Cell Death and Differentiation (1997) 4, 479–486

Inhibitors of arachidonic acid metabolism reduce DNA and nuclear fragmentation induced by TNF plus cycloheximide in U937 cells

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Received 29.11.96; revised 6.3.97; accepted 10.4.97
Edited by P. Cohen

Abstract

U937 human myeloid leukemia cells are induced to apoptosis by tumour necrosis factor (TNF) plus cycloheximide (CHX). We have analysed the effect of various inhibitors of the arachidonic acid (AA) metabolism on several features of this process. The formation of high molecular weight and oligonucleosomal DNA fragments as well as nuclear fragmentation were reduced by inhibitors of 5-lipoxygenase (BWA4C and BWB70C), 5-LO activating protein (MK-886), and cytosolic PLA_2 ($AACOCF_3$). None of these agents blocked the morphological changes detected by microscopy or flow cytometry, phosphatidylserine exposure on the cell surface or Caspase 3-like activation. AA also induced nuclear fragmentation at a concentration of 1–20 μM. However, the mechanisms by which these inhibitors act, remain unexplained since there was no 5-LO expression in the U937 cells and no AA release followed their stimulation with TNF plus CHX.

Keywords: arachidonic acid, cytosolic phospholipase A_2, 5-lipoxygenase, TNF, U937, apoptosis

Abbreviations: MW, molecular weight; CHX, cycloheximide; TNF, tumour necrosis factor, 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase activating protein; $cPLA_2$, cytosolic PLA_2; PS, phosphatidylserine; AA, Arachidonic acid; ICE, interleukin-1β converting enzyme; 5-HPETE, 5-hydroperoxy-eicosatetraenoic acid; 5-HETE, 5-hydro-eicosatetraenoic acid LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; PGH-1, prostaglandin-1 synthase or cyclo-oxygenase-1; PGH-2, prostaglandin-2 synthase or cyclo-oxygenase-2; DEVD-AMC, Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin; DEVD-CHO or aldehyde, Ac-Asp-Glu-Val-Asp-CHO

Introduction

Tumour necrosis factor (TNF, also known as cachectin or TNF_α) is a polypeptide mediator of inflammation and cellular immune responses (Aggarwal and Natarajan, 1996). Upon binding to its cell surface receptor, TNF initiates apoptosis and the transcription of protective genes by processes that are not yet well understood (Beyaert and Fiers, 1994). Apoptosis is an intrinsic suicide mechanism that occurs as consequence of physiological or pathological processes (Fraser and Evan, 1996). It can be induced by diverse stimuli which cause characteristic morphological and biochemical changes leading to cell death (Steller, 1995).

TNF induces the activation of interleukin-1β-converting enzyme (ICE)-like proteases such as CPP32 (caspase 3) which appear to be essential for the cell death mechanism (Tewari *et al*, 1995; Zhivotovsky *et al*, 1996). TNF also induces a number of cell signals that include activation of cytosolic Ser/Thr protein kinases (Beyaert and Fiers, 1994), and phospholipase A_2 (PLA_2) (Neale *et al*, 1988; Hollenback *et al*, 1992; McEwan, 1996), ceramide production and superoxide radical generation (Aggarwal and Natarajan, 1996). The ability of TNF to induce AA release and activate the enzymes involved in its metabolism, has prompted the suggestion that this pathway may be an important regulator of cell survival (Schutze *et al*, 1992; Tang *et al*, 1996; Voelkel-Johnson *et al*, 1996).

Arachidonic acid (AA) metabolism begins with $cPLA_2$ catalysing the release of AA from cell membrane phospholipids (Dennis, 1994). 5-Lipoxygenase (5-LO) catalyses the conversion of AA to 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) which can be degraded either to 5-hydroxyeicosatetraenoic acid (5-HETE) or leukotriene A_4 (LTA_4), that can then be metabolised to LTB_4. Other important contributors to AA metabolism include 5-LO activating protein (FLAP), which is required for optimal cellular leukotriene synthesis (Claesson *et al*, 1992) and prostaglandin (PGH) synthases (also known as cyclo-oxygenases) which catalyse the conversion of AA to prostaglandins and thromboxanes (Hamberg *et al*, 1975). The effect of various PLA_2, lipoxygenase (LO) and prostaglandin synthase (PGH-synthase) inhibitors is unclear, since they have been associated both with induction of (Anderson *et al*, 1993, 1995; Tsujii and Dubois, 1995; Tang *et al*, 1996) and protection from apoptosis (Chang *et al*, 1992; Agarwal *et al*, 1993; O'Donnell *et al*, 1995).

To investigate the link between AA metabolism and apoptosis, we have studied the events that occur when U937 cells are exposed to TNF plus cycloheximide (CHX). Previous studies have used non-specific inhibitors such as NDGA, which is an antioxidant that can inhibit both LO and

PGH synthases (Chang *et al*, 1992; O'Donnell *et al*, 1995). Instead, we have studied the effect of the until now considered specific 5-LO inhibitors BW A4C and BW B70C. We have also used MK-886, a FLAP inhibitor that interferes with substrate binding to 5-LO (Gillard *et al*, 1989; Ford-Hutchinson *et al*, 1994), and $AACOCF_3$, a specific inhibitor of $cPLA_2$ (Riendeau *et al*, 1994). Here, we report that these compounds inhibit nuclear but not cytoplasmic features of apoptosis induced by TNF plus CHX in U937 cells.

