Involvement of oxidants and oxidant-generating enzyme(s) in tumour-necrosis-factor-α-mediated apoptosis: role for lipoxygenase pathway but not mitochondrial respiratory chain

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Cellular signalling by the inflammatory cytokine tumour necrosis factor α (TNFα) has been suggested to involve generation of low levels of reactive oxygen species (ROS). Certain antioxidants and metal chelators can inhibit cytotoxicity and gene expression in response to TNFα in numerous cell types. However, neither the source nor function of TNFα-induced oxidant generation is known. Using specific inhibitors, we ruled out involvement of several oxidant-generating enzymes [cyclo-oxygenase (indomethacin), cytochrome *P*-450 (metyrapone), nitric oxide synthase (N^G-methyl-L-arginine), NADPH oxidase (iodonium diphenyl), xanthine oxidase (allopurinol), ribonucleotide reductase (hydroxyurea)] in TNFα-mediated apoptosis of the murine fibrosarcoma line, L929. We also demonstrated no role for mitochondrial-derived radicals/respiratory chain in the lytic pathway using specific inhibitors/uncouplers (rotenone, KCN, carboxin, fluoroacetate, antimycin, malonate, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) and chloramphenicol-derived respiration-deficient cells. Significant ROS (H_2O_2, $O_2^{-\cdot}$) generation was not observed in response to TNFα in L929 cells using four separate assays. Also, prevention of intracellular H_2O_2 removal by inhibition of catalase did not potentiate TNFα-mediated cell death. These data suggest that neither H_2O_2 nor $O_2^{-\cdot}$ plays a direct role in TNFα cytotoxicity. Finally, we suggest a central role for lipoxygenase in TNFα-mediated lysis. Three inhibitors of this radical-generating signalling pathway, including an arachidonate analogue (5,8,11,14-eicosatetraynoic acid), could protect cells against TNFα. The inhibitor nordihydroguaiaretic acid is also a radical scavenger, but it could not protect cells from ROS toxicity at concentrations that effectively prevented TNFα killing. Therefore protection by nordihydroguaiaretic acid cannot be due to scavenging of cytotoxic H_2O or $O_2^{-\cdot}$. The lipoxygenase product, (12*S*)-hydroxyeicosatetraenoic acid, was also significantly protective. As this analogue can act as a substrate for certain lipoxygenases, this effect may be due to prevention of generation of physiological products.

INTRODUCTION

The inflammatory cytokine tumour necrosis factor α (TNFα) was originally described by its ability to cause apoptotic cell death in several mammalian tumour lines, for example the human HL-60 and U937 [1], and the murine fibrosarcomas L929 [2,3] and WEHI [4]. Cellular effects of TNFα are diverse and include transcription factor activation (e.g. NF-κB) and induction of numerous gene products (e.g. interleukin 6, interleukin 1 and granulocyte–macrophage colony-stimulating factor), but cytotoxicity itself is independent of both transcription and translation [5]. Inclusion of transcription or translation inhibitors in cytotoxicity assays leads to much greater sensitivity of cells to TNFα, suggesting that gene induction plays a major role in cellular protection against this cytokine. A role for reactive oxygen species (ROS), in particular superoxide ($O_2^{-\cdot}$) or H_2O_2, in TNFα-mediated lysis was postulated [6,7] following the observation that mitochondrial superoxide dismutase (MnSOD) expression could influence the sensitivity of cells to TNFα. More recently, a role for mitochondrial oxidants has been suggested [8,9]. However, available data on the effects of mitochondrial inhibitors on lysis are contradictory [8,10], and it is unclear whether mitochondrial damage is critical and causative or merely a post-lethal event [11–13]. Certain antioxidants can modulate both transcription and cytotoxicity in response to TNFα; however, indices of free-radical damage only increase at a late stage in killing [10,14]. This suggests that radical-mediated cell damage is not the primary cause of cell death. The most significant protective antioxidants are free-radical scavengers [10,14,15], metal chelators [16,17] or thiol ligands [18]. All of these have many effects on cellular systems distinct from inhibition of production of Fenton chemistry intermediates (see [19,20] for reviews).

Production of large amounts of $O_2^{-\cdot}$ (80–100 nmol/min per 10^7 neutrophils) is confined to phagocytic cells as part of the body's defence mechanism against disease processes [21]. However, generation of small amounts, at 1–2% of the phagocyte level, in response to TNFα has been demonstrated in a variety of cell types, including fibroblasts [22,23], kidney mesangial cells [24] and endothelial cells [25]. Although the involvement of the phagocyte enzyme NADPH oxidase has been suggested for some of these [26,27], in many cases neither the source nor function of this $O_2^{-\cdot}$ is known. Cellular sources of $O_2^{-\cdot}$ are numerous and include mitochondrial respiratory chain enzymes, cytochrome *P*-450 systems, nitric oxide synthase, xanthine oxidase, NADPH oxidase, lipoxygenases, cyclo-oxygenase and DNA synthesis enzymes (ribonucleotide reductase). For many of these, involvement in the TNFα-mediated generation of ROS has never been investigated.

Abbreviations used: ROS, reactive oxygen species; TNFα, tumour necrosis factor α; SOD, superoxide dismutase; NDGA, nordihydroguaiaretic acid; PDTC, pyrrolidinedithiocarbamate; TTFA, thenoyltrifluoroacetone; NMMA, N^G-methyl-L-arginine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCPIP, 2,6-dichlorophenol-indophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; ETYA, 5,8,11,14-eicosatetraynoic acid; DCF, 2′,7′-dichlorofluorescein diacetate; mtDNA, mitochondrial DNA; SDH, succinate dehydrogenase; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; NBT, Nitro Blue Tetrazolium.

