signalling for death by its cytoplasmic death domain motif and TNFR2 modulating death responses. TNFR2 is also capable of proliferative cellular actions, but how the subtype signalling accomplishes these responses is still not clear [16]. It is now evident that both receptors can activate intracellular signal pathways independently, but can also co-operate both at the level of ligand binding and at the level of signal transduction [15]. Membrane-bound TNF activates both TNFR1 and TNFR2, whereas soluble TNFs (as are most TNFs commonly used in experiments) can only efficiently activate TNFR1 [17], with

Abbreviations used: [¹⁴C]-SAPC, stearyl-arachidonoyl-[¹⁴C]phosphatidylcholine; cPLA₂, cytosolic phospholipase A₂; fura 2/AM, fura 2 acetoxymethyl ester; [³H]arachidonic acid, [5,6,8,9,11,12,14,15-³H(N)]arachidonic acid; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C; TNF, tumour necrosis factor- α ; TNFR, TNF receptor; TNFR1, type I 55 kDa TNFR; TNFR2, type II 75 kDa TNFR.

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agonistic mutant TNFs and antisera helping to act as TNFR2 stimuli [16].

TNFRs activate an array of kinases that are capable of activating downstream effector enzymes such as cPLA₂ [5]. For example, TNFR1 by its interaction with TNFR-associating factors and various adaptor proteins can activate PKCs, MAPK, p38MAPK and c-Jun N-terminal kinase (JNK; all of which have been implicated in the phosphorylation and stimulation of cPLA₂). Recent evidence, however, has indicated that TNFR2 does not efficiently stimulate MAPK or p38MAPK, but only activates JNK pathways [18], which probably do not contribute to cPLA₂ phosphorylation [5]. Therefore our aim was to determine the ability of both TNFR subtypes in activating cPLA₂ enzyme and determining the signalling pathways by which these events occurred.

MATERIALS AND METHODS

Materials

Recombinant human TNF was purchased from R & D Systems (Abingdon, Oxfordshire, U.K.). Cytokine's biological activity was confirmed by measurement in the L929 cytotoxicity assay by comparing cytotoxic activity of TNF standards (kindly provided by Dr Meenu Wadhwa, National Institute of Biological Standards and Controls, Potters Bar, Herts. U.K.). Each batch of TNF was confirmed to have at least 2×10^7 WHO units/mg. Subtype-specific polyclonal and monoclonal agonistic antisera were a gift from Wim Buurman (University of Maastricht, The Netherlands). Anti-human cPLA₂ antisera [19] were obtained from The Binding Site Limited (Birmingham, U.K.). Phospho-specific MAPK and p38MAPK antisera were purchased from New England Biolabs (Hitchin, Herts., U.K.). Pan MAPK and p38MAPK antisera used as a control were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). [5,6,8,9,11,12,14,15-³H(N)]arachidonic acid ([³H]arachidonic acid) and stearoyl-arachidonoyl-[¹⁴C]phosphatidylcholine ([¹⁴C]-SAPC) were purchased from Amersham Biosciences. Compounds PD98059 and SB203580 were obtained from Merck Biosciences Ltd (Nottingham, U.K.). Caspase inhibitors were acquired from Bachem (U.K.) Ltd (St. Helens, U.K.). All other materials were from BDH-Merck Ltd (Poole, Dorset, U.K.) or from the Sigma Chemical Co. (Poole, Dorset, U.K.) and were of the highest grade obtainable.

Cell culture

HeLa cells were co-transfected with the cDNA that encodes human TNFR2 (gift from Werner Lesslauer, Yale University, New Haven, CT, U.S.A.) plus the pBABE Hygromycin-resistance cDNA vector. The resulting stable clone HeLa-TNFR2 was generated as described in [18]. Cells were grown in Dulbecco's modified Eagle's medium with 10% (v/v) foetal bovine serum, 1 mM L-glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin supplemented with 100 µg/ml Hygromycin B (Roche Diagnostics Ltd, Lewes, E. Sussex, U.K.).