Results

Induction of apoptosis

TNF plus CHX induced features of apoptosis in U937 cells, occurring simultaneously with trypan blue exclusion over a 5 h time course. U937 cells were analyzed by FACS (Figure 1). Changes in forward scatter (FSC) and side scatter (SSC) were apparent after 2 h incubation with TNF plus CHX. The number of cells in gate 1 (to the right) decreased, with correspondent increasing values in gate 2 (to the left), which included cell fragments and cells with diminished size (Figure 1A). From 10 000 cells that were originally in gate 1, there was a decrease to 5956 ± 1575 cells at 5 h, which was significantly different ($n=4$; $P<0.05$) when compared to cells incubated with ethanol alone. However, the effect of incubating U937 cells with TNF plus CHX and the inhibitors was not different from the effect induced by TNF plus CHX alone (Figure 1B).

PS exposure

PS exposure was detected using FITC-Annexin binding assay, after 2 h treatment of U937 with TNF plus CHX. Again, no inhibitory effect on annexin binding was obtained when TNF plus CHX-stimulated cells were incubated in the presence of the inhibitors as illustrated with AACOCF3 in Figure 2.

Caspase 3-like activity

TNF plus CHX stimulated an increase in Caspase 3-like enzyme activity in U937 cells after 90 min (Table 1), which is inhibitable by DEVD-CHO, a specific inhibitor of Caspase 3-like enzymes (Vanags *et al*, 1996). Inhibitors of AA metabolism were incubated with U937 cells in culture stimulated with TNF and CHX, but none of the inhibitors had a significant effect on Caspase 3-like enzyme activity (Table 1).

5-LO, FLAP and $cPLA_2$ inhibitors reduce nuclear and DNA fragmentation

TNF plus CHX induced nuclear fragmentation which was increased over the 5 h time course (Figure 3). However, the

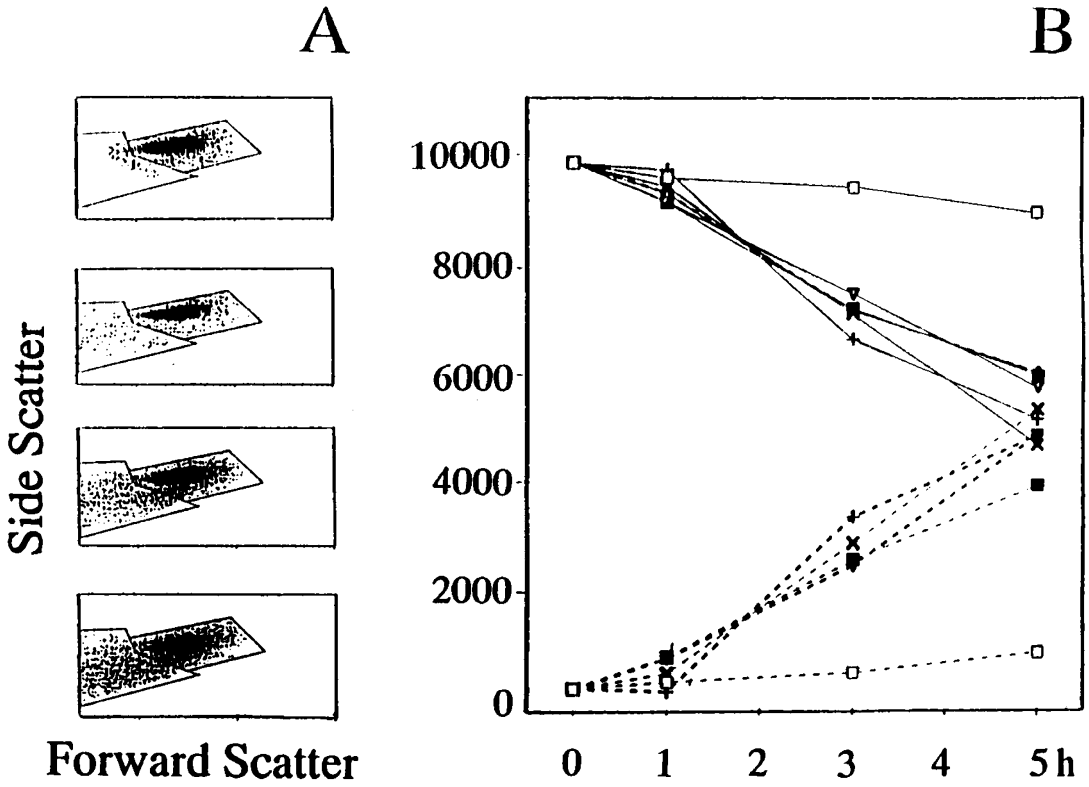


Figure 1 Cellular fragmentation and shrinkage. U937 cells were collected at different time points during incubation with TNF (12 ng/ml) plus CHX (1 μg/ml), and analyzed by flow cytometry. (**1A**) shows dot plots corresponding to 0, 1, 3 and 5 h (from top to bottom). 10 000 cells were analyzed by linear FSC *vs* logarithmic SSC. Gate 1 (to the right) includes cells with the initial morphology, while Gate 2 (to the left) includes cells with altered size and density. (**1B**) shows cell numbers in Gate 1 (continuous lines) and Gate 2 (discontinuous lines) during the 5 h incubation with ethanol (□) or TNF plus CHX, alone (■) or in the presence of BW A4C (X), BW B70C (△), MK-886 (+), and $AACOCF_3$ (▽).

number of cells with nuclear fragmentation was decreased when they were incubated in the presence of 5-LO, FLAP and $cPLA_2$ inhibitors. This inhibition was dose dependent, with maximal effect at 10 μM concentration of each one of the inhibitors ($P<0.05$, Figure 4).

