Specificity of endogenous fatty acid release during tumor necrosis factor-induced apoptosis in WEHI 164 fibrosarcoma cells

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Abstract Recombinant tumor necrosis factor alpha (rTNF-α)-induced release of endogenous fatty acids was examined in WEHI 164 clone 13 fibrosarcoma cells using a highly sensitive HPLC method. The initial rTNF-α-induced extracellular release of endogenous fatty acids was dominated by 20:4n–6, 22:4n–6, 24:4n–6, and 18:1n–9 showing relative rates of 2.9, 0.9, 1.1, and 1.0, respectively. Release of endogenous AA and DNA fragmentation occurred simultaneously and preceded cell death by approx. 2 h. Methyl arachidonoyl fluorophosphonate and LY311727, specific inhibitors of Ca^{2+}-dependent cytosolic PLA_2 ($cPLA_2$) and secretory PLA_2 ($sPLA_2$), respectively, neither blocked rTNF-α-induced cytotoxicity or endogenous AA release. However, both inhibitors reduced rTNF-α-induced release of other endogenous fatty acids. In comparison, the antioxidant butylated hydroxyanisole (BHA) completely inhibited the rTNF-α-induced cytotoxicity as well as AA release mediated through the TNF receptor p55, while the very similar antioxidant butylated hydroxytoluene had no effect. BHA did not inhibit recombinant $cPLA_2$ or $sPLA_2$ enzyme activity in vitro. Furthermore, stimulation of cells with rTNF-α for 4 h did not increase $cPLA_2$ enzyme activity. The data indicate that neither $cPLA_2$ or $sPLA_2$ mediate rTNF-α-induced apoptosis and extracellular AA release in WEHI cells. The results suggest that a BHA-sensitive signaling pathway coupled to AA release is a key event in TNF-induced cytotoxicity in these cells.—Brekke, O-L., E. Sagen, and K. S. Bjerve. **Specificity of endogenous fatty acid release during tumor necrosis factor-induced apoptosis in WEHI 164 fibrosarcoma cells.** ***J. Lipid Res.*** **1999.** 40: **2223–2233.**

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Tumor necrosis factor (TNF) plays a role in inflammation, is cytotoxic to several cancer cells, and enhances growth in normal fibroblasts (1), but it is only partly known why TNF have different effects in different cells. The two specific TNF receptors (TNFR)s p55 and p75 (reviewed in ref. 2), have different intracellular domains suggesting that they activate different intracellular signaling mechanisms. TNFR p55 mediates the cytotoxicity signal in most (3, 4), and the p75 receptor in some cell lines (5). TNFR p55 contains a region near its intracellular C-terminus called the death domain. Binding of TNFR-associated factor 2 to the activated TNFR signals the activation of NF-κB as well as c-Jun N-terminal kinase (JNK), also called stress-activated protein kinase (SAPK), which seems to be separately regulated (6). Activation of κB elements mediates the transcriptional activation of the zinc finger protein A20 (7), which protects against TNF-cytotoxicity. Manganous superoxide dismutase (8) and the major heat shock protein hsp70 (9) also protect against TNF-induced cytotoxicity. Furthermore, TNF stimulates the synthesis of hydroxyl radicals (10) and increase lipid peroxidation (11). TNF-stimulation of reactive oxygen intermediates leads to necrotic cell death in L929 cells (12). Activation of certain proteolytic caspases is probably involved in protection against TNF-induced free radicals and necrosis in L929 cells (13).

TNF also activates other signal transduction pathways including phosphatidylcholine specific phospholipase C, protein kinase C, and phospholipase A_2 (PLA_2) (14). Several

Abbreviations: AA, arachidonic acid; [^{3}H]AA, [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid; Ab-p55, hamster agonistic monoclonal antibody clone 55R-593 against mouse TNF-receptor p55; Ab-p75, rat agonistic monoclonal antibody clone HM102 against mouse TNF-receptor p75; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; $cPLA_2$, Ca^{2+}-dependent cytosolic group IV phospholipase A_2; FCS, fetal calf serum; FCS-M, 10% (v/v) heat-inactivated fetal calf serum in RPMI-1640 medium supplemented with 2 mm l-glutamine and 40 mg/l gentamicin; HAc, acetic acid; HPLC, high performance liquid chromatography; JNK, c-Jun N-terminal kinase; LY311727, 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy)propane phosphonic acid; MAFP, methyl arachidonoyl fluorophosphonate; MAPK, mitogen-activated protein kinase; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); PBS, Dulbecco's phosphate-buffered saline without calcium and magnesium; rTNF-α, recombinant tumor necrosis factor alpha; SAPK, stress-activated protein kinase; $sPLA_2$, Ca^{2+}-dependent secretory group II phospholipase A_2; TNF, tumor necrosis factor; TNFR p55 and p75, 55 and 75 kilodalton TNF receptors; WEHI cells, WEHI 164 clone 13 murine fibrosarcoma cells.

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studies indicate that TNF-α-induced cytotoxicity is associated with release of arachidonic acid (AA), probably due to activation of phospholipases (11,15–17). The TNF-induced PLA_2 activity is probably coupled to G-proteins in osteoblast-like cells (18). TNF-resistant C3HA cells can be made sensitive to TNF cytotoxicity by inhibitors of transcription and translation, with a simultaneous increase in PLA_2 activity (19). Three calcium-dependent cellular PLA_2s have been described. The calcium-dependent 85 kilodalton cytosolic group IV PLA_2 ($cPLA_2$) is highly selective for AA in the *sn*-2 position and is activated by phosphorylation at micromolar Ca^{2+} concentrations (reviewed in ref. 20). The two closely related secretory PLA_2 ($sPLA_2$) enzymes, group II and V, act extracellularly at millimolar Ca^{2+} and are not specific for unsaturated fatty acids in the *sn*-2 position (reviewed in ref. 21). In addition, calcium-independent PLA_2 enzymes have been described. TNF stimulates $sPLA_2$ in mesangial cells (22) and TNF plus interleukin-1β induced delayed $sPLA_2$ synthesis in rat 3Y1 fibroblasts (23). However, TNF induces $cPLA_2$, but not $sPLA_2$ gene expression in bronchial epithelial cells (24). In comparison, TNF induces $sPLA_2$, but not $cPLA_2$ mRNA in osteoblasts (25), indicating cell-specific responses. Hayakawa et al. (26) showed that L929 clone C12 cells resistant to TNF-induced necrosis do not release AA after challenge with TNF due to a lack of $cPLA_2$. The fatty acid released after TNF stimulation of PLA_2 may act as intracellular signal substances, as several fatty acids can activate protein kinase C (reviewed in ref. 27) and AA activates MAPK (28). However, stimulation of AA release by serum is in itself not cytotoxic to L929 cells (17), suggesting that AA release is a necessary but not sufficient event in TNF-induced cytotoxicity. TNF-induced growth enhancement in FS-4 fibroblasts is also associated with enhanced release of AA (29). The same study showed that TNF is cytotoxic towards human FS-4 fibroblasts in the presence of 50 μm AA and that indomethacin inhibited this toxicity, suggesting that cyclooxygenase metabolites of AA were involved (29).

Eicosanoids are probably also involved in the TNF-induced modulation of gene transcription since TNF regulates CSF-1 mRNA levels in HL-60 cells through prostaglandin E_2 synthesis (30). Furthermore, TNF enhances c-fos mRNA through the lipoxygenase metabolite 5-HPETE in TA1 cells (31). Inhibitors of the cyclooxygenase and lipoxygenase pathways reduce TNF-induced cytotoxicity in some cell lines, but in WEHI 164 clone 13 cells (WEHI cells) these metabolites are probably not involved (32). This suggests that the involvement of AA metabolites may be different in different cell lines. Previous studies on TNF-induced cytotoxicity and release of fatty acids have only used prelabeling of cells with radiolabeled fatty acids, mostly AA. The result is that only TNF-induced AA and AA metabolites can be detected as the release of other endogenous fatty acids cannot be analyzed. However, Thorne et al. (33) showed that TNF enhanced the release of $[^3H]$AA and $[^3H]$16:0 in adenovirus-infected C3HA cells, in agreement with selective activation of $cPLA_2$.

We have recently developed a highly sensitive HPLC method to quantitate agonist-induced release of endogenous fatty acids in cultured cells at the pmol level (34). This study is the first to examine the specificity of endogenous fatty acid release and the involvement of $cPLA_2$ and $sPLA_2$ during TNF-induced apoptosis in WEHI cells. We stimulated WEHI cells with recombinant TNF-α (rTNF-α) or agonistic antibodies against TNFR p55 or p75 in the presence or absence of inhibitors with high selectivity towards $cPLA_2$ or $sPLA_2$ and measured the release of endogenous fatty acids, $[^3H]$AA release, and TNF-induced cytotoxicity. We found that rTNF-α stimulates the release of several endogenous fatty acids including AA, 22:4n–6, 24:4n–6, 18:1n–9, 16:0, and 18:0, which suggests that TNF do not selectively activate $cPLA_2$ in WEHI cells. Furthermore, rTNF-α stimulation of WEHI cells did not increase $cPLA_2$ enzyme activity. TNF-induced cytotoxicity was still associated with endogenous AA release. The antioxidant butylated hydroxyanisole (BHA) blocked the TNF-induced cytotoxicity and AA release signal mediated through the TNFR p55 but did not inhibit $cPLA_2$ or $sPLA_2$ enzyme activity in vitro. Specific $sPLA_2$ and $cPLA_2$ inhibitors neither inhibited TNF-induced cytotoxicity nor AA release indicating that these enzymes do not mediate TNF-induced AA release and apoptosis in WEHI cells. The presence of another BHA-sensitive signaling pathway coupled to AA release is discussed.