[³H]Arachidonic acid release

Cells were grown to confluency in 24-well culture plates and analysed for [³H]arachidonic acid release essentially as described in [13]. Each cell was then isotopically labelled in serum-free medium supplemented with 0.5 µCi/ml [³H]arachidonic acid (specific activity of 12 Ci/mmol). Cells were left to incorporate the [³H]arachidonic acid into their membrane lipids for 13–14 h,

after which the radioactive medium was discarded, followed by four washes with serum-free medium. Cells were then incubated with the indicated concentrations of drugs for 1 h in a total volume of 1 ml of serum-free medium per well. After treatment, the medium from each well was carefully removed with a Pasteur pipette to avoid disrupting any cell from the plates. The medium was centrifuged at 1600 g for 3 min at 4 °C to pellet any detached cells, and assessed for [³H]arachidonic acid release by liquid-scintillation counting.

Phosphatidylcholine-vesicle assay

The cPLA₂ activity was also assessed using phosphatidylcholine vesicles. HeLa-TNFR2 cells were grown to confluence in 6-well plates and then detached using cell-dissociation solution. The detached cells were spun down in a bench-top centrifuge and then washed once in PBS to remove traces of the medium and dissociation solution. The cells were pelleted once again and lysed by sonication in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 0.2 mM PMSF, 2 µg/ml leupeptin and 1 mM bezamidine. After sonication, the protein content of the total cell lysates was measured and adjusted. The enzymes being assayed therefore comprise both the non-phosphorylated and phosphorylated forms from the total cell lysate (membranes and cytosol). Equal amounts of protein samples were then incubated in a reaction buffer [1 mM Tris (pH 8.8)/50 mM CaCl₂] to a final volume of 89 µl. For each reaction, 1 µl of [¹⁴C]-SAPC was dissolved in 1 µl of DMSO. After the addition of 10 µl of reaction buffer, the mixture was placed in an ultrasonication water bath for 5 min at 37 °C, to allow the formation of phosphatidylcholine vesicles.

The cell lysates and reaction buffer mixtures were then supplemented with 11 µl of phosphatidylcholine vesicles, and incubated for 30 min at 37 °C. cPLA₂ within the cell lysates liberates [¹⁴C]arachidonic acid from the radiolabelled phospholipid, which can then be measured as a function of the activity of cPLA₂. Each reaction was performed in duplicate, and each experimental set contained a control containing the reaction buffer only.

[¹⁴C]Arachidonic acid was extracted from lysate mixtures by the method of Dole and Meinertz [20]. To each lysate, 500 µl of extraction buffer (0.1 vol. H₂SO₄/1 vol. heptane/4 vol. propan-2-ol) was added. The mixture was shaken vigorously and incubated at room temperature (25 °C) for 5 min. After this period, 200 µl of distilled water and 300 µl of heptane were again added. The mixture was again shaken to allow thorough mixing and then left for 5 min when a phase separation was visible. From the upper phase containing the [¹⁴C]arachidonic acid, 300 µl was removed and added to a clean test tube. Next, 50 mg of silica gel was added and the mixture was shaken and then pelleted by centrifugation. The supernatant (200 µl) was assessed for ¹⁴C radioactivity by liquid-scintillation counting.

Western-blot analysis

HeLa-TNFR2 cells were analysed for phospho-specific MAPK and p38MAPK activities by Western blotting as described previously [18]. The phosphorylation-induced shift in cPLA₂ mobility was assessed by Western blotting of samples that were treated the same way, except that they were electrophoresed on a full-length Hoeffer gel unit (1.5 mm spacers) by SDS/PAGE [8% (w/v) resolving gel at pH 8.8 rather than at pH 8.4]. Phosphorylated and non-phosphorylated forms of protein were separated by electrophoresis for 5 h at 200 V to separate completely the two forms of cPLA₂ proteins, which were then

visualized by cPLA₂ (1:100) primary antisera and Protein-G/horseradish peroxidase (1:1000) secondary antisera followed by *o*-dianisidine/H₂O₂ staining.