Treatment with TNF plus CHX also induced the formation of high MW and oligonucleosomal DNA fragments. DNA fragmentation was inhibited when the cells were incubated in the presence of BWA4C, BWB7OC, and MK-886 in a range of concentrations between 0.1 and 10 μM (Figure 5A and B). High MW DNA fragments were decreased in the presence of 1 μM $AACOCF_3$, but not in the presence of ethanol alone. Incubating the cells with TNF, CHX or the inhibitors alone did not result in DNA fragmentation (not shown).

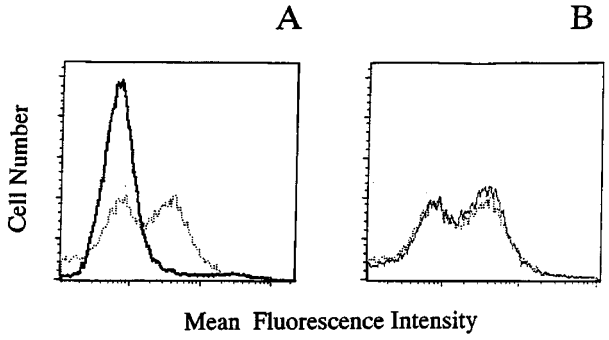


Figure 2 PS exposure on the surface of U937 cells. (**A**) Annexin V-FITC binding from U937 cells not treated (continuous line) or treated with TNF plus CHX (dotted line). (**B**) Annexin V-FITC binding of TNF plus CHX treated cells in the presence (continuous line) or in the absence (dotted line) of $AACOCF_3$.

Table 1 DEVD-AMC cleavage in U937 cells

	AMC released (pmoles/min)
Medium	2.0±0.5
Ethanol	3.0±1.0
TNF plus CHX	24.0±12.0
TNF plus CHX and BW A4C	26.0±11.0
TNF plus CHX and BW B70C	21.0±11.0
TNF plus CHX and MK-886	24.0±13.0
TNF plus CHX and $AACOCF_3$	20.0±9.0

DEVD-AMC cleavage was assessed in a kinetic assay monitoring pmol of AMC released over time. V_0 maximum (pmoles/min) was estimated from linear regressions ($r^2>0.99$) after U937 cells were incubated with the inhibitors (10 μM). Data are mean±s.e. of three experiments done in triplicate.

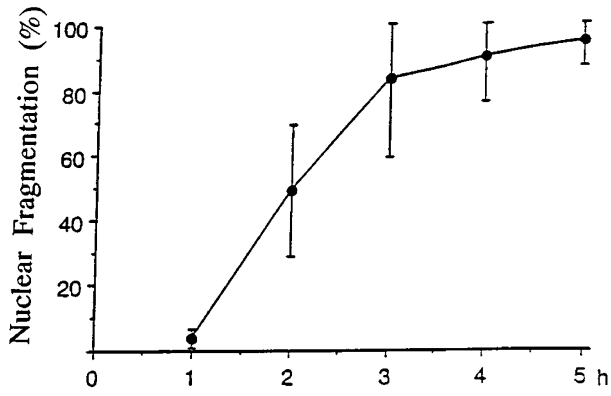


Figure 3 Percentage of nuclear fragmentation induced in U937 cells during 5 h time course incubation with TNF (12 ng/ml) plus CHX (1 μg/ml).

Enzyme expression by RT–PCR

The expression of the key enzymes for AA degradation was analysed at transcriptional level. Our U937 cell clone was positive for β actin, $cPLA_2$, FLAP, LTA_4 and PGH-1