MATERIALS AND METHODS

Materials

RPMI-1640, Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS), l-glutamine, trypsin solution (0.025%), and fetal calf serum were obtained from Gibco (Life Technologies, Paisley, UK). Fatty acid-free bovine serum albumin (BSA), BHA, butylated hydroxytoluene (BHT), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). Fatty acids were obtained from Sigma, Larodan Fine Chemicals Ab (Sweden) or Nu Chek Prep (Elysian, MN). Fatty acids and antioxidants were dissolved in 96% ethanol from Arcus Produkter A/S (Oslo, Norway) and stored under N_2 at −70°C and −20°C, respectively, to prevent oxidation. Gentamicin sulfate and 12-epi-scalaradial were obtained from Schering Corp. (Kenilworth, NJ) and Biomol (Plymouth Meeting, PA), respectively. Methyl arachidonoyl fluorophosphonate (MAFP) and arachidonoyl trifluoromethyl ketone ($AACOCF_3$) were both obtained from Cayman Chemical Company (Ann Arbor, MI). The specific $sPLA_2$ inhibitor 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy)propane phosphonic acid (LY311727), which interacts non-covalently with the active site of the enzyme, was generously provided by Dr. G. Camejo, Astra Hässle (Gothenburg, Sweden) and was dissolved in ethanol. Both [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (specific activity 210 Ci/mmol) and [methyl-^{3}H]thymidine were purchased from Amersham (Buckinghamshire, UK). Flow-Scint III and Optifluor® were obtained from Packard (Downer's Grove, IL). Recombinant murine rTNF-α (specific activity 8×10^7 U/mg) was a generous gift from Genentech Inc. (South San Francisco, CA). The agonistic hamster monoclonal antibody clone 55R-593 against mouse TNFR p55 (Ab-p55) was obtained from Genzyme (Cambridge, MA.). The agonistic rat monoclonal antibody clone HM102 against mouse TNFR p75 (Ab-p75) was from HyCult biotechnology b.v (Uden, The Netherlands).

Cell culture

The highly TNF sensitive WEHI 164 clone 13 murine fibrosarcoma cell line was cultured as previously described (32). Cells were grown at 37°C in 10% (v/v) heat-inactivated fetal calf serum in RPMI-1640 medium supplemented with 2 mm l-glutamine and 40 mg/l gentamicin (FCS-M) as previously described (32). Ethanol at a final concentration of 0.05% (v/v) did not inhibit cell growth.

Measurement of rTNF-α-induced release of endogenous fatty acids

Unless otherwise indicated, WEHI cells were seeded at a density of 0.5×10^6 cells per well in 60-mm petri dishes from Costar (Cambridge, MA) using 1.5 ml FCS-M. After 4 h, cells received 1.5 ml FCS-M with or without 50 μm fatty acid dissolved in ethanol as indicated. Preincubation with 18:2n–6 or 20:4n–6 did not affect cell growth (32, 34). After 44 h further incubation, the medium was removed and cells were washed four times with 2 ml RPMI-1640 to remove extracellular fatty acids. rTNF-α and/or inhibitors were then added in 2 ml RPMI-1640 containing 0.1 g/l fatty acid-free BSA. At the times indicated, the culture medium was collected, centrifuged, and stored at −70°C. Fatty acids were extracted, derivatized with 1-pyrenyldiazomethane from Molecular Probes (Eugene, OR), and analyzed using HPLC with fluorescence detection essentially as described (34). The detection limit was approx. 20 fmol using a Hewlett-Packard HPLC instrument, compared to approx. 1 pmol using the Waters instrument (34).

Cell survival assay

Briefly, 2×10^3 cells were transferred into microtiter wells (Costar) using 100 μl FCS-M/well. After 4 h incubation, 100 μl FCS-M was added. After 48 h total incubation, the medium was changed and rTNF-α or agonistic antibody was added in the presence or absence of inhibitors as indicated. The incubation was continued for another 22 h and the MTT assay was performed as described previously (32).

Assay of extracellular [^{3}H]AA release and HPLC analysis of [^{3}H]AA metabolites

WEHI cells were seeded in 60-mm petri dishes or 35-mm wells (Costar) at a density of 0.5 or 0.23×10^6 cells/well, and using 3 or 1.5 ml FCS-M, respectively. After 24 h the medium was changed to FCS-M containing 1% FCS and [^{3}H]AA (1 mCi/l) and the incubation continued 24 h. Thereafter, cells were washed four times to remove extracellular [^{3}H]AA as described previously (17). Cells received inhibitors approx. 30 min before rTNF-α stimulation as indicated in FCS-M containing RPMI-1640 without phenolic red to avoid quenching. [^{3}H]AA and metabolites were extracted using C_{18} columns and analyzed by reversed phase HPLC essentially as described previously (35) using culture media from 60-mm wells. Fractions (0.5 ml) of the HPLC eluant were mixed with Flow-Scint^R III (Packard) and counted in a LKB Wallac 1211 Rackbeta counter to increase assay sensitivity.

Incorporation of [^{3}H]AA into cell phospholipids

Cells were seeded in 60-mm wells and the medium was changed to 1% FCS-M containing [^{3}H]AA (1 mCi/l) as described above. Cell total lipids were extracted after 0, 1, 3, 6, and 24 h further incubation using a modified Bligh and Dyer technique essentially as described previously (36). Phospholipids and neutral lipids were separated by TLC using $CHCL_3$–MeOH–HAc–H_2O 81:10:45:3 (v/v/v/v) and n-hexane–diethyl ether–HAc 80:20:2 (v/v/v) containing BHT as solvents, respectively. Spots were identified using iodine vapor, scraped into scintillation vials, and counted (36).

DNA fragmentation assay

In brief, cells were incubated for 24 h and then received [^{3}H]thymidine (1 mCi/l). After 24 h incubation, cells were washed four times and stimulated with rTNF-α as indicated while still in log-phase. Radioactive ^{3}H-labeled DNA fragments were separated from intact DNA and quantitated using liquid scintillation counting essentially as described by Wright, Zheng, and Zhong (37).

Other methods

Cells were counted using a Coulter counter ZF. Cell viability was assessed using trypan blue exclusion and results were expressed as % dead cells. Protein was analyzed using Coomassie brilliant blue (38). The statistical analysis was performed using SigmaStat for Windows from Jandel Scientific Software (Erkrath, Germany). Student's *t*-test was also performed using the Statmed program obtained from Nycomed (Oslo, Norway).

RESULTS

Time course and specificity of rTNF-α-induced release of endogenous fatty acids

The time course of rTNF-α-induced extracellular release of 8 different endogenous fatty acids is shown in **Fig. 1**. These fatty acids comprised approx. 80% of the endogenous fatty acids released. After 2 h, rTNF-α increased the release of 20:4n–6, 22:4n–6, 24:4n–6, and 18:1n–9 with 285, 90, 105, and 100 pmol/well above the respective spontaneous release, respectively. In contrast, there was no rTNF-α-stimulated release of 16:0, 18:0, 14:0/16:1, or 20:3n–6 up to this time point. However, after 4 h a slight increase was seen in 16:0, 18:0, and 14:0/16:1. This indicates that although TNF stimulated the release of several different fatty acids, the initial release was relatively specific for 20:4n–6 and its chain elongation products. The rTNF-α-induced release of 18:1n–9, 22:4n–6, and 24:4n–6 were all similar and approx. 35% of 20:4n–6. The initial spontaneous release of 20:4n–6, 22:4n–6, and 24:4n–6 was clearly more rapid than that of the saturated and monounsaturated fatty acids which showed a lag of at least 30 min before appearing extracellularly. This initial rapid spontaneous release leveled off after approx. 60 min. rTNF-α stimulated a delayed release of the saturated and monounsaturated fatty acids after 4 h, suggesting that the late TNF-induced fatty acid release reflects recruitment of secondary lipolytic mechanisms (39).

