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Release of arachidonic acid induced by tumor necrosis factor-α in the presence of caspase inhibition: Evidence for a cytosolic phospholipase A_2α-independent pathway

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ABSTRACT

Stimulation of L929 cells with tumor necrosis factor-α (TNFα) caused cell death accompanied by a release of arachidonic acid (AA). Although the inhibition of caspases has been shown to cause necrosis in TNFα-treated L929 cells, its role in the TNFα-induced release of AA has not been elucidated. The release of AA is tightly regulated by phospholipase A_2 (PLA$_2$). To find out the mechanisms underlying the TNFα-induced release of AA, we investigated the relationship between TNFα stimulation and PLA$_2$ regulation with and without zVAD, an inhibitor of caspases. In the present study, we found that treatment with TNFα and zVAD stimulated release of AA and cell death in C12 cells (a variant of L929 cells lacking α type of cytosolic PLA$_2$ (cPLA$_2$α)). Stimulation with TNFα/zVAD also caused the release of AA from L929-cPLA$_2$α-siRNA cells. Treatment with pyrrophenone (a selective inhibitor of cPLA$_2$α) completely inhibited the TNFα-induced release of AA, but only partially inhibited the TNFα/zVAD-induced response in L929 cells. The TNFα/zVAD-induced release of AA from C12 and L929-cPLA$_2$α-siRNA cells was pyrrophenone-insensitive, but inhibited by treatment with butylated hydroxyanisole (BHA, an antioxidant). Treatment with dithiothreitol, which inactivates secretory PLA$_2$ activity, decreased the amount of AA released by TNFα/zVAD. TNFα/zVAD appears to stimulate release of AA from C12 cells in a cPLA$_2$α-independent, BHA-sensitive manner. The possible roles of secretory PLA$_2$ and reactive oxygen species from different pools in the release of AA and cell death were discussed.

1. Introduction

Treatment of cells with tumor necrosis factor-α (TNFα) induces various responses such as proliferation, differentiation, and cell death. Treatment of mouse fibrosarcoma L929 cells with TNFα induces a relatively slow death in which signs of both apoptosis and necrosis can be observed [1–4]. TNFα-induced responses including cell death in L929 cells were mediated by the 55-kDa TNFα receptor (p55, TNF-R1), although TNFα can bind to another distinct cell surface receptor, 75-kDa

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TNF-R2 (p75), which is also expressed in L929 cells [2]. Under conditions where apoptosis is either not initiated or inhibited, TNFα induces a caspase-independent programmed cell death in various cells including L929 cells. Treatment with benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD), a caspase inhibitor with broad specificity, dramatically potentiated TNFα-mediated cell death in L929 cells, although TNFα alone had a marginal effect on the activities of caspases [2,5–8]. Yu et al. [3] reported that treatment of L929 cells with zVAD or caspase-8-specific RNA interference caused autophagic cell death. A similar enhancement of cell death by inhibition of the caspase pathway has been shown in HT29 adenocarcinoma cells, NIH3T3 cells, human U937 monocytoids, and mouse RAW macrophages treated with several death-receptor ligands [3,9]. Cauwels et al. [10] showed that pretreatment with zVAD can sensitize mice to the lethal effect of TNFα via activation of p55. These reports suggest that inhibition of the caspase pathway activates and/or enhances TNFα-induced pathways leading to cell death. However, the mechanism of zVAD-induced toxicity has not been entirely elucidated.

In L929 cells, TNFα-evoked responses such as cell death and the accumulation of reactive oxygen species (ROS) are mediated by the activation of phospholipase A_2 (PLA_2) and/or arachidonic acid (AA) [1,11,12]. Group IV-A (α type) cytosolic PLA_2 ($cPLA_2\alpha$) selectively releases AA from glycerophospholipids [13,14]. The L929 variant C12, which expresses undetectable levels of $cPLA_2\alpha$, is resistant to the TNFα-induced release of AA and cell death, and transfection of C12 cells with $cPLA_2\alpha$ cDNA recovered TNFα-induced responses [1,11]. These reports show that the release of AA mediated by $cPLA_2\alpha$'s activation is critical for TNFα-induced cell death. However, the roles of other types of PLA_2 in TNFα-induced responses including the release of AA and cell death, and the effects of zVAD on these responses when $cPLA_2\alpha$ has been depleted, have not been well elucidated.

RNA interference is a gene silencing mechanism whereby double-stranded small interfering RNA (siRNA) induces degradation of mRNA in a sequence-specific manner. In the present study, we demonstrated a stable and sequence-specific silencing of mouse $cPLA_2\alpha$ gene expression in L929 cells, and established several cell sub-cultures (L929-$cPLA_2\alpha$-siRNA cells) by using the vector-based RNA interference. We investigated the effects of zVAD on the TNFα-induced release of AA and cell death in L929 cells, C12 cells, and L929 cells lacking $cPLA_2\alpha$. Treatment of C12 cells with TNFα caused release of AA and necrotic cell death in the presence of the caspase inhibitor zVAD. The release of AA induced by TNFα/zVAD was observed in L929-$cPLA_2\alpha$-siRNA cells. Treatment with pyrrophenone (a selective inhibitor of $cPLA_2\alpha$) did not inhibit the release induced by TNFα/zVAD in C12 and L929-$cPLA_2\alpha$-siRNA cells, and inhibited partially, but not completely, the release in L929 cells. Transfection with cDNA encoding human $cPLA_2\alpha$ into L929-$cPLA_2\alpha$-siRNA cells did not enhance the release induced by TNFα/zVAD. These findings suggest that treatment with TNFα when caspases are inhibited by zVAD causes release of AA in a $cPLA_2\alpha$-independent manner in L929 and C12 cells. The roles of secretory PLA_2 and ROS in the release of AA and cytotoxicity were discussed.

2. Experimental procedures

2.1. Materials

[5,6,8,9,11,12,14,15-^{3}H]AA (7.92 TBq/mmol) and 1-palmitoyl-2-[^{14}C]-arachidonic phosphatidylcholine (1776 MBq/mmol) were purchased from Amersham (Buckinghamshire, UK) and PerkinElmer (Boston, MA, USA), respectively. Human TNFα and zVAD were obtained from Pepro Tech EC Ltd. (London, UK) and Biomol (Plymouth Meeting, PA, USA), respectively. Butylated hydroxyanisole (BHA, a lipid soluble antioxidant), 4β-phorbol myristate acetate (PMA), *N*-acetyl-cysteine (NAC), A23187, and *tert*-butyl hydroperoxide (solution, B2633) were purchased from Sigma (St. Louis, MO, USA). Pyrrophenone was kindly provided by Dr. Hanasaki (Shionogi Pharm. Ltd., Osaka, Japan). Bromoenol lactone (BEL) was acquired from Cayman (Ann Arbor, MI, USA). 1,4-Diamino-2,3-dicyano-1,4-bis-(o-aminophenylmercapto)butadiene (U0126) and 2,6-bis(1,1-dimethylethyl)-4-[[(1-ethyl)amino]methyl]phenol hydrochloride (LY231617) were obtained from Calbiochem (San Diego, CA, USA). The concentrations of reagents including inhibitors were the same as those in previous reports [15–17]. A23187, PMA, or zVAD was dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the medium was under 0.5%. The vehicle containing dimethyl sulfoxide did not cause release of AA or cell toxicity for 6 h.