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MATERIALS AND METHODS

Materials

Recombinant murine TNFα (5 μg/ml, units) and xanthine oxidase (buttermilk) were from Boehringer-Mannheim; xanthine, H_2O_2, nordihydroguaiaretic acid (NDGA), pyrrolidinedithiocarbamate (PDTC), thenoyltrifluoroacetone (TTFA), rotenone, antimycin A, myxothiazol, N^G-methyl-L-arginine (NMMA), allopurinol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lucigenin, malonate, monofluoroacetic acid, metyrapone, actinomycin D, chloramphenicol, ethidium bromide, cytochrome *c*, pyruvate, malate, succinate, decylubiquinone, 2,6-dichlorophenol-indophenol (DCPIP), digitonin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were from Sigma; (12*S*)-, (15*S*)-, (5*S*)-hydroxyeicosatetraenoic acid (HETE) and 5,8,11,14-eicosatetraynoic acid (ETYA) were from Cascade Ltd. (Reading, Berks., U.K.); curcumin, Rev-5901 (*p* isomer) and indomethacin were from Biomol; sodium salicylate was from Merck; 2′,7′-dichlorofluorescein diacetate (DCF) was from Molecular Probes; KCN and iodonium diphenyl were from Fluka. Carboxin was kindly given by Dr. M. Degli Esposti (University of Bologna, Italy).

Cell culture

L929 cells, which were originally obtained from American Type Culture Collection (Rockville, MD, U.S.A.), were a gift from Professor E. Peterhans (University of Bern, Bern, Switzerland) and were always used within the first 20 passages. They were maintained in Dulbecco's modified Eagle's medium (Gibco/BRL), supplemented with 10% heat-inactivated foetal calf serum, 2 mM glutamine, penicillin (200 units/ml) and streptomycin (100 units/ml) at 37 °C and 5% CO_2.

Cytotoxicity assay

Cells were seeded at 10^4/well into 96-well multiwell plates and allowed to adhere overnight. Actinomycin D (1 μg/ml) with or without TNFα (125 pg/ml) and known concentrations of the compound under investigation (as shown in Figure legends) were then added. After a further 24 h incubation, cell viability was assessed by measuring MTT reductase activity [28]. Briefly, MTT (0.5 mg/ml) was added and the plate incubated for 1–2 h at 37 °C and 5% CO_2 until Blue Formazan was visible. The medium was then removed and formazan solubilized with DMSO. Absorbance was read at 490–750 nm ($n = 8$ for each point).

DNA-fragmentation assay

L929 cells (1.2×10^7) were treated with TNFα (250 pg/ml) for 2–6 h, in the presence of actinomycin D (1 μg/ml). Total genomic DNA was then isolated by phenol/chloroform extraction after proteinase K and RNase digestion. It was separated on a 1.5% agarose gel and visualized under UV using ethidium bromide.

DCF assay for intracellular hydroperoxide

Cells were seeded into 96-well plates at 10^4 per well. After overnight culture, medium was removed and adherent cells were washed twice with Krebs–Ringer buffer (20 mM Hepes, 10 mM D-glucose, 127 mM NaCl, 5.5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgSO_4$, pH 7.4). Krebs buffer (100 μl) containing DCF (1 μg/ml) with or without TNFα (250 pg/ml) and the compound under investigation (as indicated on the Figures) was then added. Plates were incubated at 37 °C and 5% CO_2 and read at 15–20 min intervals on a Cytofluor 2300 plate reader, with excitation at 485 nm and emission at 530 nm ($n = 8$ for each point). To ensure that TNFα treatment of cells did not lead to alterations in intracellular availability of DCF, levels of hydrolysed probe were measured as follows: after 90 min incubation in DCF in the presence or absence of TNFα as above, cells were briefly washed once with Krebs buffer, then lysed by the addition of digitonin (0.06% in 100 μl of Krebs buffer), and hydrolysed DCF was oxidized by the addition of horseradish peroxidase (1.8 μg/ml) and H_2O_2 (100 μM). Fluorescence was then measured as above.

Assays of $O_2^{-\cdot}$ generation

Cytochrome *c* reduction

L929 cells (10^6 per cuvette) were seeded into poly-D-lysine-coated glass cuvettes and cultured overnight. Krebs–Ringer buffer (containing 80 μM cytochrome *c*) was then added and absorbance monitored at 550–540 nm using $\epsilon_{1mM} = 19.1$ [29] in an Aminco DW-2a UV–visible spectrophotometer at 37 °C, with stirring, before and after additions of TNFα (up to 5 ng/ml) in the presence or absence of superoxide dismutase (SOD) (100 μg).

Lucigenin-enhanced chemiluminescence

L929 cells (10^6 per cuvette) were seeded into poly-D-lysine-coated plastic cuvettes and cultured overnight. Krebs–Ringer buffer containing lucigenin (0.1–1 mM) was then added and luminescence monitored (model 9635 photomultiplier, EMI) at 37 °C, with stirring, before and after additions as above.

Nitro Blue Tetrazolium (NBT) reduction

L929 cells (1.6×10^5 per well) were seeded into six-well plates and cultured overnight. SOD-sensitive NBT reduction was assayed as described [30], in the presence or absence of TNFα (up to 5 ng) and SOD (100 μg).