To further examine the specificity of rTNF-α-induced fatty acid release, cells were enriched with 18:2n–6 or 20:4n–6 (**Table 1**). In non-enriched control cells, the major fatty acids released were 16:0, 18:0, and 18:1n–9. In cells enriched with 18:2n–6, the net rTNF-α-induced release of 18:2n–6 and 20:4n–6 were 2430 and 650 pmol/140 mm well, respectively. In comparison, in cells enriched with 20:4n–6, the net rTNF-α-induced release of 20:4n–6 was 13000 pmol/well. However, both in the 18:2n–6 and 20:4n–6-enriched cells, there was a considerable rTNF-α-stimulated release of other fatty acids as well. Enrichment with 18:2n–6 or 20:4n–6 decreased the rTNF-α-induced release of 16:0, 18:1n–9, and 14:0/16:1 (Table 1). Data for

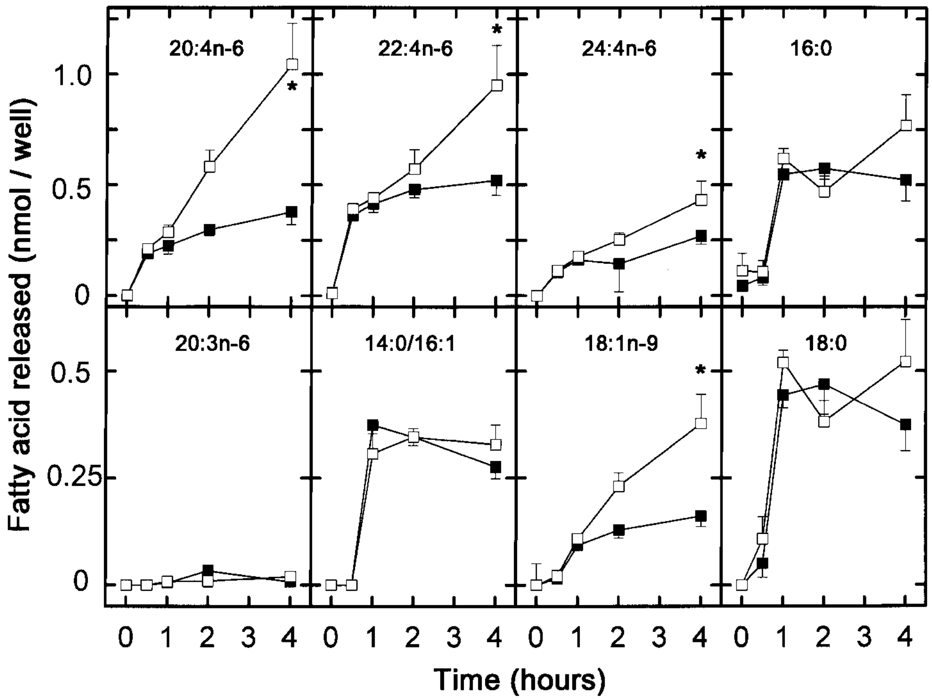

Fig. 1. Time course of rTNF-α-induced release of endogenous fatty acids in WEHI clone 13 cells. Cells were seeded in 60-mm wells and received 50 μm AA after 4 h. Incubation was then continued for another 44 h. Thereafter, the medium was changed to RPMI-1640 containing fatty acid-free BSA (0.1 g/l) with (□) or without (■) 1 μg/l rTNF-α. Fatty acids released into the medium at the indicated time intervals were analyzed after extraction and derivatization using HPLC and fluorescence detection. Results are expressed as nmol fatty acid/well and given as means ± SD of triplicates. Data from the main fatty acids are shown. * $P < 0.05$ compared to no addition using Student's *t*-test on data after 4 h incubation.

the release of n−3 fatty acids are not shown in Table 1 because the sensitivity of the fluorimetric detector was attenuated in order to see the high fatty acid concentrations in enriched cells. As shown in **Table 2**, however, the rTNF-α-induced release of 20:5n−3 and 22:6n−3 was even higher than the rTNF-α-induced release of 20:4n−6 in non-enriched cells. The conclusions were the same when the data were calculated as nmol fatty acid released/mg cell

TABLE 1. Specificity of rTNF-α-induced extracellular release of endogenous fatty acids

Fatty Acid Measured	Fatty Acid Released					
	No Addition		18:2n–6 Added		20:4n–6 Added	
	−TNF	+ TNF	−TNF	+TNF	−TNF	+TNF
	nmol/well/4 h					
16:0	1.78 ± 0.05	15.48 ± 0.94[a]	0.66 ± 0.26	2.11 ± 0.19[a]	0.93 ± 0.07	8.83 ± 0.65[a]
18:0	0.87 ± 0.04	5.94 ± 0.31[a]	1.65 ± 0.15	3.15 ± 0.66	0.78 ± 0.08	5.51 ± 0.23[a]
14:0/16:1	0.79 ± 0.08	8.69 ± 0.55[a]	1.43 ± 0.21	2.46 ± 0.03[a]	0.36 ± 0.08	3.31 ± 0.26[a]
18:1n−9	2.01 ± 0.10	22.19 ± 1.10[a]	3.01 ± 0.03	4.91 ± 0.07[a]	0.67 ± 0.02	5.51 ± 0.47[a]
18:2n−6	ND	ND	3.47 ± 0.30	5.90 ± 0.16[a]	ND	ND
20:4n−6	0.23 ± 0.007	0.90 ± 0.09[a]	1.31 ± 0.12	1.96 ± 0.15[a]	2.37 ± 0.13	15.37 ± 0.34[a]
22:4n−6	0.02 ± 0.01	0.05 ± 0.04	0.08 ± 0.05	0.39 ± 0.02[a]	2.54 ± 0.06	12.22 ± 0.10[a]
24:4n−6	ND	ND	ND	ND	1.43 ± 0.06	4.87 ± 0.13[a]
Total FA	5.3 ± 0.31	54.2 ± 3.24[a]	11.61 ± 0.001	20.9 ± 0.34	9.5 ± 0.31	58.7 ± 1.90[a]

Cells were seeded in 140-mm wells at a density of 4 × 10^6 cells in 20 ml FCS-M. Cells were preincubated with or without the indicated fatty acids (50 μm) for 44 h. The medium was then removed and cells were washed four times using RPMI-1640 to remove extracellular fatty acids. Cells were finally incubated in RPMI-1640 containing fatty acid-free BSA (0.1 g/l) with or without rTNF-α (1 μg/l). After 4 h, the cell medium was collected and centrifuged to remove loose cells. Fatty acids were extracted, derivatized with 1-pyrenyldiazomethane, and analyzed by HPLC using a low sensitivity in order to detect only the main fatty acids released. The results are given as means ± SD from one of two similar experiments performed in quadruplicate, except data for 18:2n−6 which are duplicates. Results are expressed as nmol fatty acid released/well per 4 h; ND, not detected, i.e. below the detection limit, when analyzed using the fluorescence detector at low sensitivity; FA, fatty acids.

[a] $P < 0.05$ compared to without rTNF-α in the same preincubation when tested using Student's *t*-test.

TABLE 2. Effect of inhibitors on rTNF-α-induced release of endogenous fatty acids

Fatty Acid Measured	Fatty Acid Released									
	Control (No Addition)		BHA Added		BHT Added		MAFP Added		LY311727 Added	
	−TNF	+TNF	−TNF	+TNF	−TNF	+TNF	−TNF	+TNF	−TNF	+TNF
	pmol/well									
16:0	516 ± 61	1496 ± 9[a]	400 ± 28	708 ± 15[a]	531 ± 26	1493 ± 83[a]	592 ± 11	954 ± 31[a]	666 ± 51	1244 ± 7[a]
18:0	313 ± 16	728 ± 26[a]	264 ± 36	475 ± 50[a]	328 ± 24	792 ± 67[a]	373 ± 28	497 ± 6[a]	411 ± 23	618 ± 15[a]
14:0/16:1n−9	217 ± 22	394 ± 28[a]	172 ± 16	276 ± 67	243 ± 21	379 ± 32[a]	257 ± 36	281 ± 13	292 ± 45	351 ± 5
18:1n−9	380 ± 40	1775 ± 53[a]	240 ± 20	475 ± 8[a]	385 ± 22	1570 ± 121[a]	330 ± 9	930 ± 3[a]	423 ± 37	1515 ± 55[a]
18:3n−3/n−6	1 ± 1	10 ± 3[a]	8 ± 9	2 ± 4	1 ± 1	13 ± 4[a]	6 ± 9	7 ± 2	1 ± 1	13 ± 1[a]
20:5n−3	118 ± 3	202 ± 21[a]	120 ± 8	188 ± 9[a]	136 ± 10	253 ± 9[a]	197 ± 14	184 ± 1	190 ± 17	215 ± 23
22:6n−3	147 ± 35	382 ± 160	192 ± 16	425 ± 107[a]	203 ± 53	324 ± 156	135 ± 92	109 ± 10	348 ± 118	51 ± 12[a]
18:2n−6	6 ± 2	20 ± 4[a]	10 ± 1	9 ± 8	6 ± 1	14 ± 6	8 ± 3	8 ± 1	14 ± 1	14 ± 1
20:3n−6	1 ± 1	8 ± 7	1 ± 2	ND	2 ± 0.3	ND	2 ± 2	ND	ND	ND
20:4n−6	19 ± 1	66 ± 10[a]	15 ± 3	18 ± 6	19 ± 1	91 ± 12[a]	13 ± 3	60 ± 5[a]	15 ± 4	59 ± 3[a]
22:4n−6	18 ± 2	24 ± 2[a]	17 ± 1	38 ± 18	14 ± 11	33 ± 12	15 ± 11	19 ± 5	30 ± 1	28 ± 2
Total FA	1788 ± 128	5271 ± 162[a]	1472 ± 17	2695 ± 89[a]	1924 ± 47	5154 ± 291[a]	1991 ± 138	3155 ± 108[a]	2467 ± 89	4196 ± 27[a]

WEHI clone 13 cells were seeded in 60-mm wells using FCS-M. After 48 h, cells were washed four times and incubated for another 4 h in RPMI-1640 containing BSA with or without rTNF-α (0.1 μg/l) in control medium or in medium containing the cPLA2 inhibitor MAFP (2.5 μm), the sPLA2 inhibitor LY311727 (5 μm), or the antioxidants BHA (100 μm) or BHT (100 μm) as indicated. Free fatty acids released to the culture medium were extracted, derivatized using PDAM, and analyzed using HPLC using high detection sensitivity. Results are given as means ± SD of triplicates. Minor fatty acids are not listed. FA, fatty acids; ND, not detected.