2.2. Cell culture and analysis of cell viability

L929 cells and the variant C12 cells (provided by Dr. Tsujimoto, Setsunan University, Osaka, Japan) were grown in DMEM supplemented with 5% fetal bovine serum. Cells stably expressing wild-type human $cPLA_2\alpha$ and mutant $cPLA_2\alpha$ were generated by transfection with pcDNA4/HisMax-$cPLA_2\alpha$ and pPUR encoding the puromycin resistance gene (CLONTECH, Palo Alto, CA, USA), as previously described [15]. $cPLA_2\alpha$-S228A and $cPLA_2\alpha$-D522E refer to the mutation of the Ser residue at position 228 to Ala and the Asp at position 522 to Asn in wild-type $cPLA_2\alpha$, respectively. When the cells achieved 60–80% confluence (sub-confluent stage), they were used for assays. The quantification of cell viability was based on the cleavage of a water-soluble tetrazolium salt (WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate sodium salt) by mitochondrial dehydrogenases. Cell viability was also estimated by the lactate dehydrogenase leakage method. The assays were conducted according to the manufacturer's instructions (Roche, Basel, Switzerland).

2.3. Assay for release of AA

The release of AA from cells was determined as described previously [15–17]. Briefly, cells on 12-well plates were labeled overnight with DMEM containing 0.2% serum, 0.1% fatty acid-free bovine serum albumin, and [^{3}H]AA. The labeled and washed cells were stimulated for 6 h with TNFα with and without zVAD in DMEM containing 5% serum. For the assay of AA released by PMA/A23187, the cells were stimulated for 30 min in DMEM. In some experiments, cells were cultured with the respective inhibitors such as pyrrophenone for 15 or 30 min before the assay. Then, the medium was collected and

centrifuged at 8000 × *g* for 2 min. The ^{3}H content of the supernatant was estimated, and data were calculated as percentages of all the radioactivity incorporated (20,000–40,000 dpm per well). The release of AA without stimuli was dependent on each experiment, and was 2–5% of the total incorporated in both L929 and C12 cells. It is reported that there is release of various ^{3}H-labeled membrane vesicles and fragments as components of apoptotic bodies in apoptotic lymphoid cells [18]. In some experiments, the supernatant was further centrifuged at 10,000 × *g* for 30 min in order to precipitate the pellet containing the vesicles and fragments as described previously [18]. In the vehicle- and TNFα/zVAD-treated cells (L929 and C12 cells), a large part of the radioactivity was detected in the supernatant that was collected by the high-speed centrifugation. Thus, the ^{3}H content of the supernatant appeared to be non-esterified AA and the soluble metabolites including prostanoids, not the release of membrane fragments including apoptotic bodies. For quantitative analyses of the data, in some cases, the values for the fold-increase in the release of AA were normalized as a percentage of the respective control value without stimuli.

2.4. PLA_2 assay in vitro

PLA_2 activity in vitro was measured using 1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine as the substrate [15]. For the measurement of activity, 12.5 μg of protein from cell lysate was used per tube.

2.5. Western blotting

Cell lysates (30 μg of protein per lane) were fractionated by SDS-PAGE and transferred to polyvinyldifluoride membranes. The blocked membranes were then incubated with anti-cPLA$_2$α antibody (4-4B-3C, Santa Cruz Biotech, Santa Cruz, CA, USA) and anti-β-tubulin antibody (T-4026, Santa Cruz Biotech). The immunoreactive bands were visualized using a chemiluminescent reagent (Amersham).

2.6. Knockdown of endogenous cPLA$_2$α by RNA interference in L929 cells

To suppress the expression of mouse cPLA$_2$α in L929 cells, we selected three gene sequences for RNAi (No. 1, GCGAACGAGACACTTCAAT, No. 2, GCACATCGTGAGTAATGAC, and No. 3, GGTGCATAACTTCATGCTG). We confirmed that these oligonucleotides did not have homology to human cPLA$_2$α or any other mouse gene by using a BLAST search (NCBI web-site). The oligonucleotides were obtained from Greiner Japan (Tokyo). Double-stranded siRNAs were generated using the pSilencer 2.1-U6 hygro siRNA expression vector (Ambion, Austin, TX, USA). pSilencer 2.1-U6 hygro negative control was used as a control scrambled sequence according to the manufacturer's instructions (Ambion). The plasmid constructs were prepared with a QIAGEN Plasmid Midi kit (QIAGEN, MD, USA). pSilencer 2.1-U6 hygro/mouse cPLA$_2$α was transfected with LipofectAMINE PLUS reagent (Invitrogen, CA, USA) into L929 and HEK293 cells grown in DMEM to 70% confluence in 60 mm wells, and the cells were incubated for 48 h at 37 °C. First, we examined the inhibitory effects of the three siRNAs for mouse cPLA$_2$α on HEK293 cells co-transfected with mouse and human cPLA$_2$α cDNA (Fig. 5, Panels A–D). Then, we tried to knockdown the expression of cPLA$_2$α in L929 cells using the most effective pSilencer (No. 2). Since the percentage of transfected L929 cells was low, we established stable sub-clones of L929 cells resistant to hygromycin B (500 μg/mL, Wako, Osaka, Japan) by limiting dilution, and used the sub-clonal cells maintained in the medium with hygromycin B as cPLA$_2$α knockdown cells (L929-cPLA$_2$α-siRNA cells). The level of mouse cPLA$_2$α in L929-cPLA$_2$α-siRNA cells was confirmed by immunoblotting using anti-cPLA$_2$α antibody (Fig. 5, Panel E).

2.7. Data presentation

For the release of AA, values are means ± S.E.M. from more than three independent experiments. Some data are means ± S.D. for a typical experiment, representative of two or three independent experiments. In the case of multiple comparisons, the significance of differences was determined using a one-way analysis of variance followed by the Bonferroni test. *P*-values < 0.05 were considered significant.

3. Results

3.1. TNFα-induced cell death of L929 and C12 cells in the presence of zVAD

The level of cPLA$_2$α protein in C12 cells was low (not detectable) compared with that in L929 cells, as described previously [1,15]. In the present study, we used human TNFα as an activator of the p55 TNF receptor in mouse L929 cells, since human TNFα binds to mouse p55 but not to mouse p75 [19]. Treatment with 1 nM TNFα alone caused marked cell death after 24 h in L929 cells, but not in C12 cells (Fig. 1) (Panel A). Treatment with 10 nM TNFα alone caused significant cell death after 24 h in C12 cells, although the response was much weaker than in L929 cells. The treatment of L929 cells with 10 nM TNFα for 6 h slightly but significantly caused cell death; the rate of cell survival was 90.2 ± 3.2 (% of control, $n = 3$, $P < 0.05$). Treatment with 1 nM TNFα for 6 h had no effect. It is reported that co-treatment with zVAD enhanced the toxicity of TNFα in L929 cells [2,3,5]. Under our conditions, treatment of L929 cells with 1 and 10 nM TNFα for 6 h caused ~50% and ~80% cell death in the presence of 20 μM zVAD, respectively. Treatment of C12 cells with 1 and 10 nM TNFα for 6 h did not cause cell death, but co-treatment of C12 cells with 20 μM zVAD and TNFα caused slight but significant cell death (Panel B). In the presence of 10 μM zVAD, however, treatment with 10 nM TNFα for 6 h did not affect survival in C12 cells. The same treatment caused 30–50% cell death in L929 cells depending on experiments. In the following experiments, we investigated the effect of co-treatment with 10 nM TNFα and 10 μM zVAD (TNFα/zVAD) for 6 h on responses in the cells.

Next, we investigated the effect of TNFα/zVAD on morphological changes in L929 and C12 cells. Under the phase contrast microscope, L929 cells 6 h after TNFα/zVAD treatment appeared round with large vacuoles (Fig. 2), as described previously [3]. TNFα/zVAD-treated C12 cells were also round,

but large numbers appeared to have no vacuoles. Treatment of L929 and C12 cells with zVAD alone had no effect on cell survival or morphology after 6 and 24 h (data not shown). Treatment with 50 μM BHA, a lipid soluble antioxidant, almost completely protected L929 and C12 cells 6 h after treatment with TNFα/zVAD; the rates of cell survival were 95.4 ± 5.8 (% of control, $n = 3$) and 98.2 ± 3.9% ($n = 3$) in L929 and C12 cells, respectively. Treatment with BHA alone had no effect on cell survival. Treatment of C12 cells (Fig. 2, lower panel) and L929 cells (not shown) with 50 μM BHA inhibited the morphological changes induced by TNFα/zVAD. These findings suggest that TNFα in the presence of zVAD caused cell death via BHA-sensitive pathway(s) in C12 cells lacking cPLA$_2$α.