Isolation of respiration-deficient subclones of L929 cells

Cells were cultured in Dulbecco's modified Eagle's medium containing either 50 μg/ml chloramphenicol [31] or 400 ng/ml ethidium bromide [9] with pyruvate (110 μg/ml), glucose (6 mg/ml) and uridine (5 μg/ml) [32]. At intervals (3–4 days), samples of cells were assayed for respiration deficiency. Functional criteria of respiration deficiency used include lack of respiratory enzyme activity and inability to remain viable in medium lacking supplements listed above.

Isolation of mitochondria

Mitochondria were isolated from cultured cells by the method of Rickwood et al. [33]. Briefly, cells (2×10^7–5×10^7 per sample) were harvested by treatment with trypsin, washed with Ca^{2+}/Mg^{2+}-free PBS, pelleted and frozen for 3 h at −80 °C. They were then washed once in sucrose buffer (0.25 M sucrose, 1 mM EGTA, 10 mM Hepes/NaOH, 0.5% BSA, pH 7.4) and pelleted (500 *g*; 2 min; 5 °C). The pellet was resuspended in 2 ml of sucrose buffer and homogenized in a glass/Teflon homogenizer (ten strokes at 500 rev./min). The homogenate was centrifuged at 1500 *g* for 10 min at 5 °C. The supernatant was kept aside and the pellet re-extracted as above. Finally, the two supernatants were combined and centrifuged at 10000 *g* for 10 min at 5 °C. The mitochondrial pellet was resuspended in 200 μl of sucrose buffer and mitochondrial protein (total protein minus BSA content) measured by the method of Bradford [34]. Samples were kept on ice in sucrose buffer until assay of respiratory enzymes as described below.

Spectrophotometric measurement of mitochondrial respiratory enzymes and xanthine oxidase

Where possible, mitochondrial respiratory enzyme activity was assayed in digitonin-permeabilized whole cells using cytochrome *c* as electron donor/acceptor [35,36]. For measurement of succinate dehydrogenase (SDH), it was necessary to use isolated mitochondria to avoid artificial reduction of electron acceptors by cytosolic enzymes. For assay, cells were harvested by trypsin treatment, resuspended in assay buffer (0.25 M sucrose, 20 mM Hepes, 10 mM $MgCl_2$,$6H_2O$), counted and kept on ice until assay. All assays were performed using an Aminco DW-2a UV–visible spectrophotometer at 37 °C.

Succinate–cytochrome *c* reductase activity

Succinate–cytochrome *c* reductase activity was measured by the malonate-inhibitable rate of cytochrome *c* reduction using succinate as respiratory substrate (monitored at 550–540 nm using $\epsilon_{1mM} = 19.1$ [29]). L929 cells (6×10^5) were added to 50 μM cytochrome *c* (horse heart, type 1) in 2 ml of assay buffer in a glass cuvette at 37 °C with stirring. The rate of cytochrome *c* reduction was measured in the presence of rotenone (100 nM), KCN (1 mM), FCCP (80 nM), digitonin (0.057 %) and succinate (5 mM). Malonate (10 mM) was added at the end and residual rate subtracted.

NADH–cytochrome *c* reductase activity

Activity of NADH–cytochrome *c* reductase was determined in digitonin-permeabilized cells (6×10^5) as above, using the rotenone-sensitive rate of cytochrome *c* reduction in the presence of KCN and FCCP, and with pyruvate/malate (5 mM each) as respiratory substrates. Rotenone (100 nM) was added at the end and residual rate subtracted.

Cytochrome oxidase activity

Activity of cytochrome oxidase was measured as the KCN-inhibitable rate of cytochrome *c* oxidation [36]. Briefly, cells (6×10^5) in sucrose buffer containing 50 μM ferrocytochrome *c* were mixed with rotenone (100 nM), antimycin A (5 μM) and FCCP (80 nM). Enzyme activity was initiated by the addition of digitonin (0.057 %) and rates were recorded before and after the addition of KCN (1 mM).

SDH activity

Activity of Complex II was measured as the malonate-inhibitable rate of decylubiquinone reduction [37,38] in isolated mitochondria using succinate as respiratory substrate and DCPIP as secondary electron acceptor (reduction measured at 600–780 nm). In this assay it is necessary to include small amounts of Triton X-100 to optimize partition of quinone between lipid and aqueous phases [38]. For assay, 20 μl of mitochondria isolated as described were added to a cuvette containing DCPIP (75 μM), rotenone (100 nM), FCCP (80 nM), antimycin A (5 μM), myxothiazol (40 μM), KCN (1 mM), succinate (5 mM) and EDTA (200 μM) in 2 ml of sucrose buffer at 37 °C with stirring. Reaction was initiated by the addition of decylubiquinone (50 μM). Maximum activity was achieved at 0.02 % Triton X-100. Reaction was terminated by addition of malonate (10 mM) and residual rates were recorded.

Xanthine oxidase

Activity was measured as the SOD-sensitive rate of cytochrome *c* reduction as described for $O_2^{-\cdot}$ generation, with 500 μM xanthine as substrate.

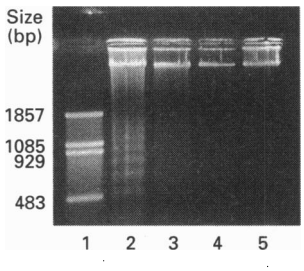

Figure 1 DNA fragmentation during TNFα-mediated lysis

L929 cells (1.2×10^7) were treated with TNFα (250 pg/ml) for 2–6 h, in the presence of actinomycin D (1 μg/ml). DNA was then isolated and electrophoresed as described in the Materials and methods section. Lane 1, markers; lane 2, DNA from cells treated for 6 h; lane 3, DNA from cells treated for 4 h; lane 4, DNA from cells treated for 2 h; lane 5, DNA from untreated controls.