[a] $P < 0.05$ compared to control cells without TNF in each inhibitor group using Student's *t*-test.

protein (34, data not shown). The fatty acids themselves did not affect cell growth (32, 34). This further supports that the enzyme(s) involved in TNF-induced fatty acid release are not at all specific for 20:4n−6.

Time course of rTNF-α-induced release from [^{3}H]AA prelabeled cells is similar to the release of endogenous AA

Figure 2A shows the time course of spontaneous and rTNF-α-stimulated release of [^{3}H]AA from prelabeled cells. At the start of the rTNF-α-induced [^{3}H]AA release, 46%, 39%, 6%, and 4% (means, n = 3) of cell lipid [^{3}H]AA were found in ethanolamine-, inositol-, choline-, and serine-containing glycerophospholipids, respectively. Figure 2 shows that both the spontaneous as well as the rTNF-α-induced release of [^{3}H]AA were similar to that seen for endogenous 20:4n−6 (Fig. 1). This suggests that the release of endogenous 20:4n−6 as well as that of preesterified [^{3}H]AA are similarly valid indicators of TNF-induced fatty acid release. The release of endogenous fatty acids was followed using fatty acid-free BSA in the culture medium while release of [^{3}H]AA was measured using FCS-M. This does not affect the conclusion above as [^{3}H]AA release was similar in both BSA and FCS-M (data not shown). Dose–response experiments showed that the [^{3}H]AA release reached a plateau after stimulation with 1 μg/l rTNF-α after 4 h (data not shown). Further, the rTNF-α-induced release of both endogenous 20:4n−6 and [^{3}H]AA from prelabeled cells and DNA fragmentation (40, Fig. 2B) started approx. 2 h before the onset of cell death (Fig. 2C). This confirms that rTNF-α-induced release of 20:4n−6 as well as of 22:4n−6 and 18:1n−9 is one of the earlier TNF signals and not secondary to cell death. However, the signals preceding this release are presently unknown. The finding that DNA fragmentation precedes TNF-induced cell death is typical of apoptosis.

We next examined to what extent the [^{3}H]AA released extracellularly by TNF had been further converted to eicosanoids. **Figure 3A** shows that the major radioactive lipid component released after 4 h stimulation with rTNF-α was [^{3}H]AA, but significant amounts of 22:4n−6 were also released. PGE_2 was only seen after rTNF-α-stimulation. This indicates that only a minor part of the [^{3}H]AA released extracellularly after 4 h is converted to eicosanoids and suggests that quantitation of endogenous AA release gives a reliable picture of TNF-induced AA release in WEHI cells.

Specific $cPLA_2$ and $sPLA_2$ inhibitors neither inhibit rTNF-α-induced cytotoxicity nor rTNF-α-induced release of endogenous fatty acids

Figure 4A shows that the specific and irreversible $cPLA_2$ inhibitor MAFP (41, 42) had no effect on rTNF-α-induced cytotoxicity. This was confirmed using the $cPLA_2$ inhibitor $AACOCF_3$ (43), which inhibits $cPLA_2$-mediated phospholipid hydrolysis by binding to the enzyme (data not shown). The specific $sPLA_2$ inhibitors LY311727 (44, Fig. 4A) and 12-epi-scalaradial (45, data not shown) did not inhibit rTNF-α cytotoxicity. In comparison, BHA inhibited rTNF-α-induced cytotoxicity completely up to rTNF-α concentrations of 0.1 μg/l as shown previously (17, 35). This indicates that selective $cPLA_2$ inhibitors do not inhibit rTNF-α-induced toxicity under conditions where BHA completely abolishes rTNF-α cytotoxicity.

We then examined the effect of MAFP, LY311727 and the antioxidants BHA and BHT on rTNF-α-induced release of endogenous fatty acids (Table 2). In rTNF-α stimulated cells, MAFP reduced the release of total fatty acids from 5271 to 3155 pmol/well, but did not affect the spontaneous release significantly. MAFP thus reduced the rTNF-α-specific fatty acid release from 3482 to 1164 pmol/well. Interestingly, MAFP did not significantly inhibit the extracellular release of 20:4n−6, but inhibited the release of 16:0, 18:0, 18:1n−9, 20:5n−3, and 22:6n−3. LY311727

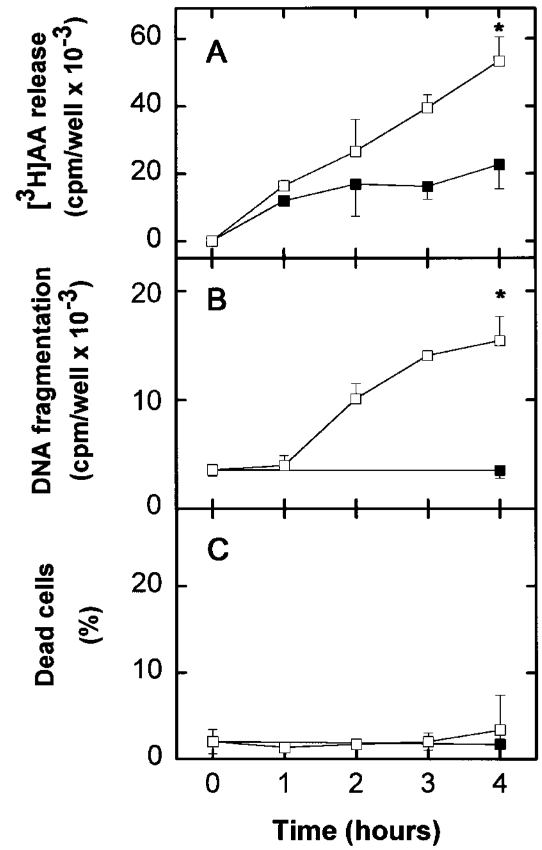

Fig. 2. Time course study indicating that rTNF-α-induced [3H]AA release (panel A) and DNA fragmentation (panel B) precedes cell death (panel C). WEHI cells were seeded in FCS-M in 60-mm wells and after approx. 24 h, the cell medium was changed to 1% FCS-M containing either A,C, [3H]AA (1 mCi/l) or B, [methyl-3H]thymidine (1 mCi/l). After 24 h further incubation, cells were washed and the medium was changed to FCS-M with (□) or without (■) 1 μg/l rTNF-α. A: Release of [3H]AA; B: 3H-labeled DNA fragmentation; and C: percent dead cells were measured as described in Experimental Procedures. Results are expressed as (A,B), cpm/well and (C), % dead cells and are given as means ± SD of triplicates, from one of two similar experiments, except from controls in B and C, which are given as means ± SD of duplicates. * $P < 0.05$ compared to no addition using Student's *t*-test on data after 4 h incubation.

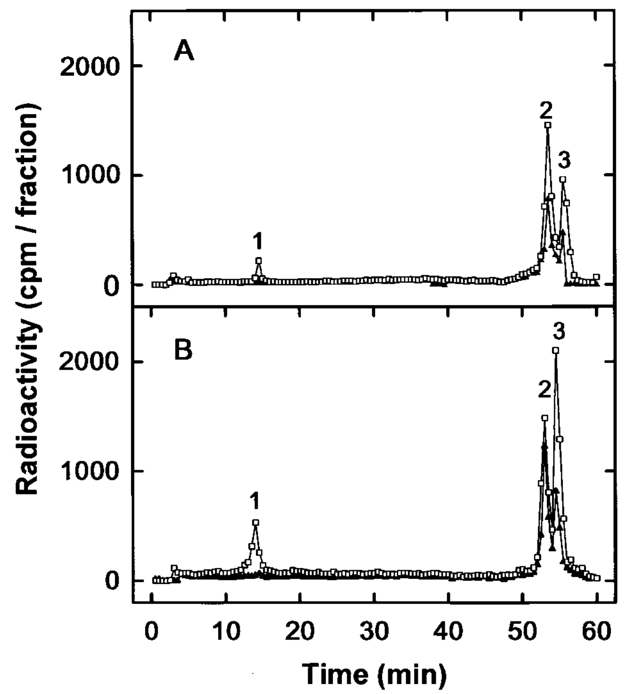

Fig. 3. rTNF-α-induced extracellular metabolites of [3H]AA in WEHI cells. Cells were prelabeled with [3H]AA for 24 h in 60-mm wells, washed, and incubated for 4 h (panel A) or 22 h (panel B) with (□) or without (▲) 1 μg/l rTNF-α. [3H]AA and metabolites were extracted and separated using reversed phase HPLC. Aliquots of 0.5 ml of the column eluate were mixed with 2.5 ml Flow-Scint III scintillation fluid and counted. Results are given as cpm/fraction per well and are from one of up to three similar experiments. Fatty acids and metabolites were identified by comparison with standards. The position of commercial standards are as follows: 1: prostaglandin E_2, **2:** 20:4n−6, **3:** 22:4n−6.

slightly increased the spontaneous release while decreasing the TNF-induced release of total fatty acids from 3482 to 1729 pmol/well. Similar to MAFP, LY311727 had no significant effect on the release of 20:4n−6, but abolished the TNF-induced release of 20:5n−3 and 22:6n−3. In comparison, BHA but not BHT totally blocked the rTNF-α-induced release of 20:4n−6 in the same way as previously shown using [3H]AA (17). BHA only partially inhibited rTNF-α-induced release of endogenous 16:0, 18:0, and 18:1n−9, but had no effect on 20:5n−3 and 22:6n−3. This suggests that BHA inhibits some enzyme(s) other than $cPLA_2$ or $sPLA_2$, and suggests that some enzyme(s) other than the AA selective $cPLA_2$ may be involved in rTNF-α-induced cell death in these cells.