3.2. *Effect of pyrrophenone on the release of AA by TNFα/zVAD from cells*

The release of AA mediated by cPLA$_2$α is crucial to the cytotoxic action of TNFα in L929 cells [1,12]. However, the mechanism(s) of the release of AA induced by TNFα/zVAD in L929 cells and the response in C12 cells have not been elucidated. Next, we examined the effect of TNFα/zVAD on the release of AA from L929 and C12 cells. Treatment with TNFα alone stimulated the release of AA after about 3 h, and the response at 6 h was enhanced about two- to three-fold in L929 cells (Fig. 3, Panel A). In C12 cells, the TNFα-induced response was undetectable or marginal (under 1.5-fold), as previously reported [1,15]. Treatment of L929 cells with 100 nM PMA plus 10 μM A23187 for 30 min caused the release of AA via activation of cPLA$_2$α [15,17]. Pretreatment of L929 cells with 10 μM pyrrophenone, a selective inhibitor of cPLA$_2$α [20], almost completely inhibited the release of AA induced not only by PMA/A23187 (Panel B) but also by TNFα (Panel C). The inhibitory effect of pyrrophenone on the TNFα-induced release was marked and significant (Panel D). Although treatment with 10 μM pyrrophenone for 1 h did not change the basal release of AA [17], the treatment for 6 h slightly but significantly inhibited the basal release in L929 cells.

Treatment with 10 μM zVAD markedly enhanced the release of AA by TNFα from L929 cells, and the response by TNFα/zVAD was increased seven- to eight-fold (Fig. 4, Panel A). Treatment with 10 μM pyrrophenone significantly inhibited the release of AA by TNFα/zVAD, but the inhibitory effect was partial (~50%) in L929 cells. Treatment with TNFα/zVAD caused the release of AA from C12 cells, and the response was significant and increased three-fold (Panel B). Treatment with 10 μM pyrrophenone did not inhibit the response in C12 cells. Treatment with zVAD alone had no effect on the release of AA from L929 and C12 cells (data not shown), as previously reported [15]. In the serum-free conditions, treatment of C12 cells with TNFα/zVAD significantly stimulated the release of AA, and a large part of the response (70–80%) was pyrrophenone-insensitive; the responses with and without 10 μM pyrrophenone were 1.7 ± 0.1-fold and 1.9 ± 0.1-fold, respectively ($n = 3$). TNFα/zVAD markedly stimulated the release of AA from L929 cells in the serum-free conditions (7.7 ± 0.3-fold, $n = 3$), and the response was partially, not completely, inhibited by 10 μM pyrrophenone (2.7 ± 0.2-fold, $n = 3$). These findings suggest that treatment with TNFα/zVAD was capable of stimulating the release of AA in a pyrrophenone-insensitive manner from C12 cells, and that a part of the L929 cell response is pyrrophenone-insensitive. The release of AA at 4 h after TNFα/zVAD treatment was pyrrophenone-insensitive in C12 cells, and the response in L929 cells at 4 h treatment, when morphological changes were not marked and cell death is not yet apparent, was partially (~50%) inhibited by pyrrophenone.

3.3. *Selection of siRNA for knockdown of mouse cPLA$_2$α in L929 cells*

We further substantiated that the release of AA occurred via a cPLA$_2$α-independent pathway by selectively targeting cPLA$_2$α expression using RNA interference. First, we examined the effects of three predesigned siRNAs for mouse cPLA$_2$α (pSilencer Nos. 1–3) on cPLA$_2$α protein (Fig. 5, Panel A) and activity (Panel B) in HEK293 cells transfected with mouse cPLA$_2$α cDNA. Treatment with pSilencer-2 or -3 markedly inhibited the expression and activity of mouse cPLA$_2$α, and treatment with pSilencer-1 had a partial inhibitory effect. Treatment with pSilencer-2 inhibited expression (Panel C) and activity (Panel D) in HEK293 cells transfected with mouse cPLA$_2$α cDNA, but not in the cells transfected with human cPLA$_2$α cDNA. These findings suggest that pSilencer-2 selectively inhibited mouse cPLA$_2$α in the cells. Treatment with pSilencer-2 almost completely decreased the expression of cPLA$_2$α protein without change of β-tubulin expression in L929 cells (Panel E). The treatment with pSilencer-2 partially inhibited PLA$_2$ activity in L929 cells (data not shown), although we could not reach a clear conclusion because of the weak (~300 dpm/tube) activity in native L929 cells under our assay conditions. Treatment with the control (mock) having a scrambled sequence had no effect on cPLA$_2$α expression in HEK293 cells or L929 cells.

3.4. *AA released by TNFα/zVAD from L929-cPLA$_2$α-siRNA cells*

Next, we established several clones of L929 cells lacking cPLA$_2$α (L929-cPLA$_2$α-siRNA cells, Nos. 10, 13, 17 and 49). Like in C12 cells, cPLA$_2$α was not expressed in these L929-cPLA$_2$α-siRNA cells (Fig. 6, Panel A). The release of AA induced by 10 nM TNFα from L929-cPLA$_2$α-siRNA cells was much less extensive than that from native L929 cells, and almost the same as that from C12 cells; the responses were increased about three-fold in L929 cells (Fig. 3D) and less than 1.5-fold in C12 and L929-cPLA$_2$α-siRNA cells. Previously, we reported that the release of AA by PMA/A23187 was almost completely mediated by cPLA$_2$α's activation in L929 cells [17]. The release of AA induced by PMA/A23187 was marginal in L929-cPLA$_2$α-siRNA cells, like in C12 cells (Fig. 6, Panel B). However, the release induced by TNFα/zVAD was marked in every clone of the L929-cPLA$_2$α-siRNA cells tested. The release of AA induced by TNFα/zVAD from clone No. 49 was significant, and the response was little inhibited by 10 μM pyrrophenone (Panel C).

Next, we tried to express wild-type human cPLA$_2$α and cPLA$_2$α-S228A in L929-cPLA$_2$α-siRNA cells using the expression vector pEB6 CAG, as described previously [15]. Human cPLA$_2$α has a catalytic center serine-228, and the mutation of this Ser residue to Ala markedly decreased the activity. Treatment with PMA/A23187, which couples with the activa-

Table 1 – Release of AA induced by TNFα/zVAD and PMA/A23187 from L929-cPLA$_2$α-siRNA cells expressing mutant cPLA$_2$α-S228A

AA release (% of total)

Exp. I	Vehicle	TNFα	TNFα/zVAD
Wild-type	2.3 ± 0.1 (1)	3.0 ± 0.1 (1.3)	12.0 ± 0.1 (5.2)
S228A	2.4 ± 0.1 (1)	4.0 ± 0.2 (1.7)	9.8 ± 0.3 (4.1)

AA release (% of total)

Exp. II	Vehicle	PMA/A23187
Wild-type	1.5 ± 0.1 (1)	5.9 ± 0.3 (3.9)
S228A	1.5 ± 0.1 (1)	1.9 ± 0.1 (1.2)