Confocal laser scanning fluorescence microscopy

Cells were fixed with ice-cold methanol for 20 s and washed with 2 ml of PBS (pH 7.2) before labelling with anti-cPLA₂ polyclonal antibody as described in [21] at a 1:100 dilution in Krebs solution [137.4 mM NaCl/5.9 mM KCl/1.2 mM CaCl₂ · (6H₂O)/1 mM MgCl₂ · (6H₂O)/11.6 mM Hepes/11.5 mM glucose] on a rocking platform for 60 min. Primary antibody was removed and replaced by a 1:100 dilution of FITC-labelled anti-sheep/goat antibody. Cells were incubated in the dark at room temperature for a further 60 min on a rocking platform and washed three times in Krebs solution before visualization of the labelled antibody. Confocal laser microscopy was performed on a Bio-Rad μ radiance system measuring fluorescence at green/blue wavelengths (480–520 nm).

[Ca²⁺]_i measurements

Calcium release from internal stores on drug treatment was determined in cells using the cell-permeable calcium indicator dye fura 2 acetoxymethyl ester {fura 2/AM; 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-*N,N,N,N*-tetra-acetic acid penta-acetoxymethyl ester}, as described previously [22]. HeLa-TNFR cells were grown on 35 mm² Petri dishes to 80 % confluence. The cells were loaded with 1 μ M fura 2/AM for 1 h at room temperature in the dark. Next, the medium was removed and replaced with a covering of Hepes-buffered Krebs solution in which the stimuli were added and the ratiometric fluorescence intensity changes were assessed.

Statistical analysis

Western blots were obtained using a Bio-Rad densitometer. The image and data were statistically analysed using Instat software (GraphPAD Software for Science, San Diego, CA, U.S.A.). Comparison between group values was made by one-way ANOVA as indicated in the text. **P* < 0.05 are cited as statistically significant.

RESULTS

Release of arachidonic acid is mediated by both TNFRs

Soluble TNF primarily activates TNFR1, but is inefficient in activating TNFR2 [17]. Thus most studies investigating TNF examine the responses of TNFR1. To activate TNFR2 effectively, the receptor subtype needs to be present in sizeable amounts and agents such as agonistic antisera have proved useful [16]. HeLa cells are a useful model for studying TNF-induced cPLA₂ activation and gene responses [6,8,13,23,24]. As HeLa cells express the TNFR1 subtype almost exclusively, we used here HeLa cells that stably express substantial amounts of TNFR2 (cells termed HeLa-TNFR2 [18]) and made use of the TNFR2-specific agonistic agents R2-TNF and MR2-1 monoclonal antisera [25]. Our results have also been mainly confirmed in the KYM-1 and U937 cell lines, which express equal amounts of TNFR1 and TNFR2.

As shown in Figure 1, HeLa-TNFR2 cells release arachidonic acid in a Ca²⁺-dependent fashion, consistent with our assays measuring cPLA₂ activity [13]. The reason for raising Ca²⁺ levels in our cPLA₂ assays is to allow observable arachidonate release

in a short time period (i.e. through phosphorylation of cPLA₂ by TNFRs) and to distinguish this from the long-term effects of TNF on cPLA₂ gene expression [13], a time period that would not require increased [Ca²⁺]_i in the cPLA₂ assays. TNF caused a concentration-dependent increase in arachidonic acid release, which was more apparent in the presence of A23187 Ca²⁺ ionophore (Figure 1B). Stimulation of TNFR1 or TNFR2 individually is achieved by treatment with R1-TNF or R2-TNF + MR2-1 antisera respectively [18]. It was observed that both TNFR1 and TNFR2 were capable of stimulating cPLA₂ activity in the presence of A23187 Ca²⁺ ionophore. This was observed not only when using arachidonic acid release as a measure of cPLA₂ action (Figure 1C), but also when using a phosphatidylcholine vesicle cPLA₂ assay (Figure 1D). With these assays we found a significant stimulation of arachidonate release by both TNFR1 and TNFR2 (TNFR2 stimulation to a lesser extent, which is similar to our observations for other TNF signalling [18]). The use here of A23187 to increase [Ca²⁺]_i is to facilitate the observation of cPLA₂ function; our assays were not particularly sensitive in the absence of Ca²⁺, where enzyme activity is low and too close to the level of detection for these systems. Ideally, we would have preferred to perform the assays in the absence of Ca²⁺ but assay sensitivity would not allow it. It was also observed (Figure 1B) that significant TNF stimulation of arachidonate was seen in the absence of A23187; however, TNFR2 stimulation of arachidonate release in the absence of A23187 was not significantly increased but required the presence of A23187 to allow the observation of its stimulatory abilities (Figures 1C and 1D). It is interesting that TNFR2 was found to be less efficient than TNFR1 in activating the cPLA₂ enzyme in HeLa-TNFR2 cells, given that we found TNFR2 stimulation by itself can cause cell death in HeLa-TNFR2 cells [18] without caspase activation [26], and it may be that TNFR2 activation of cPLA₂ as observed in the present study was probably responsible for the observed TNFR2-mediated cell death.