The effect of $cPLA_2$ inhibitors was also confirmed using [3H]AA-labeled cells. rTNF-α enhanced the extracellular release of [3H]AA to 328% of the control after 4 h (**Table 3**). The specific $cPLA_2$ inhibitors MAFP and $AACOCF_3$ had no effect on rTNF-α-induced release of [3H]AA. This further supports the observation that $cPLA_2$ is not involved in rTNF-α-induced extracellular AA release. The $sPLA_2$ inhibitor 12-epi-scalaradial slightly increased the spontaneous release of [3H]AA, while it had no effect on the rTNF-α-induced release, further supporting that $sPLA_2$ is not involved (data not shown). To further examine which mechanism(s) are involved in rTNF-α-induced release of AA, the effects of antioxidants and the cyclooxygenase inhibitor indomethacin were examined. BHA but not BHT inhibited the rTNF-α-induced [3H]AA release completely. This shows that not all antioxidants inhibit TNF-induced release of AA in these cells, and indicates that the release of [3H]AA is a critical event in rTNF-α-induced cytotoxicity as BHA but not BHT and α-tocopherol inhibited rTNF-α-induced cytotoxicity (35, Fig. 4). In comparison, indomethacin (20 μm) and α-tocopherol (100 μm) had no effect on rTNF-α-induced release of [3H]AA (data not shown).

Control experiments showed that $cPLA_2$ enzyme activity in WEHI cytosol did not change after 4 h stimulation with rTNF-α. Neither BHA nor BHT inhibited recombinant human $cPLA_2$ activity in vitro. $AACOCF_3$ and MAFP almost

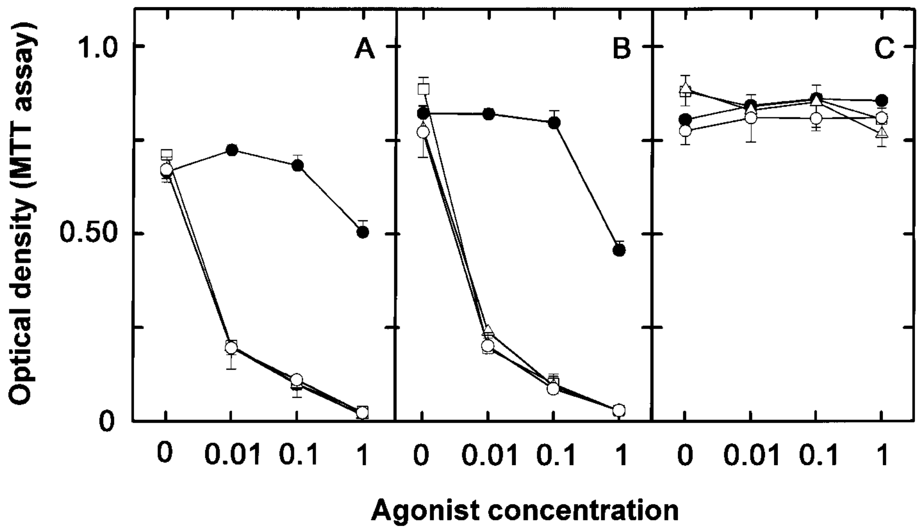

Fig. 4. Effect of the $cPLA_2$ inhibitor MAFP, $sPLA_2$ inhibitor LY311727, BHA, and BHT on cell survival after stimulating with rTNF-α or with TNFR-p55 and -p75 selective agonistic antibodies. Cells were seeded at a density of 2×10^3 cells/well in microplates. After 48 h, the medium was changed, and cells received rTNF-α (panel A), agonistic Ab-p55 (panel B), and Ab-p75 (panel C) in FCS-M containing either no addition (□), 10 μm MAFP (△), 5 μm LY311727 (▽), or 100 μm of the antioxidant BHA (●) or BHT (○). After incubation for another 22 h, optical density was measured using the MTT assay. Results are expressed as absorbance measured using the MTT assay and are given as means ± SD of quadruplicate values from one of three similar experiments.

completely blocked the recombinant $cPLA_2$, while LY311727 but not BHA or BHT blocked partially purified human $sPLA_2$ activity in vitro (data not shown).

TNFR p55 but not p75 mediates cytotoxicity and [^{3}H]AA release

We then examined the effect of specific $cPLA_2$ inhibitors on cell survival in cells stimulated with the agonistic TNFR Ab-p55 or Ab-p75. Figure 4 B and C shows that Ab-p55 but not Ab-p75 induced cytotoxicity, confirming that the TNF cytotoxicity signal is mediated through the p55 receptor in WEHI 164 cells (4). MAFP had no effect on Ab-p55-induced cytotoxicity. In comparison, BHA inhibited Ab-p55-induced cytotoxicity completely up to an Ab-p55 concentration of 0.1 mg/l. This shows that BHA inhibits the cytotoxic signal mediated through the TNFR-p55.

Figure 5 shows that only rTNF-α and Ab-p55 but not Ab-p75 stimulated the release of [^{3}H]AA. The effect of PLA_2 inhibitors and antioxidants BHA and BHT on Ab-p55-induced [^{3}H]AA release was then examined (**Fig. 6**). BHA completely inhibited Ab-p55-induced [^{3}H]AA release, while in comparison LY311727 slightly increased it. The $cPLA_2$ inhibitor MAFP and BHT reduced the Ab-p55-induced [^{3}H]AA release by 37% and 35%, respectively. Furthermore, $AACOCF_3$ increased the spontaneous release, but did not inhibit Ab-p55-induced [^{3}H]AA release (data not shown). As both rTNF-α and Ab-p55 but not Ab-p75 (Fig. 4) induced cell death, the results indicate that both cytotoxicity as well as AA release are mediated through the TNFR p55 receptor. Interestingly, this p55-mediated signal is completely and specifically inhibited by BHA, but not by specific $cPLA_2$ or $sPLA_2$ inhibitors.

TABLE 3. Effect of PLA_2 inhibitors and antioxidants on rTNF-α-induced release of [^{3}H]arachidonic acid

Addition	[^{3}H]Arachidonic Acid Release −rTNF-α	+rTNF-α
	cpm/well	
Control	10808 ± 1420[b]	35477 ± 3508[a]
MAFP (10 uM)	10861 ± 1270[b]	41584 ± 6717[a]
$AACOCF_3$ (10 uM)	13944 ± 1363[b]	35957 ± 5998[a]
BHA (100 uM)	9845 ± 2037[b]	11336 ± 533[b]
BHT (100 uM)	11288 ± 886[b]	39659 ± 4140[a]

WEHI cells were seeded at a density of 0.23×10^6 cells/well in 1.5 ml FCS-M. Approximately 24 h later, the medium was replaced with RPMI-1640 containing 1% (v/v) FCS, 2 mm l-glutamine and [^{3}H]AA (1 mCi/l) and the cells further incubated for 24 h. Thereafter, cells were washed to remove extracellular [^{3}H]AA and cells received inhibitors approx. 30 min prior to stimulation with 0.1 μg/l rTNF-α. After 4 h, the culture medium was harvested, centrifuged, and aliquots of the supernatants assayed for radioactivity. Results are expressed as cpm [^{3}H]AA released to the cell culture medium/well and are given as mean ± SD of triplicates from one of two similar experiments. Data were analyzed by one-way analysis of variance using Tukey's method for multiple comparisons.

[a] $P < 0.05$ compared to control in the absence of rTNF-α.

[b] $P < 0.05$ compared to control in the presence of rTNF-α.

DISCUSSION

This report shows that rTNF-α-induced release of endogenous fatty acids is not as specific for 20:4n–6 as previously observed in experiments using radiolabeled AA (11, 15–17). rTNF-α not only enhanced the early release of 20:4n–6, but also of 22:4n–6, 24:4n–6, and 18:1n–9 in AA-enriched cells. In non-enriched cells, the major fatty-

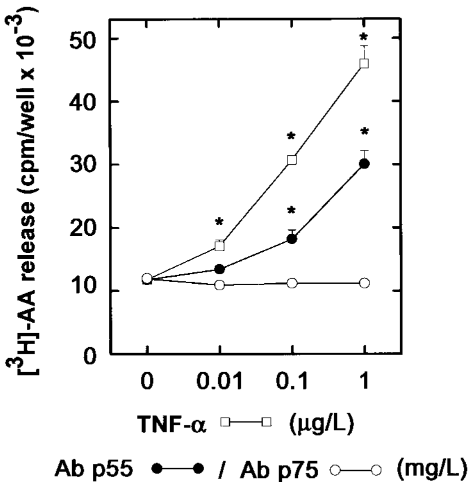

Fig. 5. Effect of increasing concentrations of rTNF-α, agonistic Ab-p55, and Ab-p75 on extracellular release of [^{3}H]AA. Cells were seeded at a density of 0.23 × 10^6 cells/well in 1.5 ml FCS-M. Approx. 24 h later, the medium was replaced with 1% (v/v) FCS-M (1 ml/well) containing [^{3}H]AA (1 mCi/l) and the cells were further incubated for 24 h. Thereafter, the cells were washed four times to remove extracellular [^{3}H]AA. Cells then received rTNF-α (□), and Ab-p55 (●) or Ab-p75 (○) as indicated. After 4 h, the culture medium was harvested, centrifuged, and aliquots of the supernatants were assayed for radioactivity using liquid scintillation counting. Results are expressed as cpm/well and are given as means ± SD of triplicates from one of two similar experiments.