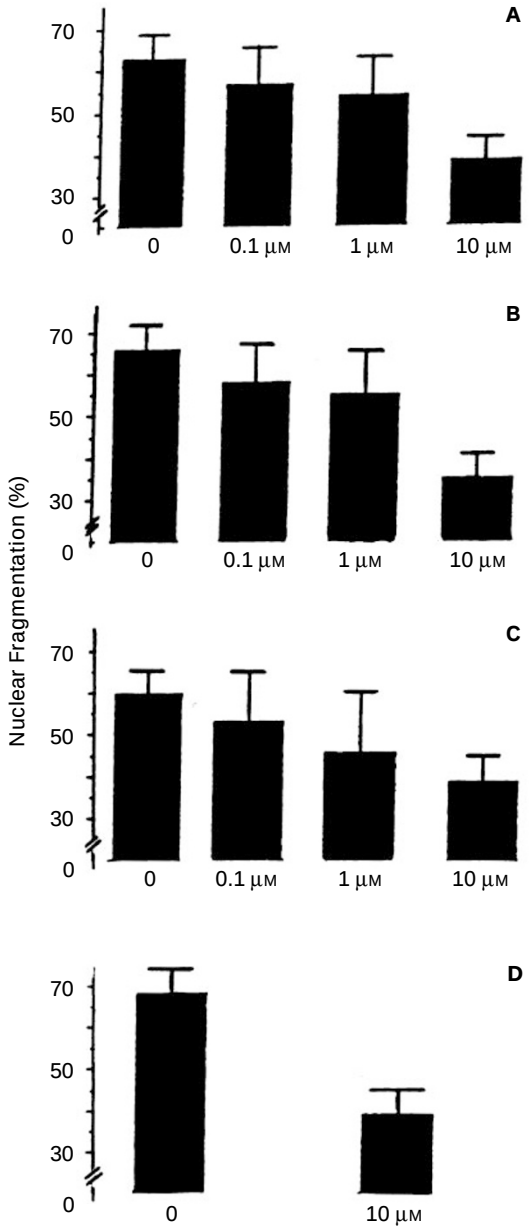
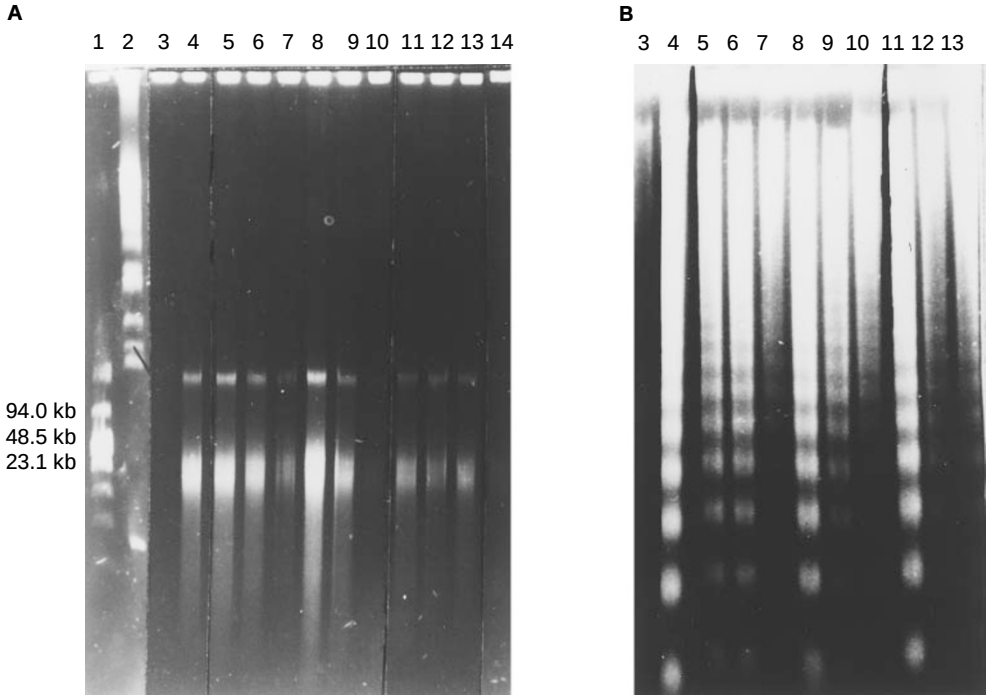


Figure 4 Percentage of nuclear fragmentation in U937 cells induced by TNF (12 ng/ml) plus CHX (1 μg/ml) after 3 h in the presence of BW A4C (**A**), BW B70C (**B**), MK-886 (**C**), or $AACOCF_3$ (**D**). Inhibition reached statistical significance ($n=4$, $P<0.05$) with every compound at 10 μM concentration.



>Figure 5 Analysis of high MW (**A**) and oligonucleosomal (**B**) DNA fragments. Lane 1 corresponds to MW marker from 0.1 to 200 kb, lane 2 to MW marker from 225 to 2200 kb, and lane 3 to U937 cells incubated with ethanol 0.1%. U937 cells were incubated with TNF (12 ng/ml) plus CHX (1 μg/ml) alone (lane 4), or in the presence of BW A4C (lanes 5, 6, 7), BW B70C (lanes 8, 9, 10), or MK-886 (lanes 11, 12, 13) at 0.1 μM, 1 μM and 10 μM. U937 were also incubated with TNF plus CHX and 1 μM $AACOCF_3$ (lane 14).

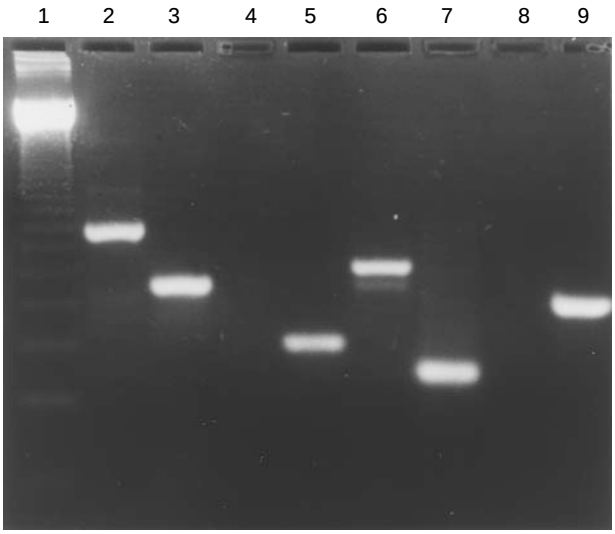


Figure 6 Analysis of cDNA from U937 cells by RT–PCR. Lane 1 corresponds to a 123 bp molecular marker, and the following lanes correspond to β actin (2), $cPLA_2$ (3), 5-LO (4), FLAP (5), LTA_4 hydrolase (6), PGH-1 synthase (7) and PGH-2 synthase (8). Positive amplification of the BL41-E95-A lymphoma cell line for 5-LO is shown in lane 9.