Phosphorylation of cPLA₂ occurs through TNFR1 only

It has been demonstrated that cPLA₂ is phosphorylated in the presence of a wide variety of agonists and that this phosphorylation can correlate with increased cPLA₂ activity [5]. Since some phosphorylated proteins show altered migration patterns by electrophoresis relative to their non-phosphorylated counterparts, we analysed the mobility of cPLA₂ in response to various TNF stimuli. In cytoplasmic extracts of resting HeLa-TNFR2 cells analysed by Western blotting, cPLA₂ protein was observed as a doublet comprising two approx. equal intensity bands (Figure 2). Treatment with increasing concentrations of TNF caused a complete shift towards the upper more slowly migrating form of cPLA₂ protein, as did activation of PKC by phorbol-12,13-dibutyrate (PDBu) treatment (Figure 2A). As can be seen from the immunoblots, the antisera used were rather clean and very specific for cPLA₂ protein with no discernible major non-specific interactions.

Treatment of the HeLa-TNFR2 cells with TNFR isotype-specific agents revealed that activation of TNFR1 alone could cause the phosphorylation of cPLA₂ (Figure 2B). Stimulation of TNFR2 did not enhance the phosphorylation state of cPLA₂ protein, as judged by Western-blot analysis. We have found that TNFR2 responses could be delayed with respect to TNFR1 effects [18]. Therefore we performed a time course of TNFR1 and TNFR2 stimuli on cPLA₂ phosphorylation (Figure 2C). TNFR1 could phosphorylate cPLA₂ for treatment times between 30 and 120 min, whereas TNFR2 was incapable of phosphorylating

Figure 1 TNFR stimulation of cPLA₂ activity

Prelabelled HeLa-TNFR2 cells were treated with the indicated concentrations of (A) A23187 or (B) TNF ($\pm 0.1 \mu\text{M}$ A23187) for 1 h before determination of [^3H]arachidonic acid release as described in the Materials and methods section. (C) [^3H]Arachidonic acid labelled HeLa-TNFR2 cells were treated for 1 h with the indicated 50 ng/ml TNFs, 2 $\mu\text{g/ml}$ MR2-1 TNFR2-specific agonistic monoclonal antisera or 0.1 μM A23187 before measurement of radiolabel release. (D) HeLa-TNFR2 cells were treated with the indicated TNFR stimuli or A23187 (concentrations as indicated in C) for 1 h. The extracts were then subjected to [^{14}C]-SAPC-labelled vesicles and cPLA₂ activity was measured as described in the Materials and methods section. Data represent the means $\pm$ S.E.M., $n \geq 3$, * $P < 0.05$.

cPLA₂ even after 2 h stimulation. Therefore it is clear that, although both TNFRs are capable of stimulating cPLA₂ activity, only TNFR1 causes the phosphorylation of the enzyme.