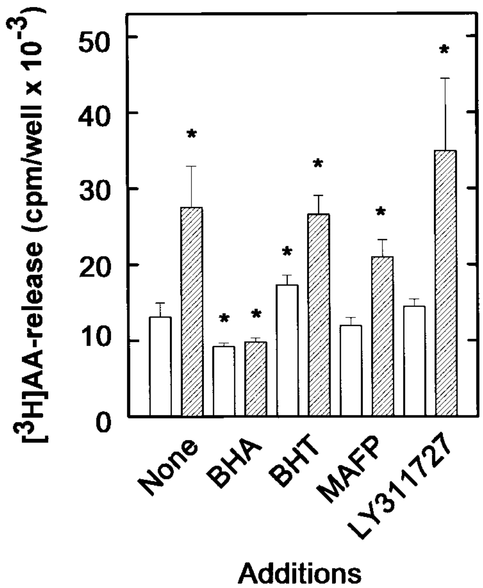

Fig. 6. Effect of specific PLA_2 inhibitors and antioxidants on TNF receptor Ab-p55-induced [^{3}H]AA release. Cells were seeded at a density of 0.23 × 10^6 cells/well in 1.5 ml FCS-M. Approx. 24 h later, the medium was replaced with 1% (v/v) FCS-M (1 ml/well) containing [^{3}H]AA (1 mCi/l) and the cells were further incubated for 24 h. Thereafter, the cells were washed four times to remove extracellular [^{3}H]AA. Cells then received FCS-M with (hatched bars) or without Ab-p55 (0.1 mg/l) (open bars) in the absence or presence of 100 μm BHA, 100 μm BHT, 10 μm MAFP, or 1 μm LY311727 as indicated. After 4 h, the culture medium was harvested, centrifuged, and aliquots of the supernatants were assayed for radioactivity using liquid scintillation spectrometry. Results are expressed as cpm/well and are given as means ± SD of six determinations from two experiments, each performed in triplicate. * $P < 0.05$ compared to no addition using Kruskal-Wallis one-way analysis of variance on ranks.

acids released were 18:1n–9, 16:0, and 18:0 while the amount of 20:4n–6 released was relatively small. rTNF-α-induced AA release and DNA fragmentation preceded cell death confirming that rTNF-α induces apoptosis in WEHI cells (40).

Several observations indicate that fatty acids like AA may act as intracellular signal molecules in TNF-induced apoptosis. Haliday, Ramesha, and Ringold (31) showed that TNF stimulated the synthesis of 5-HPETE which acted together with arachidonate itself as a signal molecule, inducing mRNA of transcription factor AP-1. The fatty acids released may activate both protein kinase C (27) and MAPK (28). It has been shown that MAPK can phosphorylate and activate $cPLA_2$ (46). Others have shown that exogenous AA increases cytosolic calcium in Balb-c 3t3 mouse fibroblasts (47), necessary for the activation of calcium-dependent phospholipase(s). Preincubation of WEHI cells with AA and certain other unsaturated fatty acids increases the sensitivity to TNF-induced cytotoxicity (32), and increased rTNF-α-induced release of endogenous AA (Table 1). Preincubation with 18:2n–6 showed none of these effects (32, Table 1). The decreased rTNF-α-induced release of saturated and monounsaturated fatty acids after enrichment with 18:2n–6 or 20:4n–6 may either be due to saturation of the fatty acid release mechanism or to some change in specificity during the reacylation process. The present report shows that although AA comprised only a minor part of the endogenous fatty acids released extracellularly by rTNF-α (Tables 1 and 2), its release was associated with rTNF-α toxicity (17, Fig. 2, Table 3). BHA inhibited both rTNF-α cytotoxicity and endogenous AA release completely, while specific $cPLA_2$ as well as $sPLA_2$ inhibitors neither inhibited rTNF-α cytotoxicity nor AA release. This indicates that $cPLA_2$ does not mediate rTNF-α-induced apoptosis in WEHI cells. Other phospholipases therefore appear to be involved. This could be an unknown phospholipase A_2. It could also involve the formation of diglyceride or phosphatidic acid after initial activation of phospholipase C or D, respectively, with the subsequent release of fatty acids from these intermediates (14). However, the specific effects of BHA still indicate that AA release is a key signal in rTNF-α-induced cell death, confirming previous reports (11, 15, 17).

The initial rTNF-α-induced extracellular release of endogenous fatty acids was not specific for 20:4n–6 as 22:4n–6, 24:4n–6, and 18:1n–9 were all released at considerable rates (Fig. 1, Tables 1 and 2). We have previously shown that these fatty acids are mainly released from phosphatidylcholine (34). However, the initial rate of AA release was approx. 2.7- to 3.3-times higher than these other fatty acids, AA thus comprising approx. 49% of the major endogenous fatty acids released by TNF after 2 h (Fig. 1). The specificity for AA after 4 h was even lower in both AA-enriched (Fig. 1, Table 1) and non-enriched cells

(Tables 1 and 2). The net rTNF-α-induced release of both 20:5n−3 and 22:6n−3 was higher than the net release of AA in non-enriched cells (Table 2). Interestingly, preincubation with 20:5n−3 and 22:6n−3, but not 18:3n−3 enhanced TNF cytotoxicity in WEHI cells (32). Palombella and Vilcek (48) also reported that TNF stimulates the release of [^{3}H]AA and [^{3}H]16:0 in BALB/c 3t3 cells. Furthermore, our data indicate that the TNF-induced release of 22:4n−6 is not caused by enriching the cells with AA as it was also found in non-enriched cells (Fig. 3, Tables 1 and 2). Release of 24:4n−6 was detected only in AA-enriched cells, probably because it is nearly absent from cell membrane phospholipids in non-enriched cells (32,34). The relatively high amount of 24:4n−6 found extracellularly after TNF stimulation could possibly be explained by its slow reesterification into cell phospholipids (49). Only a minor part of the AA released after 4 h TNF stimulation was further metabolized to prostaglandin E_2 (Fig. 3). The data indicate that TNF, also named cachectin, activates lipolytic mechanism(s) which is not specific for AA in WEHI cells. In comparison, Thorne et al. (33) showed that TNF-induced cytotoxicity in adenovirus-infected C3HA cells is associated with extracellular release of [^{3}H]AA and [^{3}H] 16:0, as expected when $cPLA_2$ is involved.

We found that $cPLA_2$ activity was present in WEHI cytosol, but did not increase after TNF stimulation (data not shown), as also found in other cell types in which $cPLA_2$ mRNA levels were studied (25). TNF-induced activation of caspases in WEHI-S cells results in both cleavage of $cPLA_2$ and enhanced AA release (50), suggesting that TNF-induced activation of $cPLA_2$ is involved in some cells. In this study, the $cPLA_2$ inhibitor MAFP and the $sPLA_2$ inhibitor LY311727 reduced TNF-induced release of total endogenous fatty acids (Table 2), but had no effect on TNF-induced release of endogenous AA (Tables 2 and 3). As the same inhibitors had no effect on TNF-induced cytotoxicity, inhibition of $cPLA_2$ and $sPLA_2$ is not sufficient to prevent TNF cytotoxicity. BHA, which nearly completely abolished TNF toxicity (Fig. 4), also completely inhibited the release of endogenous AA but had no effect on 20:5n−3 and 22:6n−3 (Table 2). BHA reduced the TNF-induced release of 16:0, 18:1n−9, and several other fatty acids comparable to MAFP. However, MAFP had no effect on either TNF cytotoxicity or AA release indicating that it is the BHA-sensitive AA release that is associated with TNF-induced cytotoxicity. The lack of an effect of MAFP on TNF-induced extracellular release of AA is probably not due to an inadequate cellular uptake as it significantly reduced the release of total fatty acids (Table 2) at concentrations known to inhibit $cPLA_2$ (41). The same report showed that $cPLA_2$ is only partly responsible for agonist-induced extracellular release of AA in macrophages (41). Although MAFP had no effect on TNF-induced release of AA, it reduced Ab-p55-induced release of [^{3}H]AA by 37% (Fig. 6). Interestingly, both MAFP and LY311727 completely inhibited the TNF-induced release of 20:5n−3 and 22:6n−3 (Table 2). MAFP inhibits both $cPLA_2$ and the calcium-independent cytosolic PLA_2 in macrophages (41, 51), suggesting that neither of these enzymes seems to be involved in TNF-induced apoptosis in the WEHI cells. This is apparantly in contrast to the report by Hayakawa et al. (26) showing that $cPLA_2$ is necessary in TNF-induced cytotoxicity and AA release in L929 cells. However, TNF induces necrotic cell death in most L929 cell clones (13, 52), although an atypical type of apoptosis has also been described (53). This is, however, different from the typical rTNF-α-induced apoptosis in WEHI cells (40, Fig. 2). The report by Atsumi et al. (54), showing that $cPLA_2$ is in fact cleaved and inactivated during FAS-mediated apoptosis in U937 cells, supports the observation that $cPLA_2$ is not always involved in apoptotic cell death. Our data show that in contrast to the specific $cPLA_2$ and $sPLA_2$ inhibitors, the antioxidant BHA completely inhibits both TNF-induced cytotoxicity as well as AA release (17, 35) mediated through the TNFR p55 (Figs. 4 and 6). This paper shows that BHA also completely blocks the TNF-induced release of endogenous AA (Table 2). The mechanisms by which BHA exerts its effects are still unknown, but could be explained either by inhibition of some AA-specific phospholipase, by blocking the activation of such a phospholipase, or by inducing protective mechanism(s) against TNF cytotoxicity. We found that BHA neither inhibits $cPLA_2$ nor $sPLA_2$ in vitro (data not shown). BHA probably does not act through a general protection against oxidative stress as the structurally very similar antioxidant BHT as well as α-tocopherol had no protective effects against TNF toxicity (35, Fig. 4) and did not reduce TNF-induced AA release (17, Table 3). However, BHA has been shown to inhibit TNF-induced mitochondrial reactive oxygen intermediates and cytotoxicity more efficiently than BHT in L929 cells (55). Yu, Tan, and Kong (56) showed that BHA rapidly activated cellular protection mechanism(s) through activation of the specific MAPK, extracellular signal-regulated protein kinase 2 (ERK2) in HeLa and Hep G2 cells. ERKs have been associated with cell growth, proliferation, or transformation. This is supported by the observation that BHA inhibits cell growth and is cytotoxic to WEHI and L929 cells at high concentrations (35). Furthermore, both BHA (56) and TNF (57) activate JNK-1, which is part of the SAPK cascade and participates in apoptosis (57, 58). A shift in the coregulation of the ceramide-induced SAPK cascade involving JNK-1 and the cytoprotective MAPK cascade involving ERK-1 and ERK-2 has been proposed to regulate the balance between induction of apoptosis or cell protection (58). We therefore speculate that BHA may activate protection mechanism(s) against its own toxicity, which also protects against TNF cytotoxicity. Because AA and 18:1n−9 rapidly induced sphingomyelin hydrolysis in HL-60 cells and AA itself activated sphingomyelinase activity in vitro (59), another explanation could be that BHA inhibition of TNF-induced AA release may block both TNF-induced ceramide synthesis and apoptosis. This is supported by Jarvis et al. (40) who showed that TNF stimulates ceramide synthesis in WEHI cells. One recent report shows that prolonged activation of JNK-1 is necessary for the TNF-induced initiation of apoptosis in rat mesangial cells and that a mitogen-activated phosphatase-1 may be involved in protecting cells from TNF-induced apoptosis by preventing prolonged JNK activation (57). We have previously shown that BHA inhibits TNF-induced cytotoxicity, but does not block the early TNF-induced NF-$_\kappa$B activation in L929 cells (35) and WEHI cells (data not shown) which is involved in protective pathways against TNF cytotoxicity (7, 60). In summary, the data presented strongly suggest that $sPLA_2$ and $cPLA_2$ do not mediate TNF-induced apoptosis in WEHI cells. BHA and BHT could therefore be used as tools to elucidate the roles of the SAPK and MAPK signaling cascades in TNF-induced apoptosis. It remains to be determined which enzymes(s) are involved in the TNF-induced release of endogenous AA in WEHI cells and why this release is associated with apoptotic cell death.