synthase, whereas it was negative for 5-LO and PGH-2 synthase (Figure 6). There was no 5-LO protein detected by Western blot and no leukotriene synthesis was found by HPLC (not shown). Positive 5-LO amplification was obtained with cDNA from the BL41-E95-A lymphoma cell line (Figure 6). Positive amplification for PGH-2 was obtained with cDNA from normal human leukocytes and from human umbilical cord vein endothelial cells (not shown).

$cPLA_2$ inhibition by BW A4C, BW B70C, MK-886 and $AACOCF_3$

BW A4C and BW B70C, as well as MK-886, are considered selective inhibitors for 5-LO (Garland and Hodgson, 1994) and FLAP, respectively (Ford-Hutchinson *et al*, 1994). However, we found that the three compounds partially inhibited $cPLA_2$ activity, purified approximately 500-fold from U937 cells, at the same concentrations that were effective for inhibition of apoptosis (not shown). $AACOCF_3$ was the most potent $cPLA_2$ inhibitor, allowing only $7.9 \pm 0.2\%$ of the activity from the control, when present at 1 μM concentration. $AACOCF_3$ decreased also $cPLA_2$ activity in the presence of TNF plus CHX in total cellular homogenates (Figure 7). However, we could not find an increase in $cPLA_2$ activity in the homogenate of cells stimulated with TNF plus CHX after short (0 to 10 min; Figure 7) or long time exposure (0 to 3 h; not shown). We did neither find AA release from intact U937 cells stimulated with TNF plus CHX, both in the presence or in the absence of serum (not shown).

Apoptosis induction by AA

In order to investigate whether AA itself could induce apoptosis, we incubated U937 cells with AA at various

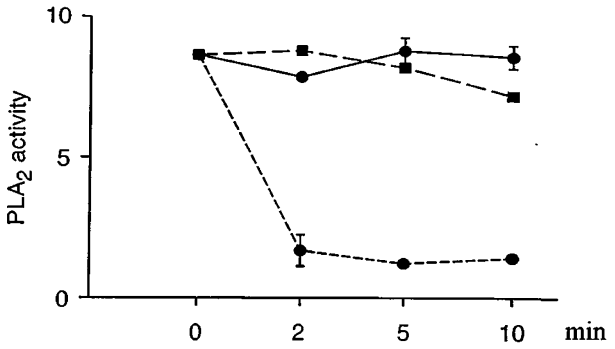


Figure 7 $cPLA_2$ activity in U937 cell homogenates. Cells were stimulated for the indicated times, and thereafter homogenised. The activity is expressed as released pmol ^{14}C-20:4/mg protein/min. Graphs correspond to $cPLA_2$ activity of untreated (●–●–●), TNF plus CHX treated (■- -■- -■) or TNF plus CHX treated cells, preincubated with 10 μM $AACOCF_3$ (●- -●- -●).

concentrations for increasing periods of time. AA induced a significant nuclear fragmentation, dose and time dependent, although not as much as that induced by TNF plus CHX (Table 2).

Table 2 Nuclear fragmentation induced by AA

	0 h	1 h	3 h	5 h
medium	5.4*	8.2	10.3	12.9
TNF+CHX		4.4	47.7	51.4
1 μM AA		2.7	11.4	14.3
10 μM AA		11.5	13.7	25.6
20 μM AA		4.6	24.4	36.5

*Percentage of cells having nuclear fragmentation, as determined by Hoechst 33342 staining. Data are from a representative experiment performed in duplicate.

Discussion

In the present study we have shown that BW A4C, BW B70C, MK-886, and $AACOCF_3$ inhibit nuclear events in U937 cells undergoing apoptosis. Since U937 cells do not produce leukotrienes and lack 5-LO (Claesson *et al*, 1993), neither 5-LO or FLAP inhibition could explain the inhibition of apoptosis by these compounds. At the concentration range used BW A4C, BW B70C and MK-886, like $AACOCF_3$ also inhibited $cPLA_2$ enzyme activity. Furthermore, we have shown that AA, the product of $cPLA_2$ activation is able to induce apoptosis in U937 cells. However, we failed in demonstrating an increase in $cPLA_2$ activity or extracellular AA release after treatment of U937 with TNF plus CHX. Although the effect of these inhibitors may help to make a distinction between nuclear and cytoplasmic events of apoptosis, their mechanisms of action remain an open question.