Phosphorylation of cPLA₂ occurs through MAPK activation

A recent study of ours on HeLa-TNFR2 cells [18] indicated that TNFR2 activates JNK but does not activate MAPK or p38MAPK pathways; however TNFR1 activated all three of these kinase pathways. Since TNFR2 does not cause the apparent phosphorylation of cPLA₂ (Figure 2), it appears that JNK does not phosphorylate cPLA₂ in HeLa cells. Both MAPK and p38MAPK (activated only by TNFR1) have been shown to phosphorylate directly cPLA₂ in various cell types [24,27–34]. Our evidence here using pharmacological tools influencing MAPK and p38MAPK indicate that both kinases are activated by soluble TNF (through TNFR1), as judged by phospho-specific antisera (Figure 3). Anisomycin and PDBu have been used as positive controls for p38MAPK and MAPK respectively. It is clear from these stimuli that only activation of MAPK by PDBu led to the phosphorylation of cPLA₂, whereas p38MAPK activation by anisomycin did not cause the phosphorylation of cPLA₂ in our cells. Use of the MAPK kinase-1 inhibitor PD98059 was able to block TNF-induced MAPK activation completely, as expected (Figure 3C). PD98059 completely blocked the basal or TNF-induced phosphorylation of cPLA₂ (Figure 3A) also, indicating that cPLA₂ phosphorylation

is mediated by MAPK. The p38MAPK inhibitor SB203580 was efficient in blocking TNF-induced p38MAPK in our cells (Figure 3B), but the inhibition of p38MAPK by SB203580 did not prevent TNF-induced cPLA₂ phosphorylation (Figure 3A). These results were obtained from studies using larger concentrations of these inhibitors (results not shown). Therefore it appears that TNF-induced MAPK activation led to the phosphorylation of cPLA₂, but the TNF-induced p38MAPK activation did not contribute to the phosphorylation of cPLA₂ seen by TNF.

Stimulated translocation and cleavage of cPLA₂

Experiments using confocal microscopy to assess the subcellular distribution of cPLA₂ indicate that TNF causes a redistribution of cPLA₂ enzyme to perinuclear regions of the cell (Figure 4A), as has been observed previously in other cell types [23,35–38]. Moreover, there is a reduction in cPLA₂ immunointensity, which is probably due to some TNF-stimulated caspase-dependent cleavage of cPLA₂ protein as reported by others. It appears that the reduction in cPLA₂ immunoreactivity is indeed caspase-dependent as inclusion of the broad-spectrum caspase inhibitor zVAD-fmk stops the TNF-induced reduction in cPLA₂ immunoreactivity without influencing stimulated subcellular redistribution of cPLA₂ (Figure 4A, lower panels). It is not clear why the observed reduction in cPLA₂ immunoreactivity seen by immunocytochemistry was not observed in Western blotting. This

Figure 2 TNFR stimulation of cPLA₂ phosphorylation

Western-blot analysis of cPLA₂ phosphorylation state in HeLa-TNFR2 cells treated (A) with the indicated concentrations of TNF or PDBu. (B) HeLa-TNFR2 cells were treated for 1 h with the indicated 50 ng/ml TNFs, 2 μg/ml MR2-1 or 0.1 μM PDBu stimulus. (C) Time course of TNFR1- or TNFR2-stimulated (50 ng/ml R1-TNF or 50 ng/ml R2-TNF plus 2 μg/ml MR2-1 respectively) HeLa-TNFR2 cells, treated for the indicated times. Representative blots are shown from an experiment repeated at least three other times independently with similar findings. con, control; wt, wild type.

may be due to the presence of an epitope that is masked in immunostaining but not masked after denaturation for Western blotting. Inhibition of caspases by zVAD-fmk does not influence TNF-induced phosphorylation of cPLA₂ (Figure 4B), which is in agreement with MAPK (but not JNK) being responsible for cPLA₂

Figure 4 Caspase sensitivity of the TNF-induced cPLA₂ subcellular redistribution and phosphorylation

(A) Confocal analysis of immunoreactive cPLA₂ subcellular distribution in HeLa-TNFR2 cells pretreated for 1 h with 20 μM zVAD-fmk and then treated for 1 h with 50 ng/ml TNF where indicated. (B) cPLA₂ Western-blot analysis of HeLa-TNFR2 cells treated the same way as in (A). Representative results from an experiment repeated at least two other times independently with similar findings. con, control.