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REFERENCES

1. Sugarman, B. J., B. B. Aggarwal, P. E. Hass, I. S. Figari, M. A. Palladino, and H. M. Shepard. 1985. Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells in vitro. *Science.* **230:** 943–945.
2. Tartaglia, L. A., and D. V. Goeddel. 1992. Two TNF receptors. *Immunol. Today.* **13:** 151–153.
3. Espevik, T., M. Brockhaus, H. Loetscher, U. Nonstad, and R. Shalaby. 1990. Characterization of binding and biological effects of monoclonal antibodies against a human tumor necrosis factor receptor. *J. Exp. Med.* **171:** 415–426.
4. Loetscher, H., D. Stueber, D. Banner, F. Mackay, and W. Lesslauer. 1993. Human tumor necrosis factor α (TNFα) mutants with exclusive specificity for the 55-kDa or 75-kDa TNF receptors. *J. Biol. Chem.* **268:** 26350–26357.
5. Bigda, J., I. Beletsky, C. Brakebusch, Y. Varfalomeev, H. Engelmann, J. Bigda, H. Holtmann, and D. Wallach. 1994. Dual role of the p75 tumor necrosis factor (TNF) receptor in TNF cytotoxicity. *J. Exp. Med.* **180:** 445–460.
6. Shi, C-S., and J. H. Kehrl. 1997. Activation of stress-activated protein kinase/c-Jun n-terminal kinase, but not NF-κB, by the tumor necrosis factor (TNF) receptor 1 through a TNF receptor-associated factor 2- and germinal center kinase related-dependent pathway. *J. Biol. Chem.* **272:** 32102–32107.
7. Krikos, A., C. D. Laherty, and V. M. Dixit. 1992. Transcriptional activation of the tumor necrosis factor α-inducible zinc finger protein, A20, is mediated by κB elements. *J. Biol. Chem.* **267:** 17971–17976.
8. Wong, G. H. W., and D. V. Goeddel. 1988. Induction of manganous superoxide dismutase by tumor necrosis factor: Possible protective mechanism. *Science.* **242:** 941–944.
9. Jäättelä, M. 1993. Overexpression of major heat shock protein hsp70 inhibits tumor necrosis factor-induced activation of phospholipase A_2. *J. Immunol.* **151:** 4286–4294.
10. Yamauchi, N., H. Kuriyama, N. Watanabe, H. Neda, M. Maeda, and Y. Niitsu. 1989. Intracellular hydroxyl radical production induced by recombinant human tumor necrosis factor and its implication in the killing of tumor cells in vitro. *Cancer Res.* **49:** 1671–1675.
11. Matthews, N., M. L. Neale, S. K. Jackson, and J. M. Stark. 1987. Tumour cell killing by tumour necrosis factor: inhibition by anaerobic conditions, free-radical scavengers and inhibitors of arachidonate metabolism. *Immunology.* **62:** 153–155.
12. Goossens, V., J. Grooten, and W. Fiers. 1996. The oxidative metabolism of glutamine. A modulator of reactive oxygen intermediate-mediated cytotoxicity of tumor necrosis factor in L929 fibrosarcoma cells. *J. Biol. Chem.* **271:** 192–196.
13. Vercammen, D., R. Beyaert, G. Denecker, V. Goossens, G. Van Loo, W. Declercq, J. Grooten, W. Fiers, and P. Vandenabeele. 1998. Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *J. Exp. Med.* **187:** 1477–1485.
14. Schütze, S., D. Berkovic, O. Tomsing, C. Unger, and M. Krönke. 1991. Tumor necrosis factor induces rapid production of 1′2′diacylglycerol by a phosphatidylcholine-specific phospholipase C. *J. Exp. Med.* **174:** 975–988.
15. Hepburn, A., J. M. Baeynaems, W. Fiers, and J. E. Dumont. 1987. Modulation of tumor necrosis factor-α cytotoxicity in L929 cells by bacterial toxins, hydrocortisone and inhibitors of arachidonate metabolism. *Biochem. Biophys. Res. Commun.* **149:** 815–822.
16. Suffys, P., R. Beyaert, D. De Valck, B. Vanhaesebroeck, F. Van Roy, and W. Fiers. 1991. Tumour necrosis factor-mediated cytotoxicity is correlated with phospholipase-A_2 activity, but not with arachidonic acid release per se. *Eur. J. Biochem.* **195:** 465–475.
17. Brekke, O-L., T. Espevik, and K. S. Bjerve. 1994. Butylated hydroxyanisole inhibits tumor necrosis factor-induced cytotoxicity and arachidonic acid release. *Lipids.* **29:** 91–102.
18. Yanaga, F., M. Abe, T. Koga, and M. Hirata. 1992. Signal transduction by tumor necrosis factor α is mediated through a guanine nucleotide-binding protein in osteoblast-like cell line, MC3T3-E1. *J. Biol. Chem.* **267:** 5114–5121.
19. Hollenbach, P. W., D. L. Zilli, and S. M. Laster. 1992. Inhibitors of transcription and translation act synergistically with tumor necrosis factor to cause the activation of phospholipase A_2. *J. Biol. Chem.* **267:** 39–42.
20. Kramer, R. M., and J. D. Sharp. 1997. Structure, function and regulation of Ca^{2+}-sensitive cytosolic phospholipase A2 (cPLA2). *FEBS Lett.* **410:** 49–53.
21. Mayer, R. J., and L. A. Marshall. 1993. New insights on mammalian phospholipase A_2(s): comparison of arachidonoyl-selective and -nonselective enzymes. *FASEB. J.* **7:** 339–348.
22. Pfeilschifter, J. M., C. Schalkwijk, V. Briner, and H. van den Bosch. 1993. Cytokine-stimulated secretion of group II phospholipase A_2 by rat mesangial cells. *J. Clin. Invest.* **92:** 2516–2523.
23. Kuwata, H., Y. Nakatani, M. Murakami, and I. Kudo. 1998. Cytosolic phospholipase A_2 is required for cytokine-induced expression of type IIA secretory phospholipase A_2 that mediates optimal cyclooxygenase-2-dependent delayed prostaglandin E_2 generation in rat 3Y1 fibroblasts. *J. Biol. Chem.* **273:** 1733–1740.
24. Wu, T., T. Ikezono, C. W. Angus, and J. H. Shelhamer. 1996. Tumor necrosis factor-α induces the 85-kDa cytosolic phospholipase A_2 gene expression in human bronchial epithelial cells. *Biochim. Biophys. Acta.* **1310:** 175–184.
25. Pruzanski, W., E. Stefanski, P. Vadas, B. P. Kennedy, and H. Van den Bosch. 1998. Regulation of the cellular expression of secretory and cytosolic phospholipases A2, and cyclooxygenase-2 by peptide growth factors. *Biochim. Biophys. Acta.* **1403:** 47–56.
26. Hayakawa, M., N. Ishida, K. Takeuchi, S. Shibamoto, T. Hori, N. Oku, F. Ito, and M. Tsujimoto. 1993. Arachidonic acid-selective cytosolic phospholipase A_2 is crucial in the cytotoxic action of tumor necrosis factor. *J. Biol. Chem.* **268:** 11290–11295.
27. Nishizuka, Y. 1992. Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. *Science.* **258:** 607–614.
28. Rao, G. N., A. S. Baas, W. C. Glasgow, T. E. Eling, M. S. Runge, and R. W. Alexander. 1994. Activation of mitogen-activated protein kinases by arachidonic acid and its metabolites in vascular smooth muscle cells. *J. Biol. Chem.* **269:** 32586–32591.
29. Hori, T., S. Kashiyama, M. Hayakawa, S. Shibamoto, M. Tsujimoto, N. Oku, and F. Ito. 1989. Tumor necrosis factor is cytotoxic to human fibroblasts in the presence of exogenous fatty acid. *Exp. Cell. Res.* **185:** 41–49.
30. Sherman, M. L., B. L. Weber, R. Datta, and D. W. Kufe. 1990. Transcriptional and posttranscriptional regulation of macrophage-specific colony stimulating factor gene expression by tumor necrosis factor. Involvement of arachidonic acid metabolites. *J. Clin. Invest.* **85:** 442–447.
31. Haliday, E. M., C. S. Ramesha, and G. Ringold. 1991. TNF induces *c-fos* via a novel pathway requiring conversion of arachidonic acid to a lipoxygenase metabolite. *EMBO J.* **10:** 109–115.