BW A4C and BW B70C, are analogues of AA containing an hydroxamic acid (BW A4C) or N-hydroxyurea (BW B70C) group linked to a lipophilic aryl unit. They seem to inhibit 5-LO by scavenging lipid peroxides at the active site of the enzyme (Garland and Hodgson, 1994). MK-886, a thiopyranindole structure is also considered a selective inhibitor of the 5-LO pathway and acts by binding to FLAP and inhibiting 5-LO translocation to nuclear membranes. MK886 has been described as not affecting 5-LO or PLA_2 (Gillard *et al*, 1989; Ford-Hutchinson *et al*, 1994), although we have shown that it does in fact inhibit $cPLA_2$ activity. This discrepancy might be due to different assay systems used for the determination of $cPLA_2$ activity. Mechanistically, it is likely that the lipophilic aryl moiety localises the inhibitors in the phase of the cell membrane at which $cPLA_2$ is active (Garland and Hodgson, 1994) and probably interferes directly with the $cPLA_2$ enzyme, independent of substrate concentration (Tanaka *et al*, 1992).

$cPLA_2$ plays an important role in basal phospholipid metabolism and signal transduction (Dennis, 1994). We found that BW A4C, BW B70C, MK-886 and $AACOCF_3$ partially inhibited basal $cPLA_2$ activity in U937 cells. Recent studies have implicated $cPLA_2$ in lymphocyte maturation (Feltenmark *et al*, 1995) and apoptosis (Gilbert *et al*, 1996). It is plausible that basal rather than TNF-stimulated $cPLA_2$ activity is an important homeostatic mechanism. The capacity of these compounds to inhibit apoptosis could be due to interference with basal $cPLA_2$ activity.

Although we did not find an increase in $cPLA_2$ activity or AA release, as consequence of TNF plus CHX stimulation, there was a clear induction of nuclear fragmentation following incubation of U937 cells with AA. Our results differ from previous reports describing an increase in PLA_2 activity and AA release after TNF plus CHX treatment in various cell types, including U937 (Neale *et al*, 1988; McEwan, 1996; Voelkel-Johnson *et al*, 1996). Our inability to detect AA release from intact cells could be explained by a rapid, transient or variable rise in AA, reincorporation of AA into cellular lipids by reacylation (Cissel *et al*, 1996), or clonal diversity (Withnall *et al*, 1995).

Other studies indicate that $cPLA_2$ and 5-LO are translocated to the nuclear membrane after activation of peritoneal macrophages by ionophore A23187 (Peters-Golden and McNish, 1993). 5-LO is primarily nuclear membrane bound in human B-lymphocytes (Jakobsson *et al*, 1995), and PGH synthase I and PGH synthase II are also localised at this site (Regier *et al*, 1993). Furthermore, it has been reported that AA released after receptor stimulation originates from nuclear membrane phospholipids (Capriotti *et al*, 1988). AA metabolites are important messengers for signalling between cells. It is also possible that AA or its metabolites have a significant role within cells by transmitting signals to the nucleus. $cPLA_2$ translocation to nuclear membranes during apoptosis could explain the lack of detectable AA in the present study.

The relative importance of nuclear and cytosolic events in the development of apoptosis remains controversial. Chromatin condensation and nuclear fragmentation can be observed in cell free systems, and membrane and cytosolic alterations occur in enucleated cells (Schultze-Osthoff *et al*, 1994; Newmeyer *et al*, 1994). Although further work is necessary to define interactions between apoptosis and AA metabolism, the ability of these inhibitors to interfere with $cPLA_2$ activity and DNA fragmentation may be an important tool to make a distinction between cytoplasmic and nuclear events that lead to cell death.

Materials and Methods

Reagents

Human TNF was a kind gift from Dr Grace Wong (Genentech Inc., San Francisco, CA). MK-886 was kindly given by Dr Anthony Ford-Hutchinson (Merck Frosst Centre for Therapeutic Research; Quebec, Canada). BW A4C and BW B70C were kindly donated by Dr Lawrence G. Garland (Wellcome Research Laboratories; Kent, UK). $AACOCF_3$, 5-HPETE and arachidonic acid were from Biomol (Plymouth Meeting, PA). CHX, poly-L-Lysine, trypan blue, propidium iodide, and phorbol 12-myristate 13-acetate were from Sigma Chemical (St. Louis, MO). Hoechst 33342 was from Molecular Probes Inc. (Eugene, OR). Apoptest was purchased from Nexins (Hoeven, Netherlands). Phenol, chloroform, DTT, HEPES and sucrose were purchased from Fluka (Buchs, Switzerland). RNAsin and Taq polymerase were from SDS (Falkenberg, Sweden). DTT, Moloney murine leukemia virus reverse transcriptase (M-MLV RT) and first strand buffer were from GIBCO BRL (Gaithersburg, MD). Random primers (pd(N)6), ultrapure dNTP and agarose NA were obtained from Pharmacia AB (Uppsala, Sweden). HPLC solvents were purchased from Rathburn Chemicals (Walkerburn UK). Monoflow 2 scintillation liquid was obtained from National Diagnostics (Manville, NJ). 1-palmitoyl 2-[1-^{14}C]arachidonyl phosphatidylcholine (PC) was obtained from New England Nuclear (Dierech, Germany). FPLC System and protein purification columns were from Pharmacia Biotech (Uppsala, Sweden). DEVD-AMC was from Bachem (Bubendorf, Switzerland).

Cell culture

The U937 human monoblastoid cell line was cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, penicillin and streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C (Sundstrom and Nilsson, 1976). Cells were kept undifferentiated by culturing for short periods and thawing a fresh aliquot of cells every three months. Cells were incubated at 1×10^6/ml in six well plates, during 5 h in the presence or in the absence of TNF (12 ng/ml) plus CHX (1 μg/ml). 5-LO, FLAP and $cPLA_2$ inhibitors were added 5 min before TNF plus CHX, at concentrations between 0.1 μM and 10 μM and were present in the correspondent cultures during the whole experiment. Cell viability was analyzed at various time points by trypan blue exclusion.