L929-cPLA$_2$α-siRNA cells (clone 49) were transfected with the vector for wild-type cPLA$_2$α and cPLA$_2$α-S228A (S228A). In Exp. I, the cells were stimulated with vehicle, 10 nM TNFα, and/or 10 μM zVAD for 6 h. In Exp. II, the cells were stimulated with vehicle or 100 nM PMA plus 10 μM A23187 for 30 min. Data (% of total incorporation) are means ± S.D. for a typical experiment repeated two times with similar results. The fold-increase induced by the reagent is shown in parentheses. In the control vector-treated cells, the release of AA was less than 1.5-fold by TNFα alone, 4–5-fold by TNFα/zVAD and 1.1-fold by PMA/A23187, like in the native L929-cPLA$_2$α-siRNA cells.

tion of cPLA$_2$α, caused the release of AA for 30 min from the cells expressing wild-type human cPLA$_2$α, but not from the cells expressing cPLA$_2$α-S228A (Table 1, Exp. II). Treatment with TNFα/zVAD markedly (four- to five-fold) stimulated the release of AA from cells expressing wild-type cPLA$_2$α and cPLA$_2$α-S228A. In the cells used, the TNFα-induced release of AA was marginal in the cells expressing wild-type cPLA$_2$α, and the response in the cells expressing cPLA$_2$α-S228A was enhanced about 1.7-fold. Our results obtained through highly specific inhibition of cPLA$_2$α using RNA interference, and with the transfection of cPLA$_2$α cDNA, show the existence of a cPLA$_2$α-independent pathway causing the release of AA induced by TNFα/zVAD in C12 (and L929) cells.

3.5. *Possible involvement of secretory PLA$_2$ in the release of AA by TNFα/zVAD*

The release of AA by TNFα/zVAD from L929 cells was inhibited by treatment with 30 μM mepacrine (a general inhibitor of PLA$_2$) or 10 μM *p*-bromophenacyl bromide, which inactivates secretory PLA$_2$ by covalent binding to the catalytic center histidine [13,21]; the values were 21.3 ± 4.5 (mean ± S.D.% of total AA incorporated) in the control cells, 9.3 ± 0.6% in the mepacrine-treated cells, and 12.3 ± 2.1% in the *p*-bromophenacyl bromide-treated cells in a typical experiment. Treatment with 10 μM BEL, a relatively selective inhibitor for Ca^{2+}-independent PLA$_2$ [22,23], did not inhibit the release of AA by TNFα/zVAD in L929, L929-cPLA$_2$α-siRNA, and C12 cells (Table 2). Treatment with 15 mM dithiothreitol, which inhibited secretory PLA$_2$ activity in vitro and the release of AA via activation of secretory PLA$_2$ in intact cells [24,25], significantly inhibited the release by TNFα/zVAD from L929 cells, and also inhibited the response in L929-cPLA$_2$α-siRNA cells. The release of AA by TNFα/zVAD in C12 cells was not inhibited by the inhibitors for kinases (1 μM herbimycin A for tyrosine kinases, 10 μM U0126 for extracellular signal-regulated kinase (ERK1/2) kinase), and the response was not changed by the depletion of protein kinase C (pretreatment with 100 nM PMA for 12 h) (data not shown), although PMA/A23187-induced release of AA mediated by cPLA$_2$α's activation was decreased by the inhibitors [17].

3.6. *Effects of oxidants and antioxidants on release of AA*

Essential to the cytotoxic action of TNFα are mitochondrial dysfunction and the generation of ROS in the mitochondria in L929 cells [26,27]. In L929 cells, the release of AA for 6 h induced

Table 2 – Effects of BEL and dithiothreitol on the release of AA by TNFα/zVAD in L929, L929-cPLA$_2$α-siRNA, and C12 cells

AA release (% of total)

Exp. I.	L929	L929-PLA$_2$α-siRNA	C12
Vehicle	2.5 ± 0.2	3.5 ± 0.3	3.4 ± 0.3
BEL (10 μM)	2.6 ± 0.2	Not determined	Not determined
TNFα/zVAD	35.1 ± 4.4	22.1 ± 0.3	12.4 ± 0.7
TNFα/zVAD + BEL	32.5 ± 2.9	22.0 ± 2.7	12.6 ± 0.3

AA release (% of total)

Exp. II.	L929	L929-cPLA$_2$α-siRNA
Vehicle	4.5 ± 0.8 (100)	(100)
DTT (15 mM)	4.1 ± 0.9 (82 ± 10)	(105, 83)
TNFα/zVAD	31.7 ± 3.9* (800 ± 175*)	(251, 400)
TNFα/zVAD + DTT	5.6 ± 0.6† (122 ± 29†)	(150, 166)

The indicated cells were pretreated with vehicle, 10 μM BEL, or 15 mM dithiothreitol (DTT) for 30 min, and then further treated with TNFα/zVAD (10 nM and 10 μM, respectively) for 6 h. In Exp. I, data are means ± S.D. for a typical experiment repeated two times with similar results. In Exp. II, data for L929 cells are means ± S.E.M. for three to four independent experiments, and data for L929-cPLA$_2$α-siRNA cells were from two independent experiments performed in triplicate. The values in parentheses are normalized as a percentage of the respective control value of AA released without stimuli. *$P < 0.05$, significantly different from the control. †$P < 0.05$, significantly different from that without DTT.

Table 3 – Effects of anti-oxidants on release of AA and cell survival in L929 cells

Treatment	TNFα/zVAD-induced responses	
	AA release (% of the control)	Cell survival (% of that without TNFα/zVAD)
+Vehicle	100	30.3 ± 5.3
+BHA (50 μM)	27 ± 4*	91.7 ± 4.3*
+NAC (5 mM)	103 ± 5	72.0 ± 3.5*
+GSH (5 mM)	Not determined	75, 72 (*n* = 2)

For the assays, labeled or unlabeled L929 cells were treated with TNFα/zVAD (10 nM and 10 μM, respectively) for 6 h. The indicated reagents were further added to the medium. The net increase of AA released by TNFα/zVAD is normalized as a percentage of the control value. For the assay of cell survival, data are presented as a percentage of the control without TNFα/zVAD. Data are means ± S.E.M. for three independent experiments. *$P < 0.05$, significantly different from the control. Data for GSH treatment were from two independent experiments.

by TNFα/zVAD (Table 3) or by TNFα alone (data not shown) was markedly inhibited by co-treatment with an antioxidant, BHA (50 μM). The release induced by TNFα/zVAD in C12 cells was almost completely inhibited by co-treatment with BHA (Fig. 7, Panel A). Co-treatment with 50 μM LY231617, another anti-oxidant [28], markedly inhibited the TNFα/zVAD-induced response (~60% inhibition). By contrast, treatment with 5 mM NAC did not inhibit the TNFα/zVAD-induced release of AA from L929 cells (Table 3) and from C12 cells (data not shown). It is reported that treatment with *tert*-butyl hydroperoxide, which is a membrane-permeant mild oxidant, stimulated the release of AA from cells in a PLA_2-dependent manner [29,30]. Treatment with 5 mM *tert*-butyl hydroperoxide for 6 h stimulated the release of AA in C12 cells, like in L929 cells (Fig. 7, Panel B). Treatment with 1 mM H_2O_2 for 6 h alone caused the release of AA in C12 cells, and the response was partially (~30%) inhibited by 30 μM mepacrine and 10 μM *p*-bromophenacyl bromide, but not by 10 μM pyrrophenone (data not shown).

Co-treatment of L929 cells with 50 μM BHA significantly decreased cell death induced by TNFα/zVAD (Table 3). Interestingly, treatment with 5 mM NAC significantly reduced TNFα/zVAD-induced toxicity in L929 cells. Treatment with 5 mM GSH also decreased cell death by TNFα/zVAD. Like the release of AA, the cell death induced by TNFα/zVAD in C12 cells was decreased by treatment with BHA, but not with GSH or NAC (data not shown). The antioxidants tested alone had no effect on the release of AA or cell survival. Treatment with 10 μM pyrrophenone slightly (10–20%) but not significantly increased cell survival in TNFα-treated L929 cells, but did not change cell death of L929 and C12 cells induced by TNFα/zVAD (data not shown).