32. Brekke, O. L., T. Espevik, T. Bardal, and K. S. Bjerve. 1992. Effect of n-3 and n-6 fatty acids on tumor necrosis factor cytotoxicity in WEHI fibrosarcoma cells. *Lipids.* **27:** 161–168.
33. Thorne, T. E., C. Voelkel-Johnson, W. M. Casey, L. W. Parks, and S. M. Laster. 1996. The activity of cytosolic phospholipase A_2 is required for the lysis of adenovirus-infected cells by tumor necrosis factor. *J. Virol.* **70:** 8502–8507.
34. Brekke, O. L., E. Sagen, and K. S. Bjerve. 1997. Tumor necrosis factor-induced release of endogenous fatty acids analyzed by a highly sensitive high-performance liquid chromatography method. *J. Lipid Res.* **38:** 1913–1922.
35. Brekke, O. L., M. R. Shalaby, T. Espevik, and K. S. Bjerve. 1992. Butylated hydroxyanisole specifically inhibits tumor necrosis factor-induced cytotoxicity and growth enhancement. *Cytokine.* **4:** 269–280.
36. Bleivik, B., R. L. White, and K. S. Bjerve. 1996. Protein kinase C inhibitors and PAF stimulate phosphatidylserine synthesis in human leukocytes. *J. Lipid Mediat. Cell Signal.* **15:** 29–43.
37. Wright, S. C., H. Zheng, and J. Zhong. 1996. Tumor cell resistance to apoptosis due to a defect in the activation of sphingomyelinase and the 24 kDa apoptotic protease (AP24). *FASEB J.* **10:** 325–332.
38. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
39. Balsinde, J., S. E. Barbour, I. D. Bianco, and E. A. Dennis. 1994. Arachidonic acid mobilization in $P388D_1$ macrophages is controlled by two distinct Ca^{2+}-dependent phospholipase A_2 enzymes. *Proc. Natl. Acad. Sci. USA.* **91:** 11060–11064.
40. Jarvis, W. D., R. N. Kolesnick, F. A. Fornari, R. S. Traylor, D. A. Gewirtz, and S. Grant. 1994. Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. *Proc. Natl. Acad. Sci. USA.* **91:** 73–77.
41. Balsinde, J., and E. A. Dennis. 1996. Distinct roles in signal transduction for each of the phospholipase A_2 enzymes present in $P388D_1$ macrophages. *J. Biol. Chem.* **271:** 6758–6765.
42. Huang, Z., S. Liu, I. Street, F. Lalibertè, K. Abdullah, S. Desmarais, Z. Wang, B. Kennedy, P. Payette, D. Riendeau, P. Weech, and M. Gresser. 1994. Methyl arachidonyl fluorphosphonate, a potent irreversible $cPLA_2$ inhibitor, blocks the mobilization of arachidonic acid in human platelets and neutrophils. *Med. Inflamm.* **3:** 307–308.
43. Street, I. P., H-K. Lin, F. Laliberté, F. Ghomashchi, Z. Wang, H. Perrier, N. M. Tremblay, Z. Huang, P. K. Weech, and M. H. Gelb. 1993. Slow- and tight-binding inhibitors of the 85-kDa human phospholipase A_2. *Biochemistry.* **32:** 5935–5940.
44. Schevitz, R. W., N. J. Bach, D. G. Carlson, N. Y. Chirgadze, D. K. Clawson, R. D. Dillard, S. E. Draheim, L. W. Hartley, N. D. Jones, E. D. Mihelich, J. L. Olkowski, D. W. Snyder, C. Sommers, and J. P. Wery. 1995. Structure-based design of the first potent and selective inhibitor of human non-pancreatic secretory phospholipase A_2. *Nat. Struct. Biol.* **2:** 458–465.
45. Potts, B. C., D. J. Faulkner, and R. S. Jacobs. 1992. Phospholipase A_2 inhibitors from marine organisms. *J. Nat. Prod.* **55:** 1701–1717.
46. Lin, L-L., M. Wartmann, A. Y. Lin, J. L. Knopf, A. Seth, and R. J. Davis. 1993. $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell.* **72:** 269–278.
47. Munaron, L., S. Antoniotti, C. Distasi, and D. Lovisolo. 1997. Arachidonic acid mediates calcium influx induced by basic fibroblast growth factor in Balb-c 3T3 fibroblasts *Cell Calcium.* **22:** 179–188.
48. Palombella, V. J., and J. Vilcek. 1989. Mitogenic and cytotoxic actions of tumor necrosis factor inBALB/c 3T3 Cells. *J. Biol. Chem.* **264:** 18128–18136.
49. Voss, A., M. Reinhardt, and H. Sprecher. 1992. Differences in the interconversion between 20- and 22-carbon (n-3) and (n-6) polyunsaturated fatty acids in rat liver. *Biochim. Biophys. Acta.* **959:** 296–304.
50. Wissing, D., H. Mouritzen, M. Egeblad, G. G. Poirier, and M. Jäatelä. 1997. Involvement of caspase-dependent activation of cytosolic phospholipase A_2 in tumor necrosis factor-induced apoptosis. *Proc. Natl. Acad. Sci. USA.* **94:** 5073–5077.
51. Lio, Y-C., L. J. Reynolds, J. Balsinde, and E. A. Dennis 1996. Irreversible inhibition of Ca^{2+}-independent phospholipase A_2 by methyl arachidonyl fluorophosphonate. *Biochim. Biophys. Acta.* **1302:** 55–60.
52. Grooten, J., V. Goossens, B. Vanhaesebroeck, and W. Fiers. 1993. Cell membrane permeabilization and cellular collapse, followed by loss of dehydrogenase activity: early events in tumour necrosis factor-induced cytotoxicity. *Cytokine.* **5:** 546–555.
53. Fady, C., A. Gardner, F. Jacoby, K. Briskin, Y. Tu, I. Schmid, and A. Lichtenstein. 1995. Atypical apoptotic cell death induced in L929 targets by exposure to tumor necrosis factor. *J. Interferon Cytokine Res.* **15:** 71–80.
54. Atsumi, G., M. Tajima, A. Hadano, Y. Nakatani, M. Murakami, and I. Kudo. 1998. Fas-induced Arachidonic acid release is mediated by Ca^{2+}-independent phospholipase A_2 but not cytosolic phospholipase A_2, which undergoes proteolytic inactivation. *J. Biol. Chem.* **273:** 13870–13877.
55. Goossens, V., J. Grooten, K. De Vos, and W. Fiers. 1995. Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. *Proc. Natl. Acad. Sci. USA.* **92:** 8115–8119.
56. Yu, R., T-H. Tan, and A-N. T. Kong. 1997. Butylated hydroxyanisole and its metabolite *tert*-butylhydroquinone differentially regulate mitogen-activated protein kinases. *J. Biol. Chem.* **272:** 28962–28970.
57. Guo, Y. L., K. Baysal, B. Kang, L. J. Yang, and J. R. Williamson. 1998. Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor-alpha in rat mesangial cells. *J. Biol. Chem.* **273:** 4027–4034.
58. Ariga, T., W. D. Jarvis, and R. K. Yu. 1998. Role of sphingolipid-mediated cell death in neurodegenerative diseases. *J. Lip. Res.* **39:** 1–16.
59. Jayadev, S., C. M. Linardic, and Y. A. Hannun. 1994. Identification of arachidonic acid as a mediator of sphingomyelin hydrolysis in response to tumor necrosis factor α. *J. Biol. Chem.* **269:** 5757–5763.
60. Beg, A. A., and D. Baltimore. 1996. An essential role for NF-κB in preventing TNF-α-induced cell death. *Science.* **274:** 782–784.