RESULTS

DNA fragmentation induced by TNFα

As the mode of killing induced by TNFα may vary with cell type

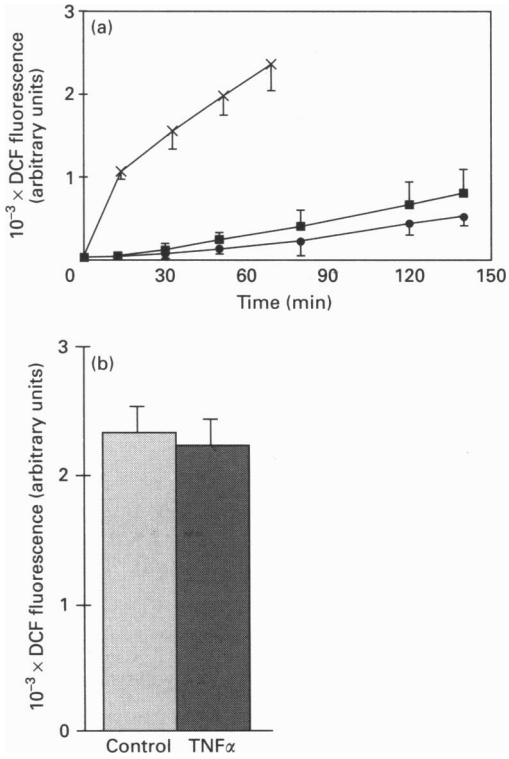

Figure 2 Effect of TNFα on DCF fluorescence

(**a**) Intracellular hydroperoxide levels. Hydroperoxide levels were determined using DCF fluorescence as described in the Materials and methods section. TNFα (250 pg/ml) was added at the same time as DCF and fluorescence measurements were taken at intervals. Results are means ± S.D. ($n = 8$). ●, Control; ■, +TNFα (250 pg/ml); ×, +195 μM H_2O_2. (**b**) Availability of DCF for oxidation. After 90 min of incubation with or without TNFα (250 pg/ml) and DCF, levels of intracellular DCF were estimated as described in the Materials and methods section. Results are means ± S.D. ($n = 8$).

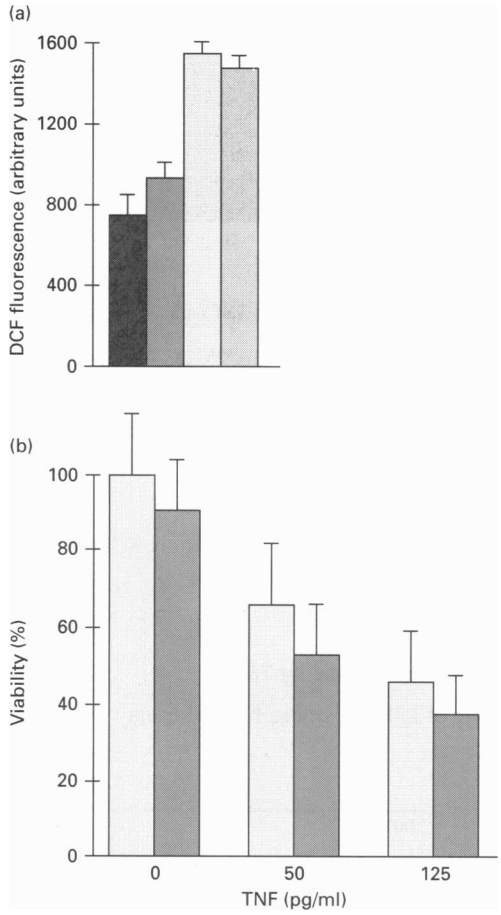

Figure 3 Effect of aminotriazole on (a) TNFα-induced DCF fluorescence and (b) TNFα-induced lysis

(**a**) TNFα (250 pg/ml) was added at the same time as DCF, with or without aminotriazole (2 mM). Data represent fluorescence at 130 min minus background due to DCF alone. Results are means ± S.D. ($n = 8$). ■, Control; ■, +TNFα (250 pg/ml); □, +aminotriazole (2 mM); ▨, +TNFα and aminotriazole. (**b**) TNFα-mediated killing was measured as described in the Materials and methods section. Aminotriazole (2 mM) was added at the same time as TNFα and viability measured after 7 h at 37 °C and 5% CO_2 using MTT reduction. Results are means ± S.D. ($n = 8$). □, Control; ■, +aminotriazole.

and cytokine dose, we examined DNA fragmentation, as a marker of apoptosis, after treatment of L929 cells with TNFα (250 pg/ml) and actinomycin D (1 μg/ml). The characteristic laddering of DNA is clearly visible after 6 h of incubation with this cytokine (Figure 1).

Effect of TNFα on oxidant production

Cellular production of ROS by L929 cells in response to TNFα was investigated using four separate assay systems. Intracellular H_2O_2 was measured with the peroxidase substrate, DCF. Small increases in fluorescence mediated by TNFα were seen, but these were not statistically significant (Figure 2a). As TNFα treatment could lead to alterations in uptake/retention of this probe, we measured levels of intracellular hydrolysed DCF in both control and cytokine-treated samples at 90 min (Figure 2b). No differences were seen, and, as total levels of available probe were well in excess of levels of oxidized probe in both control and TNFα-treated samples (Figure 2a), substrate was not limiting in this case. It is apparent that fluorescence may be slightly lower than in intact cells treated with H_2O_2 for 90 min. This most probably reflects leakage of DCF during washing of cells, as once oxidized it is poorly retained [39]. If intracellular H_2O_2 plays a role in TNFα signalling, preventing its removal should lead to potentiation of cell damage. Using the catalase inhibitor aminotriazole, we could increase basal fluorescence by 95%, but inclusion of this with TNFα neither increased fluorescence further (Figure 3a) nor potentiated TNFα-mediated lysis (Figure 3b). These data strongly argue against a role for intracellular H_2O_2 in TNFα signalling in this cell line. Three assays for $O_2^{-\cdot}$ generation (SOD-sensitive cytochrome *c* reduction, lucigenin-enhanced chemiluminescence and SOD-sensitive NBT reduction) also failed to demonstrate oxidant generation in response to TNFα (results not shown).