4. Discussion

In the present study, we found that treatment with TNFα/zVAD caused cell death (Fig. 1), morphological changes (Fig. 2), and marked release of AA (Fig. 4) within 6 h in C12 cells, a variant of L929 cells lacking $cPLA_2\alpha$. Our data show that stimulation of L929 cells with TNFα releases AA by the two pathways; preferential activation of $cPLA_2\alpha$ in normal state, and additional release of AA via $cPLA_2\alpha$-independent manner in the caspase deficient state. Our data may explain TNFα-induced, caspase-independent cells death. The release of AA and cell toxicity induced by TNFα/zVAD in the cells lacking $cPLA_2\alpha$ appeared to be due to BHA-sensitive ROS production.

4.1. *$cPLA_2\alpha$-independent release of AA induced by TNFα/zVAD from C12 cells*

Treatment with TNFα/zVAD significantly stimulated release of AA from C12 cells, although the response was weaker than that in L929 cells. It has been well established that activation of p55 with TNFα causes the activation of various kinases, which can phosphorylate and activate $cPLA_2\alpha$ [14,19,31]. Although $cPLA_2\alpha$ at the protein level was not detected by Western

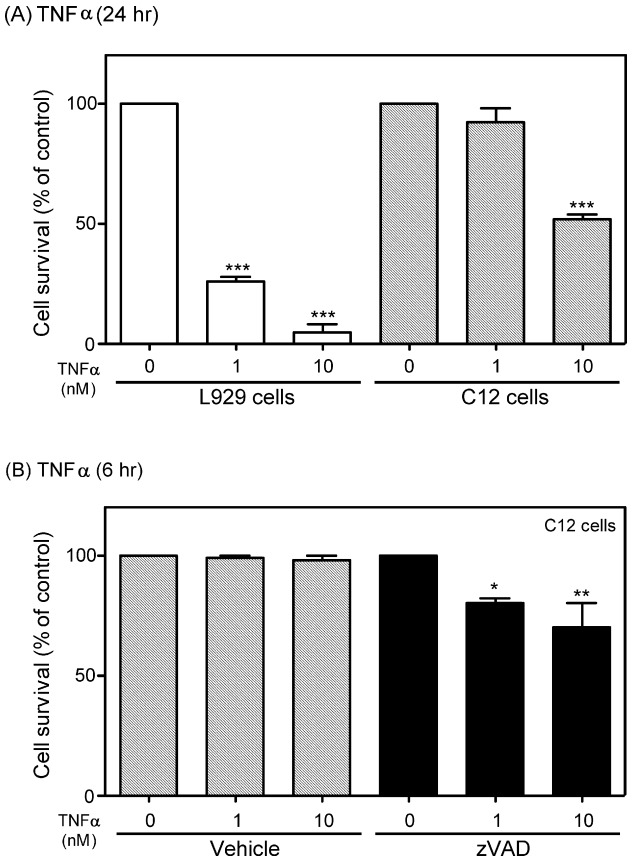

Fig. 1 – TNFα-induced cell death of L929 and C12 cells. In Panel (A) L929 and C12 cells were cultured with vehicle or 1 nM and 10 nM TNFα for 24 h. In Panel (B) C12 cells were cultured with 1 nM and 10 nM TNFα for 6 h in the presence or absence of 20 μM zVAD. Data are means ± S.E.M. for three independent experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from the control without TNFα.

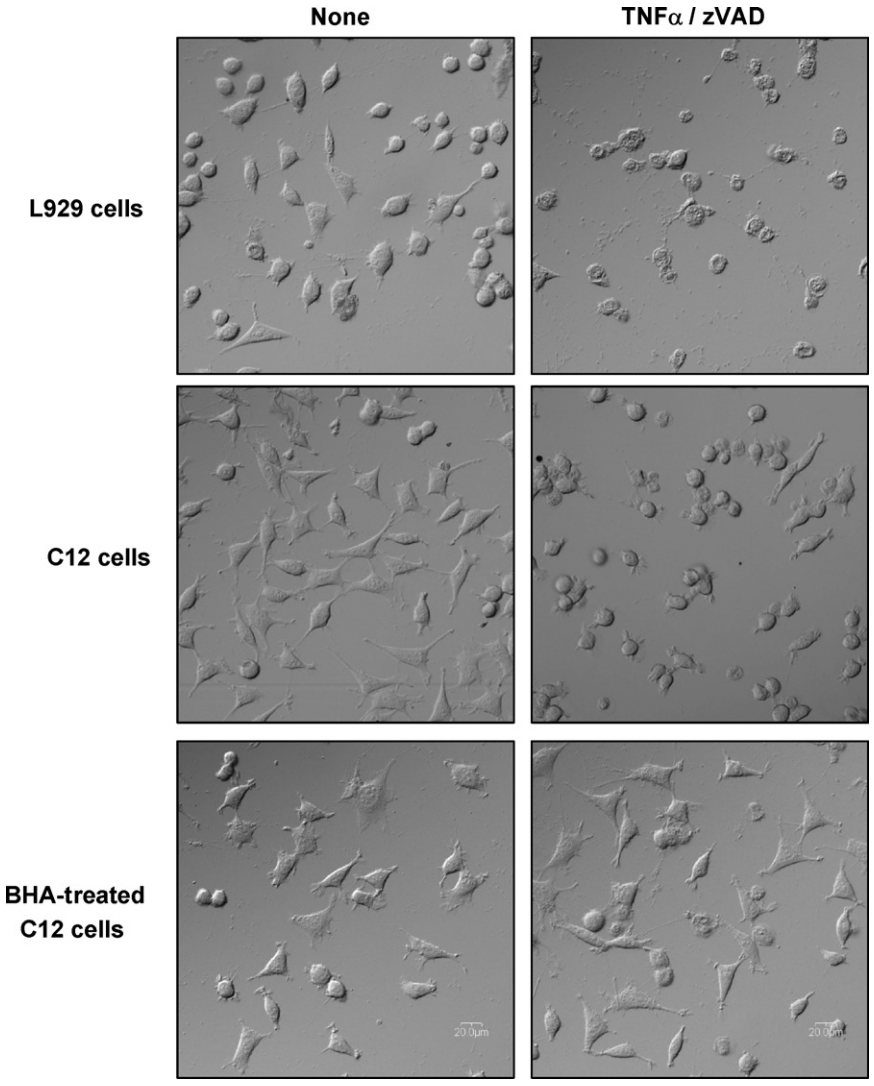

Fig. 2 – TNFα/zVAD-induced morphological changes in L929 and C12 cells. L929 and C12 cells were treated with vehicle or TNFα/zVAD (10 nM and 10 μM, respectively) for 6 h. In the lower panel, 50 μM BHA was further added to the medium. The morphological changes were observed by phase contrast microscopy. Data are from a representative experiment repeated three times with similar results.

blotting in C12 cells, it is possible that a trace amount of cPLA$_2$α is activated by TNFα/zVAD in C12 cells. The PMA/A23187-induced release of AA from L929 cells was almost completely inhibited by a selective inhibitor of cPLA$_2$α (pyrrophenone), as described previously [17]. Treatment with pyrrophenone almost completely inhibited the TNFα-induced release of AA (Fig. 3, Panel D) but only partially inhibited the TNFα/zVAD-induced release (Fig. 4, Panel A). The response by TNFα/zVAD in C12 cells was pyrrophenone-insensitive (Fig. 4, Panel B), and not modified by the inhibitors for kinase pathways including protein kinase C and ERK.