Figure 3 MAPK and p38MAPK stimulation of cPLA₂ phosphorylation

Western-blot analysis of HeLa-TNFR2 cells treated with 50 ng/ml TNF, 200 nM anisomycin or 0.1 μM PDBu stimulus. Where indicated, cells were pretreated for 1 h with 5 μM PD98059 or 5 μM SB203580 before stimulating for 30 min (1 h in cPLA₂ experiments). Cell extracts were subsequently analysed by Western blotting for (A) cPLA₂ phosphorylation or (B) p38MAPK or (C) MAPK activation. (D) Histogram of the pooled analysed data represents the means ± S.E.M., $n \geq 4$, * $P < 0.05$. con, control.

Figure 5 TNFR- and ionomycin-induced cPLA₂ subcellular redistribution

Confocal analysis of immunoreactive cPLA₂ subcellular distribution in HeLa-TNFR2 cells treated for 1 h with 50 ng/ml TNFs, 2 μ g/ml MR2-1 or 0.1 μ M ionomycin where indicated. Representative results from an experiment repeated at least three other times independently with similar findings.

phosphorylation, as another study in HeLa-TNFR2 cells had shown TNF-induced JNK activity (but not MAPK or p38MAPK activities) to be influenced by caspase 3 and caspase 6 [26]. Western blots did not show an immediate reduction in whole-cell cPLA₂ immunoreactivity at the treatment times shown here (0.5–1 h); however, a reduction in cPLA₂ immunoreactivity in HeLa-TNFR2 cells became apparent in Western blots after 4 h of TNF treatment [13].

As shown in Figure 5, stimulation of TNFR1 by R1-TNF stimulates the redistribution and caspase-dependent cleavage of cPLA₂ protein, similar to the effects of wild-type TNF. Curiously, stimulation of TNFR2 by R2-TNF plus MR2-1 agonistic monoclonal antibody stimulus does not result in the caspase-dependent cleavage of cPLA₂, but this receptor subtype can cause the subcellular redistribution of the enzyme. A Ca²⁺ stimulus brought about by ionomycin Ca²⁺ ionophore treatment results in the subcellular redistribution and translocation of cPLA₂ to perinuclear regions of HeLa-TNFR2 cells.

Role of Ca²⁺

Since the translocation of cPLA₂ is a Ca²⁺-sensitive process [39], we investigated the effects of TNFR stimulation on cytosolic Ca²⁺

Figure 6 TNFR regulation of [Ca²⁺]_i in HeLa-TNFR2 cells

Measurement of [Ca²⁺]_i in fura 2/AM-loaded HeLa-TNFR2 cells treated at zero time with TNFR1-specific (50 ng/ml R1-TNF) or TNFR2-specific (50 ng/ml R2-TNF and 2 μ g/ml MR2-1) stimuli. Where indicated 0.1 μ M ionomycin was applied to the cultured cells. Dashed lines indicate plateau levels in TNFR1- and TNFR2-stimulated samples. The results are from a representative experiment repeated at least two other times independently with similar findings.

Figure 7 Time course of TNFR2 stimulation of cPLA₂ phosphorylation in the presence of A23187

Western-blot analysis of cPLA₂ phosphorylation state in HeLa-TNFR2 cells treated for the indicated times with 0.1 μ M A23187 or 0.1 μ M A23187 and 50 ng/ml R2-TNF plus 2 μ g/ml MR2-1. A representative blot is shown from an experiment repeated at least two other times independently with similar findings.