Involvement of mitochondria: inhibition of respiratory enzymes and sensitivity to TNFα

Recent work using cells reported to be respiration-deficient suggested a role for mitochondrially derived oxidants in TNFα signalling [9]. The protein synthesis inhibitor chloramphenicol which binds to the 70S ribosome inhibits mitochondrial but not cytosolic mRNA translation in mammalian cells [31]. Using this treatment, we generated cells with 50–80% reduced activity of mitochondrial DNA (mtDNA)-encoded respiratory enzymes (Table 1) which remained viable for eight to ten passages. SDH, which is not mtDNA-encoded, did not decrease, and in two of the lines was higher than the parental L929 cells. These lines remained fully sensitive to TNFα throughout chloramphenicol treatment (Table 1). This indicates that NADH dehydrogenase, ubiquinone–cytochrome *c* reductase and cytochrome oxidase are not required for TNFα cytotoxicity. As chloramphenicol-treated cells only lacked mtDNA-encoded enzymes, we could not rule

Table 1 Activities of mitochondrial respiratory enzymes and TNFα-sensitivity of chloramphenicol-treated L929 cells

Three separate populations of L929 cells were cultured with chloramphenicol (50 μg/ml) (C1, C2, C3). Succinate–cytochrome *c* reductase, NADH–cytochrome *c* reductase and cytochrome oxidase were measured using digitonin-permeabilized whole cells, and succinate dehydrogenase activity was determined on mitochondria isolated as described in the Materials and methods section. Numbers in parentheses are percentages of enzyme activity in control L926 cells.

	Control	C1	C2	C3
Succinate–cytochrome *c* reductase (nmol of cytochrome *c* reduced/min per 10^7 cells)	54.9	0 (0)	1.7 (3)	6.6 (12)
NADH–cytochrome *c* reductase (nmol of cytochrome *c* reduced/min per 10^7 cells)	7.8	0 (0)	0 (0)	0 (0)
Cytochrome oxidase (nmol of cytochrome *c* oxidized/min per 10^7 cells)	86.5	48.7 (56)	48.8 (56)	20.2 (23)
SDH (nmol of DCPIP reduced/min per mg of protein)	65.0	65.2 (100)	119.0 (183)	125.3 (192)
TNF sensitivity (% viability after 24 h with 50 pg/ml TNFα using MTT reductase activity)	3.3	4.7	5.4	1.8

Table 2 Effect of enzyme inhibitors on TNFα-mediated cytotoxicity of L929 cells

Viability was measured as described in the Materials and methods section at 24 h (results are means for eight samples). All compounds except fluoroacetate were added at $t = 0$ along with TNFα (125 pg/ml) and actinomycin D. Fluoroacetate was added to cells 5 h before TNFα/actinomycin D.

Compound	Action	Concentration	Viability (%)	
			−TNF	+TNF
None	–	–	100	5.8
Mitochondrial inhibitors/uncouplers				
Rotenone	NADH dehydrogenase inhibitor	500 nM	70.1	5.0
Malonate	Succinate dehydrogenase inhibitor	5 mM	63.9	5.0
Carboxin	Succinate dehydrogenase inhibitor	100 μM	94.3	3.9
Antimycin A	Cytochrome bc_1 complex inhibitor	5 μM	84.3	5.9
FCCP	Mitochondrial uncoupler	80 nM	94.0	5.8
KCN	Cytochrome oxidase inhibitor	1 mM	85.0	6.3
Fluoroacetate	Aconitase inhibitor	5 mM	100.7	5.5
Inhibitors of radical-generating enzymes				
Iodonium diphenyl	NADPH oxidase inhibitor	400 μM	55.0	2.5
Indomethacin	Cyclo-oxygenase inhibitor	100 μM	99.0	2.0
Metyrapone	Cytochrome P-450 inhibitor	500 μM	94.7	4.4
NMMA	Nitric oxide synthase inhibitor	100 μM	79.0	5.8
Allopurinol	Xanthine oxidase inhibitor	100 μM	107.0	6.2
Hydroxyurea	Ribonucleotide reductase inhibitor	1.5 mM	120.0	7.0