Next, we used vector-based RNA interference to establish a L929 cell line in which the expression of mouse cPLA$_2$α was stably suppressed. pSilencer No. 2 was quite specific, inhibiting the expression and activity of mouse, but not human, cPLA$_2$α (Fig. 5). Like in C12 cells, the release of AA induced by PMA/A23187 in the L929-cPLA$_2$α-siRNA cell clones was much less extensive than that in L929 cells (Fig. 6, Panel B). Previously, we reported that the release of AA by PMA/A23187 in L929 cells was almost completely dependent on cPLA$_2$α [17]. Transient transfection with the wild-type, but not an inactive-type (S228A), of human cPLA$_2$α cDNA restored the PMA/A23187-evoked response in L929-cPLA$_2$α-siRNA cells (Table 1). Transfection with wild-type human cPLA$_2$α did not change the amount of AA released by TNFα/zVAD in L929-cPLA$_2$α-siRNA cells. It is reported that cPLA$_2$α can be proteolytically inactivated by various caspases [21,31,32]. Administration of zVAD inhibited TNFα-induced cleavage of cPLA$_2$α in mice liver in vivo [10]. Thus, treatment of L929 cells with zVAD may stimulate release of AA via inhibition of TNFα-induced proteolysis of cPLA$_2$α. By contrast, TNFα-induced activation of caspases resulted in the cleavage and activation of cPLA$_2$α in some cell types including murine fibrosarcoma WEHI-S cells [33]. In preliminary experiments, we examined the effect of transfection of C12 cells with the vector pEB6 CAG-cPLA$_2$α-D522E, in which Asp522 of human cPLA$_2$α was

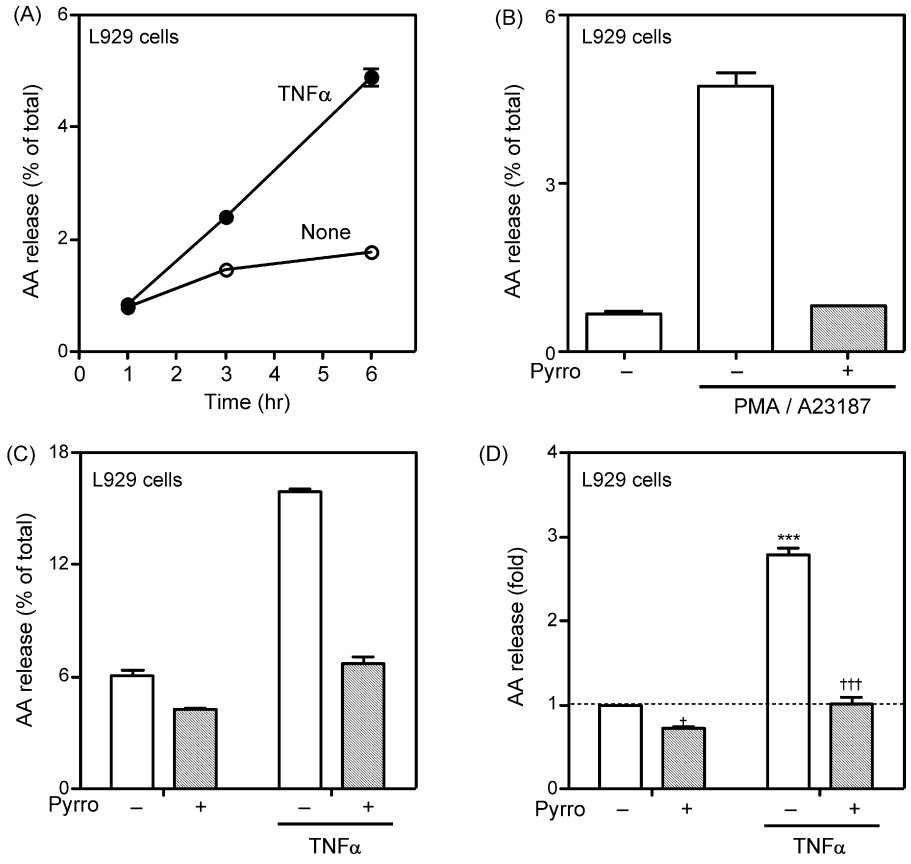

Fig. 3 – Release of AA induced by TNFα and its inhibition by pyrrophenone in L929 cells. In Panel (A) labeled L929 cells were treated for the period indicated with vehicle (○) or 10 nM TNFα (●). In Panel (B) labeled L929 cells were incubated with 10 μM pyrrophenone (Pyrro) for 30 min, and then treated with vehicle or 100 nM PMA plus 10 μM A23187 for 30 min. In Panels (C) and (D), labeled L929 cells were treated with vehicle or 10 nM TNFα in the presence or absence of 10 μM pyrrophenone for 6 h. In Panels (A)–(C), data are means ± S.D. from a representative experiment repeated two times with similar results. Quantitative data concerning the amount of AA released by TNFα are shown in Panel (D). The release of AA without stimulation was dependent on each experiment, and the amounts were 2–5% of the total incorporated. The values of fold-increase are normalized as percentages of the control value without TNFα. Data are means ± S.E.M. for three independent experiments. ***$P < 0.001$, significantly different from the control without TNFα. †$P < 0.05$, †††$P < 0.001$, significantly different from that without pyrrophenone.

replaced by Asn and was not cleaved by caspase-3 and caspase-8 [32], on the amount of AA released by TNFα. The value for C12 cells transfected with the mutant was the same as that for the cells transfected with wild-type cPLA$_2$α, and co-addition of zVAD enhanced the release to a similar degree (data not shown). These findings suggest that TNFα/zVAD at least partially stimulates release of AA in a cPLA$_2$α-independent manner from L929 cells, and the release in C12 cells is not mediated by cPLA$_2$α.

4.2. *Possible role of secretory PLA$_2$ in the release of AA by TNFα/zVAD*

Under our conditions, general inhibitors of PLA$_2$ such as mepacrine inhibited the TNFα/zVAD-induced release of AA. The release of AA by TNFα/zVAD in L929 and L929-cPLA$_2$α-siRNA cells was inhibited by dithiothreitol (Table 2), which decreases secretory PLA$_2$ activity by reducing disulfide bridges in the molecules [24,25]. It is reported that ROS including H_2O_2 and *tert*-butyl hydroperoxide stimulated the release of AA via PLA$_2$-mediated pathways in cells, not by cell toxicity [17,22,30,34]. Treatment with *tert*-butyl hydroperoxide, which is shown to stimulate the release of AA via type IIA-secretory PLA$_2$ in rabbit platelets [29], caused the release of AA from C12 and L929 cells to a similar degree (Fig. 7). Treatment of C12 cells (and L929 cells) with H_2O_2 alone for 6 h stimulated release of AA in a pyrrophenone-insensitive manner, as described in Section 3. These findings and reports suggest a possible role for secretory PLA$_2$ in the TNFα/zVAD-induced release of AA from C12 cells.