concentrations in HeLa-TNFR2 cells. Stimulation of most cell types by TNF did not result in any discernible increase in [Ca²⁺]_i [22]. It is true also of TNFR1 stimulation in HeLa-TNFR2 cells (Figure 6). However, to our surprise, TNFR2 stimulation resulted in a consistent, delayed, slight increase in [Ca²⁺]_i levels approx. 30 min after TNFR2 stimulation (Figure 6B). This increase in [Ca²⁺]_i levels was extremely modest (< 50 nM) and was sustained throughout the rest of the experiment. Whether this increase in Ca²⁺ levels is enough to allow the observed cPLA₂ redistribution (Figure 5), or whether it is a consequence of some other TNFR2-mediated signalling effect, is not clear. cPLA₂ requires Ca²⁺ for activity and TNFR signalling does not lead to any significant increase in [Ca²⁺]_i in cell types used in the present study; however, the basal resting levels of [Ca²⁺]_i (> 100 nM; Figure 6) were sufficiently elevated basal Ca²⁺ levels to allow cPLA₂ activity to occur. Increasing Ca²⁺ levels with A23187 in our cPLA₂ assays was to allow observable arachidonate release in a short time period (i.e. through phosphorylation of cPLA₂ by TNFRs) and to distinguish this from the long-term effects of TNF on cPLA₂ gene expression (a time period that would not require A23187-increased [Ca²⁺]_i in the cPLA₂ enzyme activity assays).

Since Ca²⁺ seemed to play an important role in TNFR2-mediated cPLA₂ activity, we investigated whether increased [Ca²⁺]_i may reveal TNFR2-stimulated phosphorylation of cPLA₂. As can be clearly seen from Figure 7, increasing [Ca²⁺]_i levels

with A23187 did not by itself cause phosphorylation of cPLA₂. Nor did the presence of the calcium ionophore unveil any TNFR2-stimulated phosphorylation of cPLA₂ protein as both Ca²⁺ and TNFR2 activation did not affect the phosphorylation status of the lipase.

DISCUSSION

The present study demonstrates that both TNFR subtypes are capable of activating cPLA₂. The activation of cPLA₂ by TNFR1 is by the previously defined pathways described by others [5]. In other words, TNFR1 causes the activation of MAPK, which phosphorylates cPLA₂ at its Ser⁵⁰⁵ or Ser⁷²⁷ motifs and enhances activation of the lipase in a Ca²⁺-dependent fashion. As part of the activation profile of cPLA₂, it was seen to translocate to membranous and perinuclear regions, where it encounters its substrate arachidonyl-containing phosphatidylcholine. We found that such a translocation of cPLA₂ occurred when TNFR1 was stimulated. TNFR1 was also capable of activating the destructive caspase proteases, which was observed in the present study as an apparent reduction of cPLA₂ immunoreactivity through presumed cleavage. In contrast, TNFR2 stimulation led to the redistribution and translocation of cPLA₂, without the observation of phosphorylation or caspase cleavage of cPLA₂ protein. TNFR2 activation did, however, cause significant activation of cPLA₂ enzyme activity (Figure 1). Therefore significant stimulation of cPLA₂ activity did not require Ser⁵⁰⁵ phosphorylation or caspase cleavage of the enzyme, as TNFR2 did not signal for these events but still achieved a marked activation of cPLA₂ enzyme.

Activation of kinases by TNFs that are capable of phosphorylating cPLA₂ enzyme suggests that our results are in keeping with the current thinking on kinase stimulation by TNFRs [16]. Kinases that have been shown to phosphorylate and activate cPLA₂ are MAPK, p38MAPK and PKC [5]. Phosphorylation of cPLA₂ by p38MAPK has been observed in platelets [30–32,40], but it is not clear whether this form of kinase stimulation of cPLA₂ is a general phenomenon or restricted to such cells, especially since TNF-induced cPLA₂ phosphorylation in platelets may be MAPK-independent [33]. We do not see an effect of TNF-stimulated cPLA₂ phosphorylation and its pharmacological manipulation that is consistent with a role for p38MAPK (Figure 3). We do, however, observe a role for MAPK in the TNFR1-stimulated phosphorylation of cPLA₂. Such phosphorylation of cPLA₂ by MAPK occurs through TNFR1 activation but not through TNFR2 activation, entirely consistent with our previous findings in HeLa-TNFR2 cells in which only TNFR1, but not TNFR2, was capable of MAPK and p38MAPK activation [18]. A unique death domain-containing adapter molecule, MAPK-activating DD protein, links TNFR1s to the MAPK subfamily of kinases and cPLA₂ [41]. Both TNFRs can efficiently activate JNK, but a role for this kinase in TNFR-stimulated cPLA₂ activity is unlikely, as cPLA₂ phosphorylation by JNK has not been observed and also the shift in lipase phosphorylation state (Figure 2) is through a MAPK-selective site. There may be a TNFR2-stimulated phosphorylation of cPLA₂ that we do not measure in our Western blot analyses and such a phosphorylation that causes the lipase to be activated cannot be ruled out. Likewise, a role for TNFR1- or TNFR2-activated PKC phosphorylation of cPLA₂ cannot be ruled out, as we would not observe PKC-specific phosphorylation by Western blotting of cPLA₂, and TNFR2-stimulated PKC activity has not been reported. Moreover, increasing of [Ca²⁺]_i does not unveil TNFR2-mediated phosphorylation of cPLA₂ protein (Figure 7), suggesting that some other mechanism is probably responsible for TNFR2-stimulated cPLA₂ activity.