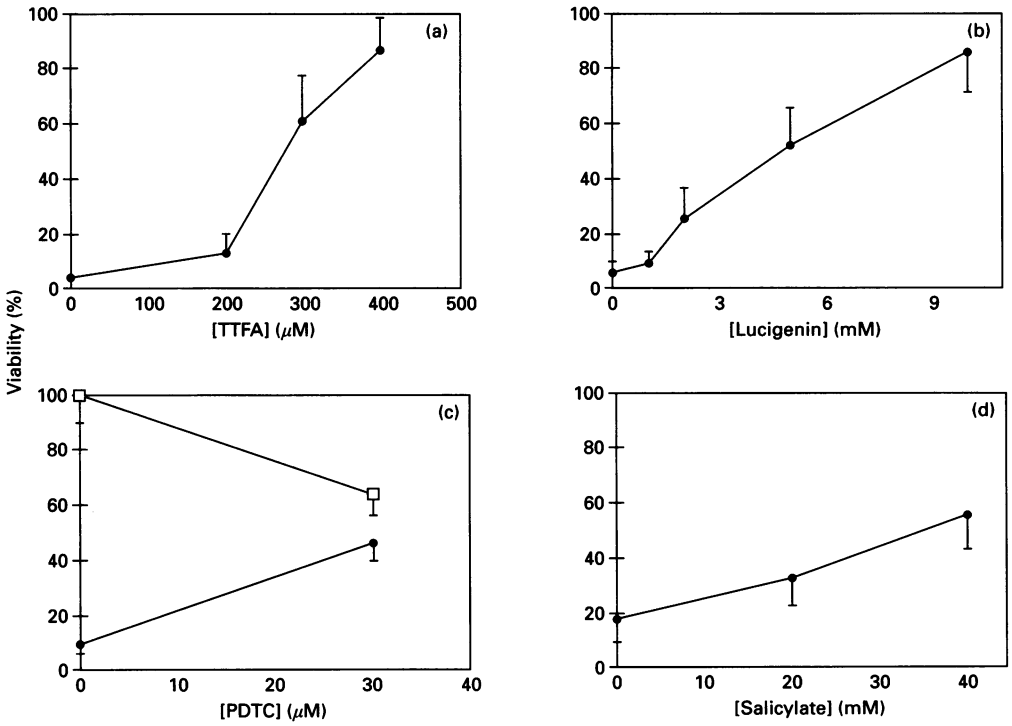

Figure 4 Protection of L929 cells against TNFα-mediated lysis by some radical scavengers and chelators

Cell viability was measured at 24 h as described in the Materials and methods section. Except for PDTC, concentrations used did not affect cell viability in the absence of TNFα (125 pg/ml). Results are means ± S.D. ($n = 8$). In (c), □, Control; ●, +TNFα.

out the involvement of SDH using this technique. Ethidium bromide has been successfully used to deplete mtDNA-encoded proteins in yeast [40], but in mammalian cells few true respiration-deficient cell lines are known (see [32] for an example). Using this treatment, we were repeatedly unable to generate stable respiration-deficient L929 cells. A transient decrease in mtDNA-encoded enzyme activities was observed, but within 3 weeks the cells had regained their full complement of respiratory enzymes and did not require uridine/pyruvate supplementation (results not shown).

To examine whether damage to/inhibition of the mitochondrial respiratory chain could be a significant causal event in TNFα-

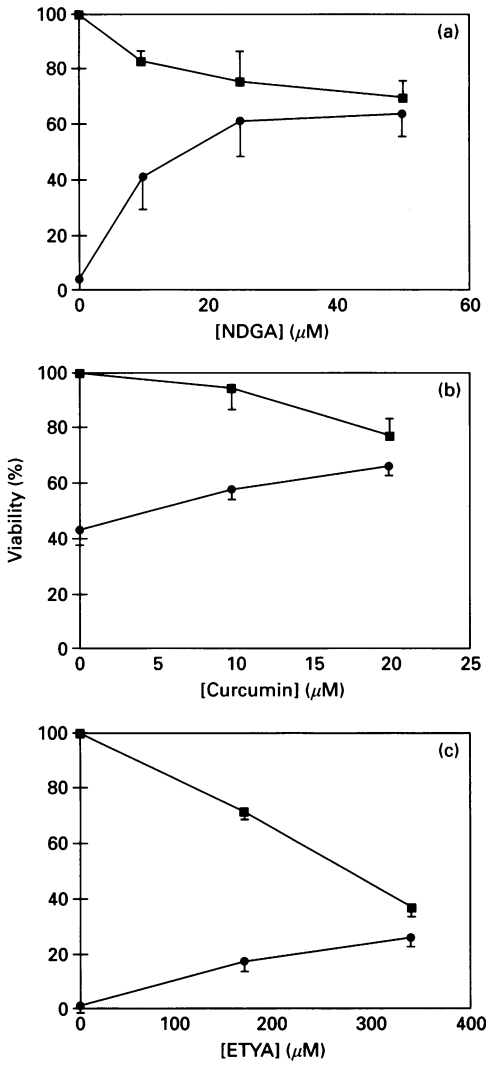

Figure 5 Effect of lipooxygenase inhibitors on TNFα-mediated lysis of L929 cells

Viability in the presence or absence of TNFα (125 pg/ml) was determined at 24 h (**a**, **c**) or 9 h (**b**) as described in the Materials and methods section. Results are means ± S.D. ($n = 8$). ■, Control; ●, +TNFα.

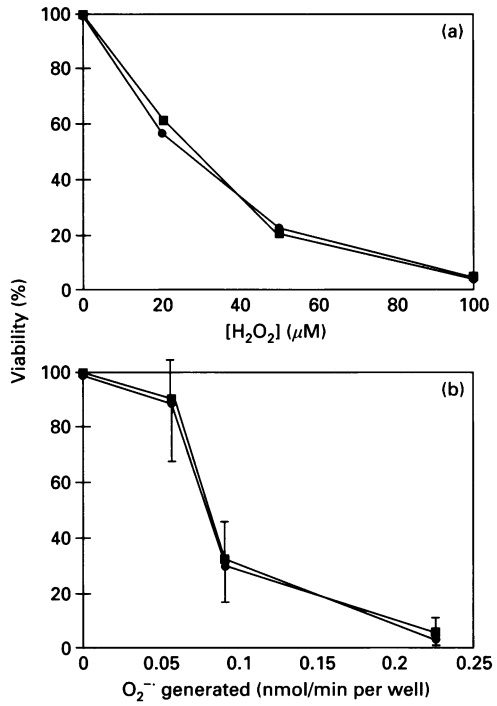

Figure 6 Effect of NDGA on ROS-mediated killing of L929 cells

Viability was determined at 24 h as described in the Materials and methods section. Actinomycin D (1 μg/ml) was present in all samples. (**a**) H_2O_2 with (■) or without (●) 20 μM NDGA was added at $t = 0$. (**b**) Xanthine (500 μM) and xanthine oxidase with (■) or without (●) 20 μM NDGA was added at $t = 0$. Xanthine oxidase activity was first calibrated using SOD-inhibitable rate of cytochrome *c* reduction. Results are means ± S.D. ($n = 8$).