It is reported that treatment of L929 cells with TNFα alone increased ROS production moderately, and that inhibition of caspases by pharmacological reagents including zVAD markedly enhanced ROS production in TNFα-stimulated L929 cells [2,5,7,9]. The responses with and without zVAD were inhibited by an antioxidant, BHA. In this study, the release of AA by TNFα alone in L929 cells and the responses by TNFα/zVAD in both L929 and C12 cells were markedly and significantly

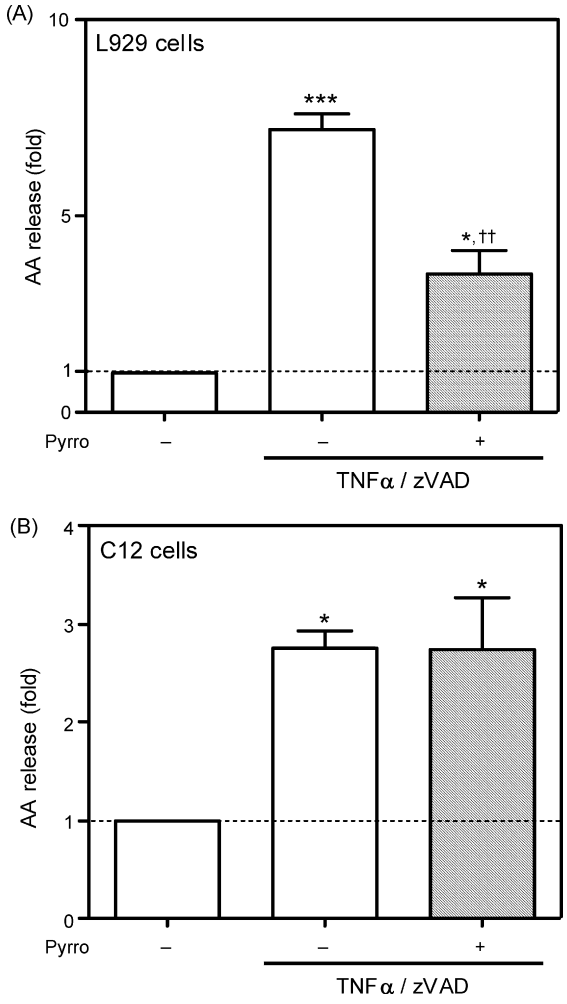

Fig. 4 – Pyrrophenone-sensitive and -insensitive release of AA induced by TNFα/zVAD. L929 cells (Panel A) and C12 cells (Panel B) were pretreated with vehicle or 10 μM pyrrophenone (Pyrro) for 30 min, and then treated with vehicle or TNFα/zVAD (10 nM and 10 μM, respectively) for 6 h. The release of AA is normalized as a percentage of the respective control value. Data are means ± S.E.M. for seven to nine independent experiments. *P < 0.05, ***P < 0.001, significantly different from the control. ††P < 0.01, significantly different from that without pyrrophenone.

inhibited by BHA. Also, the response in L929 cells was inhibited by another antioxidant, LY231617. These findings suggest that BHA-sensitive ROS may regulate TNFα-induced release of AA both in the presence and absence of zVAD in L929 (and C12) cells. It is probable that moderate amount of ROS preferentially activates cPLA$_2$α, and large amount of ROS activates both cPLA$_2$α and secretory PLA$_2$ in L929 cells. Caspases, especially caspase-8, come out both as a signal transducer for NF-κB during the early response to stimuli and as a pivotal molecule for death signaling [35,36]. The expression of ROS-related proteins may be changed by caspase inhibition. There is another possibility. Ceramide increased the activity of secretory PLA$_2$ in vitro [37], and that sPLA$_2$ activity in cells is sensitive to the dynamics of membranes such as levels of

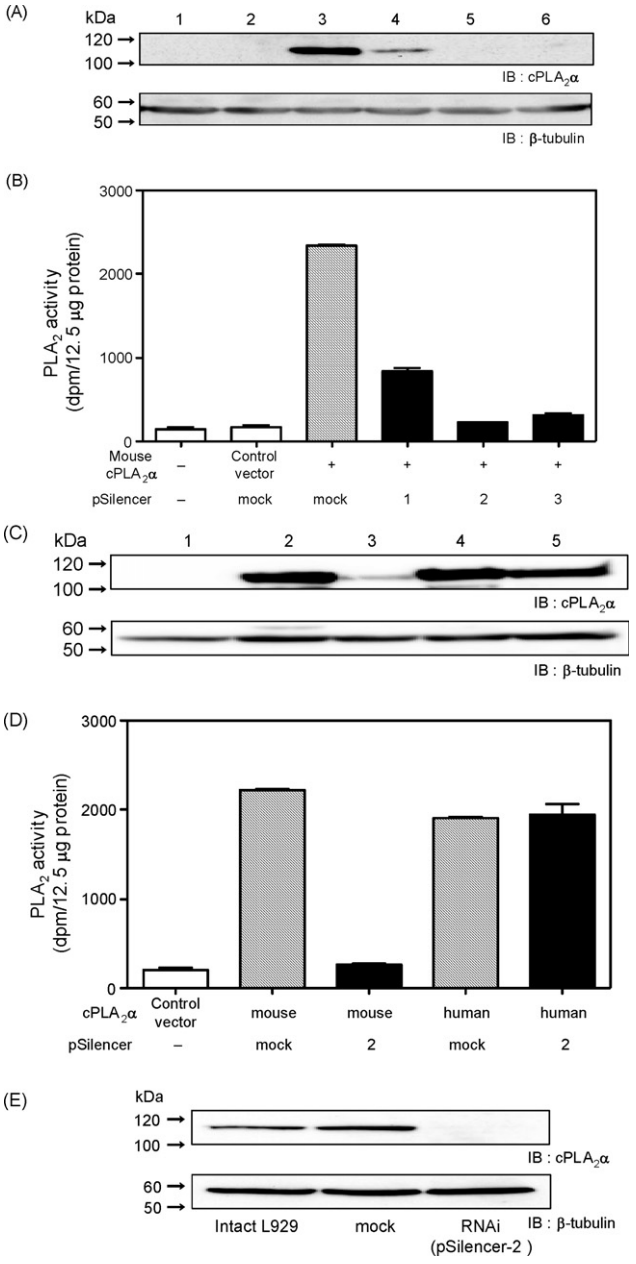

Fig. 5 – The effects of siRNAs for mouse cPLA$_2$α on levels of cPLA$_2$α in HEK293 and L929 cells. In Panels (A) and (B), HEK293 cells were transfected with vectors for mouse cPLA$_2$α and/or the indicated antisense oligonucleotides (pSilencer Nos. 1–3). The sample in each lane in Panel (A) corresponds to each column in Panel (B). In Panels (C) and (D), HEK293 cells were transfected with vectors for mouse and human cPLA$_2$α and/or pSilencer No. 2 of mouse cPLA$_2$α. The sample in each lane in Panel (C) corresponds to each column in Panel (D). Panels (A) and (C) show protein levels of cPLA$_2$α (upper panels), and β-tubulin (lower panels) for the control. Panels (B) and (D) show the enzyme activities. In Panel (E), L929 cells were transfected with control vector or vector for pSilencer No. 2, and protein levels of cPLA$_2$α (upper panel) and β-tubulin (lower panel) were estimated. Data are from a typical experiment repeated two or three times.