Flow cytometry

After incubation with or without TNF plus CHX and the various inhibitors, cells were fixed with 4% PFA, at various time points. Samples were analyzed by flow cytometry (FACS) using a Becton Dickinson FACScan flow cytometer equipped with a 15 mW, 488 nm air cooled argon laser. 10 000 cells were acquired for each determination, with a rate lower than 400 events/sec.

Phosphatidylserine (PS) exposure

PS exposure during apoptosis was quantitated by measuring the binding of annexin V-FITC with the Apoptest binding kit (Dachary-Prigent *et al*, 1993). Cells were stained with annexin V-FITC and propidium iodide (PI, 100 μg/ml) and analyzed by flow cytometry for the simultaneous determination of PS exposure and cell membrane integrity.

Caspase 3-like activation

Caspase 3-like enzyme activity was measured by the ability to cleave the peptide substrate Asp-Glu-Val-Asp (DEVD) linked to amino-4-methylcoumarin (AMC) in a fluorometric assay modified from Nicholson (Nicholson *et al*, 1995). Cell lysate and substrate were combined in a standard ICE reaction buffer (100 mM HEPES, 10% sucrose, 5 mM DTT, 0.1% CHAPS; pH 7.25). Cleavage was monitored by AMC liberation using 355 nm excitation and 460 nm emission wave lengths. Fluorescence units were converted to pmoles of AMC using a standard curve with increasing doses of AMC against fluorescence (Thornberry *et al*, 1992).

Nuclear fragmentation

U937 cells were harvested, pelleted and resuspended in 4% paraformaldehyde (PFA) in PBS, in order to spread 0.3×10^6 cells on poly-L-lysine (100 μg/ml) treated glass microscope slides that were left to dry. Cells were rehydrated and stained with Hoechst 33342 (10 μg/ml) for 10 min, rinsed with water, dried in the dark and covered with glycerol/PBS (50/50) and a coverslip. At least 200 cells were analyzed by examining the fluorescent nuclei with a fluorescence microscope.

High MW and oligonucleosomal DNA fragments

The presence of High MW DNA fragments was analyzed with field inversion gel electrophoresis (Brown *et al*, 1993). After incubation, 10^6 cells were pelleted, resuspended in 50 μl PBS and mixed with 50 μl of 1% low-melting-point agarose. The agarose plugs were digested for 48 h with pronase (1 mg/ml) at 50°C. High MW DNA fragments were electrophoresed and photographed with UV illumination. Analysis of oligonucleosomal DNA fragments was performed as described by Sorenson (Sorenson *et al*, 1990). After incubation 10^6 cells were pelleted, resuspended in 10 μl PBS, 5 μl RNAse (50 mg/ml) and 5 μl loading buffer (0.25% bromophenol blue, 30% glycerol) and 10 μl of sample were loaded in a 1.8% agarose gel, with a precast digestion gel containing 2% SDS and 1 mg/ml proteinase K in 0.8% agarose. The gel was electrophoresed overnight at 20 V followed by 3 h at 90 V, and stained with ethidium bromide.

RNA extraction, cDNA and PCR amplification

Total RNA was isolated (Chomczynski and Saachi, 1987) and quantitated by spectrophotometry. 2 μg RNA were reverse transcribed and tested for PCR amplification with specific primers (Feltenmark *et al*, 1995). 2 μl of cDNA were mixed with 46 μl of the amplification mixture containing Taq polymerase buffer, 0.2 mM dNTP, 0.5 μM each of 5′ and 3′ primers, 2 U Taq polymerase, and 0.5 mM $MgCl_2$ (for 5-LO and FLAP) or 1.0 mM $MgCl_2$ (for β actin, $cPLA_2$, LTA_4 hydrolase, PGH synthase 1 and PGH synthase 2). PCR amplification was run for 25 cycles (β actin, LTA_4 hydrolase and FLAP) or 34 cycles ($cPLA_2$, 5-LO, PGH synthase 1 and PGH synthase 2) with 60°C as annealing temperature (Feltenmark *et al*, 1995).

β actin	5′-GAGGAGCACCCCGTGCTGCTGA-3′	784 bp
	5′-CTAGAAGCATTTGCGGTGG-3′	
$cPLA_2$	5′-TTGCAAACTGCCTCAGCATCAG-3′	554 bp
	5′-CTCTAGTCCTCCGTTCAAGGAAC-3′	
5-LO	5′-ACCATTGAGCAGATCGTGGACACGC-3′	488 bp
	5′-GCAGTCCTGCTCTGTGTAGAATGGG-3′	
FLAP	5′-GGCCATCGTCACCCTCATCAGCG-3′	352 bp
	5′-GCCAGCAACGGACATGAGGAACAGG-3′	
$LTA_{4\ hydrolase}$	5′-GAACAACTGCTTGGAGGACCAGAG-3′	624 bp
	5′-TGCAGTCACGGGATGCATGCTTGC-3′	
$PGH_{synthase\ 1}$	5′-CCTACACCTCCTTCCAGGAGCTC-3′	285 bp
	5′-AGACCAGCTTCTTCAGTGTGGCCG-3′	
$PGH_{synthase\ 2}$	5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′	305 bp
	5′-AGATCATCTCTGCCTGAGTATCTT-3′	