mediated killing, lysis was measured in the presence of several inhibitors (Table 2). Inclusion of rotenone, carboxin, antimycin A, KCN or the uncoupler FCCP did not prevent cell killing. In addition, antimycin A and KCN (both of which inhibit distal to ubiquinone) did not potentiate lysis (results not shown). Only the SDH inhibitor TTFA significantly suppressed cytotoxicity (Figure 4a). However, this compound is a metal chelator which inhibits at least three other intracellular enzymes [41–43]. In contrast, the specific SDH inhibitors carboxin and malonate had no effect (Table 2). The aconitase inhibitor fluoroacetate, which inhibits succinate production, depriving SDH of substrate, was also without effect on lysis. None of the respiratory-chain inhibitors exhibited cytotoxicity towards L929 cells comparable with that of TNFα under identical conditions (i.e. in the presence of actinomycin D), indicating that inhibition of respiration is unlikely to be a significant event in TNFα-mediated lysis. These data suggest that mitochondrial respiratory enzymes do not play a direct role in TNFα-mediated signalling in L929 cells.

Effects of inhibitors of oxidant generation/generating systems on TNFα cytotoxicity

To investigate the participation of other known oxidant-generating enzymes in TNFα-mediated lysis, we measured cytotoxicity in the presence of specific inhibitors (except iodonium diphenyl, a known inhibitor of certain flavoenzymes [44]) (Table 2). No protection was observed on inclusion of inhibitors for cyclo-oxygenase (indomethacin), cytochrome *P*-450 (metyrapone), nitric oxide synthase (NMMA), NADPH oxidase (iodonium diphenyl), xanthine oxidase (allopurinol) or ribonucleotide reductase (hydroxyurea). Three compounds did protect against lysis: the metal chelator/thiol ligand PDTC (Figure 4c), the anti-inflammatory agent salicylate (Figure 4d) and the chemiluminescent $O_2^{\cdot-}$ probe lucigenin (Figure 4b).

Effects of lipoxygenase inhibitors on TNFα sensitivity of L929 cells

Several inhibitors of the lipoxygenase pathway prevented TNFα-mediated lysis. Both NDGA and curcumin protected at concentrations close to IC_{50} values [45,46] (Figure 5). However, as both of these can act as radical scavengers, we sought additional evidence using alternative inhibitors. The arachidonate analogue ETYA protected at 168 μM. A specific 5-lipoxygenase inhibitor Rev-5901 (*p* isomer), which prevents translocation of this

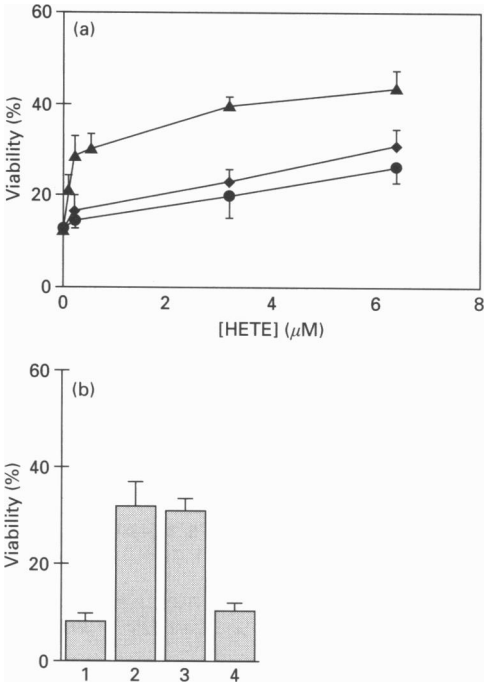

Figure 7 Effect of HETEs on TNFα-mediated lysis

(**a**) Cell lysis (125 pg/ml TNFα) in the presence of various concentrations of (12*S*)- (▲), (15*S*)- (●) or (5*S*)- (◆) HETE was measured at 13 h as described in the Materials and methods section. Concentrations of HETEs used did not affect viability in the absence of TNFα. (**b**) Effect of calphostin C on (12*S*)-HETE protection against lysis. Viability (125 pg/ml TNFα) was measured at 13 h as described in the Materials and methods section. TNFα (125 pg/ml) was present in all samples. 1, Control; 2, +calphostin C (62 nM) and (12*S*)-HETE (1.6 μM); 3, +(12*S*)-HETE (1.6 μM); 4, +calphostin C (62 nM). Results are means ± S.D. ($n = 8$).

isoenzyme to its docking site 5-lipoxygenase-activating protein, did not protect cells against lysis (up to 200 μM; results not shown). This suggests an involvement of lipoxygenases other than 5-lipoxygenase (see the Discussion section) in the cytotoxic process.