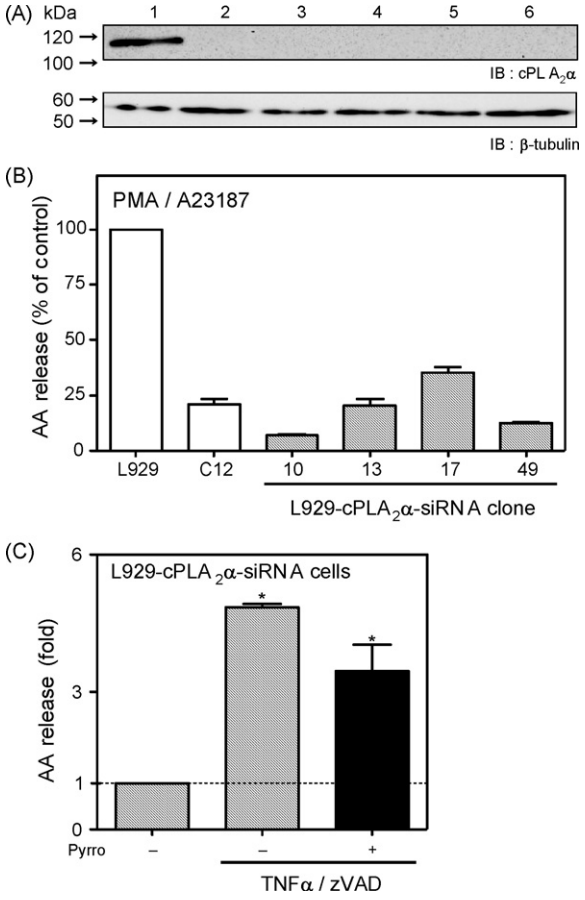

Fig. 6 – The release of AA from stable clones of L929 cells transfected with the vector for pSilencer No. 2. Panel (A) shows cPLA$_2$α (upper) and β-tubulin (lower) levels in native L929 cells (lane 1), C12 cells (2), and L929-cPLA$_2$α-siRNA cells (lanes 3–6, Nos. 10, 13, 17 and 49 clone, respectively). In Panel (B), L929, C12, and L929-cPLA$_2$α-siRNA cells were stimulated with PMA/A23187 (100 nM and 10 μM, respectively) for 30 min. The net increase of AA (% of control) induced by PMA/A23187 in the cells is shown as a percentage of that in L929 cells. Data in Panels (A) and (B) are from a representative experiment repeated two times with similar results. In Panel (C), labeled L929-cPLA$_2$α-siRNA cells (clone 49) were pretreated with vehicle or 10 μM pyrrophenone for 30 min, and then further incubated with TNFα/zVAD (10 nM and 10 μM, respectively) for 6 h. Data are means ± S.E.M. for three independent experiments. *P < 0.05, significantly different from the control. Similar results were obtained for other clones.

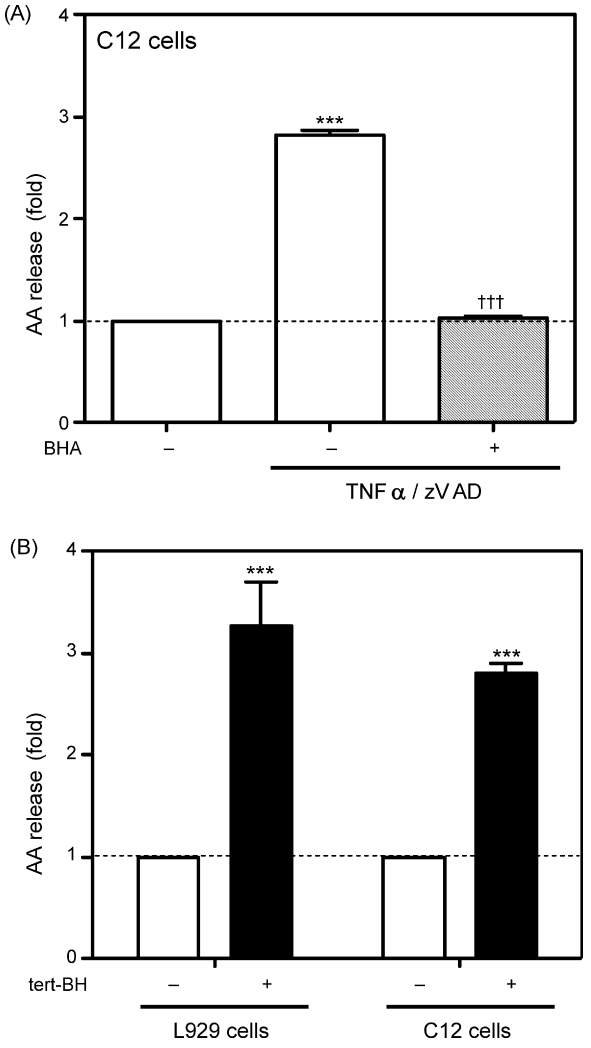

Fig. 7 – Effects of BHA and *tert*-butyl hydroperoxide on release of AA from C12 cells. In Panel (A), labeled C12 cells were incubated with TNFα/zVAD (10 nM and 10 μM, respectively) in the presence or absence of 50 μM BHA for 6 h. In Panel (B), labeled L929 and C12 cells were incubated with 5 mM *tert*-butyl hydroperoxide (*tert*-BH) for 6 h. Data are means ± S.E.M. for three to four independent experiments performed in triplicate. *P < 0.05, significantly different from the control.

ceramide [24,38]. It is reported that treatment of L929 cells with TNFα increased levels of ceramide [1,12,39], and that zVAD markedly enhanced the response 3 h after the treatment [6]. The role of ceramide in the TNFα/zVAD-induced release of AA and/or ROS production remains to be elucidated. The amounts of AA released by TNFα/zVAD in L929, C12, and L929-cPLA$_2$α-siRNA cells were not affected by a cell-permeable inhibitor of Ca^{2+}-independent PLA$_2$ (BEL). However, we cannot exclude a possible role of the enzyme at present, since it is reported that BEL changed phospholipid metabolism via inhibition of phosphatidate phosphohydrolase-1 [40], and that ROS including H_2O_2 induced the release of AA via mainly a Ca^{2+}-independent PLA$_2$-mediated pathway in several cells [22,34].

4.3. *Different effects of BHA and NAC on release of AA and cell death induced by TNFα/zVAD*

Oxidative/redox conditions including GSH metabolism, ROS production, and lipid-peroxidation are dependent on subcellular compartments in cells including L929 cells [41–43]. It is reported that cell death induced by stimuli was inhibited by lipid soluble antioxidants including BHA, whereas water-soluble antioxidants including NAC and GSH turned out to be

ineffective in several cell types [44,45]. Treatment with BHA inhibited TNFα/zVAD-induced morphological change (data not shown) and cell death (Table 3) in L929 cells, as reported previously [2,5,26]. In the present study, treatment with BHA inhibited the release of AA induced by TNFα/zVAD from L929 (Table 3) and C12 cells (Fig. 7), and morphological change in C12 cells (Fig. 2). Treatment with LY231617, another lipid soluble antioxidant, markedly decreased the amount of AA released by TNFα/zVAD in L929 cells. By contrast, treatment with NAC did not inhibit the release of AA induced by TNFα/zVAD in L929 and C12 cells. NAC increases intracellular cysteine pools necessary for cytosolic GSH synthesis and has a limited effect at preventing lipid-peroxidation in membranes [46]. Culture with NAC or GSH decreased cell death 6 h after TNFα/zVAD treatment in L929 and C12 cells, but did not affect the release of AA triggered by the stimuli in this study. Similarly, treatment with GSH inhibited cell death but not the release of AA induced by TNFα in L929 cells [47]. It is proposed that lipid soluble antioxidants including BHA, not water-soluble antioxidants including NAC and cytosolic GSH, act as ROS scavenger that can enter mitochondria [2,26]. Thus, TNFα with zVAD may cause increases of ROS levels in two types of pools; a BHA-sensitive and GSH-insensitive pool such as mitochondria and/or intracellular organelles that are essential for the release of AA, and a GSH-sensitive and cytosolic pool regulating cell death. Recently, it was reported that TNFα induced an unfolded protein response in a ROS-dependent fashion, but exogenous addition of H_2O_2 caused no such response [4]. ROS in different pools may have different functions in cells, and GSH-insensitive ROS appeared to be crucial to the release of AA induced by TNFα/zVAD in C12 and L929 cells.

Exogenous treatment with μM concentrations of AA has been shown to be toxic to many cell types because of production of ROS and/or change of the mitochondrial membrane potential, although the reactivity was dependent on conditions [48,49]. We previously reported AA-induced cell toxicity in neuronal cells [50], but AA at 20 μM did not show a marked toxic effect on L929 and C12 cells in the presence of serum (data not shown). In addition to the identification of ROS pools, the target pathways and/or molecules for AA-mediated cell toxicity should be determined. ROS, secretory PLA_2 and cytokines including TNFα are proposed to play roles in the pathophysiology of various diseases including atherosclerosis [51], and the existence of patients with caspase-8 deficiency state is reported [35,36]. Our findings may be useful in elucidating the pathological effects of TNFα, and the release of AA and ROS in cells.

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