Purification of $cPLA_2$

Liquid chromatography was performed on a Pharmacia FPLC System. All buffers were made fresh, passed through a 0.22 μm filter and degassed before use. U937 cells were sonicated twice for 5 s. The cell homogenate was centrifuged at $10^5 \times g$ for 60 min to obtain cytosolic and membrane fractions. The $10^5 \times g$ supernatant, from 25 to 30 mg of protein, obtained from U937 cell homogenate, was applied to a Resource-Q 6 ml column pre-equilibrated with buffer A [20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA and 0.5 mM DTT]. After washing, a gradient was developed from 0.2 M to 0.6 M NaCl in buffer A at a flow rate of 3 ml/min. Fractions of 1.5 ml were collected and 15 μl aliquots were assayed for $cPLA_2$ activity. $(NH_4)_2SO_4$ (1 M final concentration) was added to pooled, active fractions from the Resource-Q column and applied to a Phenyl-Superose HR 5/5 column pre-equilibrated with 1 M $(NH_4)_2SO_4$ in buffer A. Proteins were eluted in a stepwise gradient from 1 M to 0 M $(NH_4)_2SO_4$ at a flow rate of 0.3 ml/min. Fractions of 0.5 ml were collected and 15 μl aliquots of these were assayed for $cPLA_2$ activity. Thus purified, pooled active fractions were further analyzed for $cPLA_2$ content by Western blot. This partially purified $cPLA_2$ was used for activity studies with the different inhibitors. Protein concentrations were measured with a kit against BSA as standard protein (Bio-Rad, USA), essentially according to Lowry *et al*. (1951).

PLA_2 assay

PLA_2 activity was assayed with 1-palmitoyl 2-[1-^{14}C]arachidonyl phosphatidylcholine (PC) as standard substrate. The phospholipid was dried under nitrogen and resuspended in assay buffer [80 mM glycine, (pH 9.0), 5 mM $CaCl_2$, 5 mM DTT, 1 mg/ml albumin and 10% glycerol] to yield a final concentration of 2 μM PC. This preparation (57 nCi) was subsequently sonicated in a water bath for 10 min at 4°C. The reaction was initiated by adding either homogenate or purified PLA_2 and the reaction proceeded for 30 min at 37°C. The reaction was terminated with two volumes of methanol with 0.5% acetic acid and 40 μM stearic acid. Precipitated proteins and cell debris were removed by centrifugation ($800 \times g$, 10 min). The resulting supernatants were applied to a octadecyl reversed-phase disposable column (Chromabond C18, 100 mg; Macherey-Nagel, Duren, Germany). After washing, bound materials were eluted with 500 μl methanol. The samples were analyzed in a reverse-phase (RP) HPLC system equipped with a Radial-Pak cartridge (5×100 mm) packed with 4 μm Novapak C18 material, guarded by Novapak C18 column (Waters associated; Milford, MA, USA). The mobile phase was methanol : water : trifluoroacetic acid (85 : 15 : 0.007) and the flow rate was 1.2 ml/min. Analysis was performed by comparing retention times with authentic standards. Radioactivity was detected with a β-RAM HPLC flow-through monitor system (Inus System Inc., USA). Quantitative determination was performed by peak area integration.

AA release from intact U937 cells

Cells were adjusted to 2×10^6/ml in RPMI 1640 and labelled with 0.2 μCi/ml of (5,6,8,9,11,12,14,15-^{3}H)-AA (New England Nuclear, Germany) for 90 min at 37°C. The cells were washed twice to remove unincorporated ^{3}H-AA, and resuspended at 1×10^7/ml. Typically, 80 to 90% of the added ^{3}H-AA was incorporated into the cells. Cell culture was stopped at various times either with two volumes of 0.5% acetic acid and 40 μM stearic acid in methanol, or with 5 ml acidified (HCl) Folch solution (choloroform : methanol, 2 : 1). Released fatty acids were extracted and analyzed by RP-HPLC. Alternatively, AA release was measured by GC-MS. Briefly, the reactions were stopped with 5 ml Folch solution. The samples were acidified by HCl and butylhydroxytoluene was added as anti-oxidant. Deuterium-AA (d8-AA) (5.9 nM) was added as an internal standard and the samples were vigorously mixed. The chloroform phase was collected and dried under nitrogen. The fatty acids were resuspended in SP-HPLC mobile phase of hexane : isopropanol : acetic acid (99.5 : 0.5 : 0.02 vol) and separated by SP-HPLC on a Nucleosil 55 column. AA was collected, dried, resuspended in ether and analyzed by GC-MS.

Statistical analysis

One way ANOVA and Mann Whitney-U were used as parametric and non-parametric tests to analyze the data.

Acknowledgements

This work has been supported by the Swedish Medical Research Council (O3X-07135), the Swedish Council for Work Life Research, the Lars Hiertas Foundation and the Karolinska Institute. We are indebted to Dr Grace Wong for the human TNF, Dr Anthony Ford-Hutchinson for the MK-886, Dr Lawrence G Garland for the BW A4C and BW B70C compounds, and Dr D Nicholson for DEVD-CHO.

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