Another aspect of TNF signalling that is mainly mediated by TNFR1 and not by TNFR2 is the activation of caspase proteases [16]. Indeed, upstream initiator caspase-8 is directly linked to TNFR1 (through TRADD and FADD) via its death domain motif, a sequence not present in TNFR2. Whether caspase-mediated cleavage of cPLA₂ is responsible for enhancing or decreasing cPLA₂ activity is not clear as conflicting reports have been found [7–12]. Our observed reduction in cPLA₂ immunoreactivity is consistent with a caspase-mediated effect (sensitive to a broad-spectrum caspase blocker), which is seen with the TNFR1 stimulus only.

Although TNFR2 does not cause the caspase-mediated cleavage of cPLA₂, it does cause the subcellular redistribution of cPLA₂ protein (Figure 5). Maximal activation of cPLA₂ proceeds by a two-step mechanism [5]. Activation by kinase phosphorylation, with maximal activation requiring MAPK phosphorylation at Ser⁵⁰⁵, plus elevated calcium concentrations allow the C2-calcium/lipid-binding domain to signal the lipase to be targeted towards the plasma membrane and perinuclear subcellular regions. Since we observed redistribution of cPLA₂ by TNFR2 stimulation, we investigated the role of calcium in TNFR2-stimulated HeLa-TNFR2 cells (Figure 6). We observed previously that TNF stimulation does not affect calcium concentrations [22]. This is due to weak activation of TNFR2 by soluble TNFs [17,42]. To activate TNFR2 efficiently, we have used the most effective combination of R2-TNF and MR2-1 agonistic monoclonal antisera [18]. In HeLa-TNFR2 cells, TNFR2 stimulation leads to a small but consistent increase in [Ca²⁺]_i of < 50 nM (Figure 6). Whether this is due to a TNFR2-activated increase in Ca²⁺, or is merely a consequence of TNFR2-stimulated cPLA₂ redistribution, is not clear, but it is of interest since TNFRs are not normally associated with the regulation of Ca²⁺.

The role of TNFR1 and TNFR2 signalling in a variety of TNF-mediated responses, including apoptosis, is yet to be determined [16,43]. TNFR1 undoubtedly causes death under certain circumstances, but the role of TNFR2 in cell death or proliferation, or the role of TNFR1 in cellular responses other than cell death, is not clear [16]. It is clear that cPLA₂ plays a role in certain types of cell death induced by TNF and other cytotoxic agents [7,10,44–50]. Indeed, in the present study, a cPLA₂ inhibitor caused TNF-induced apoptotic cell death in HeLa-TNFR2 cells (results not shown). Death probably involves the cPLA₂-controlled conversion of arachidonic acid into leukotriene products and eventually reactive oxygen species that damage the cell and signal its nucleus to initiate cytotoxic proceedings [51–53]. Another possibility may be that cPLA₂ action stimulates ceramide generation, which also contributes to the cytotoxic response, as was found to be the case with the L929 cell line treated with TNF [54].

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