Effect of lipoxygenase inhibitor NDGA on ROS-mediated killing of L929 cells

As NDGA is also a radical scavenger, we examined whether protection of cells by this compound could be due to scavenging of toxic ROS rather than lipoxygenase inhibition. Under conditions in which TNFα induces major lysis, NDGA (20 μM) did not protect against ROS (H_2O_2 or the $O_2^{\cdot-}$-generating system xanthine/xanthine oxidase)-mediated killing even where total cell death was below 10% (Figure 6). This indicates that protection of L929 cells against TNFα by NDGA is not due to removal of cytotoxic H_2O_2 or $O_2^{\cdot-}$.

Effect of HETEs on TNFα-mediated lysis of L929 cells

HETEs are stable secondary products of lipoxygenase activity generated from the corresponding hydroperoxyeicosatetraenoic acids (HPETEs) by glutathione peroxidase. We investigated the effects of these on TNFα-mediated lysis of L929 cells. Although non-toxic alone, low concentrations of (12*S*)-HETE [and to a much lesser extent (15*S*)- and (5*S*)-HETE] were capable of significant protection against TNFα-mediated lysis (Figure 7a). As most known biological effects of (12*S*)-HETE are mediated via subsequent protein kinase C activation [47], we examined the effects of the specific protein kinase C inhibitor calphostin C on this protection. As shown (Figure 7b), calphostin C had no effect on either TNFα-induced lysis itself or the protection afforded by (12*S*)-HETE.

DISCUSSION

Several lines of evidence suggest a role for ROS in TNFα signalling. Using a variety of techniques, we have shown the lack of involvement of mitochondrial respiratory enzymes, cyclo-oxygenase, NADPH oxidase, xanthine oxidase, ribonucleotide reductase, cytochrome *P*-450 or nitric oxide synthase in TNFα-mediated apoptosis of L929 cells. In contrast, several lipoxygenase inhibitors could provide significant protection. Many inhibitors of this pathway have antioxidant activity; therefore we included the arachidonate analogue ETYA, a competitive inhibitor, in our investigations. This compound also inhibits cyclooxygenases. However, lack of protection by indomethacin, a potent and specific prostaglandin H synthase inhibitor, rules out involvement of this enzyme. Protection by NDGA (shown also in [48–50]) did not appear to involve removal of cytotoxic H_2O_2 or $O_2^{\cdot-}$, as concentrations that effectively prevented TNFα-mediated lysis could not prevent toxicity of either species. The lipoxygenase product (12*S*)-HETE significantly protected, independently of protein kinase C (implicated in most known biological effects of this isomer [47]). As HETEs can also function as alternative substrates for lipoxygenases [51], this may suggest a form of product inhibition, where prevention of generation of physiological products occurs by competition with arachidonate/linoleate.

Lipoxygenases are a group of ubiquitous intracellular enzymes that catalyse the initial reactions of leukotriene generation. A variety of free-radical intermediates, including alkyl, peroxyl, hydroxyl and superoxide radicals, have been detected during their activity *in vitro* [52–55], and scavenging of these leads to inhibition of physiological product formation. Although we saw no H_2O_2 or $O_2^{\cdot-}$ generation in response to TNFα, our data with radical scavengers (curcumin, NDGA, lucigenin) do support the involvement of ROS. This agrees with several reports of protection by scavengers, such as butylated hydroxyanisole [14], butylated hydroxytoluene [10], the spin trap TEMPOL [56] and MnSOD transfection [7]. Although MnSOD is a mitochondrial enzyme, intracellular location of the transfectant was not established and cytosolic expression cannot be ruled out. Inhibition of lipoxygenase *in vitro* by SOD has been shown [57]. In addition, both butylated hydroxyanisole and butylated hydroxytoluene can prevent $O_2^{\cdot-}$ generation by lipoxygenase [49]. Protection of cells by salicylate is distinct from cyclo-oxygenase inhibition, as indomethacin did not protect. Instead, this may be due to radical scavenging [58], as high concentrations were required. A lack of protection using the specific 5-lipoxygenase inhibitor Rev-5901 (*p* isomer) was observed; however, activation of this enzyme requires intracellular Ca^{2+} mobilization, not usually associated with TNFα signalling [59]. In addition expression of 5-lipoxygenase is restricted to myeloid cells, whereas other isoenzymes have been reported in a variety of fibroblast lines [60,61]. An involvement of lipoxygenase(s) or a similar system in TNFα signalling would explain both the inhibitory effects of radical scavengers and the low level of oxidant generation.

Prevention of TNFα signalling by metal chelators such as desferrioxamine [10,16,17] is known. Here, we confirm inhibition of lysis using two chelators, the thiol ligand PDTC, previously demonstrated to inhibit TNFα-mediated lysis of TA1 cells [18],

and TTFA [8]. In addition to preventing radical-chain reactions, chelators can inhibit enzymes by interacting with active-site metals. Lipoxygenases, which contain redox-active non-haem iron, are known to be inhibited by this class of compounds, particularly desferrioxamine [62,63].

Inhibition of mitochondrial respiratory enzymes during lysis is known, but it is unclear whether this is a central event or a consequence of the killing process [12,13]. Our data indicate that respiratory inhibition is probably secondary to TNFα-mediated lysis. Recently, Schulze-Osthoff et al. [8,9] proposed a critical role for early mitochondrial damage involving increased electron leakage at ubiquinone, leading to intracellular oxidant (H_2O_2, $O_2^{-\cdot}$) generation during TNFα-mediated lysis of L929 cells. In contrast, we did not observe protection using rotenone or potentiation using antimycin A. As Schulze-Osthoff et al. [8] also did not observe potentiation of toxicity using the Complex IV inhibitor, KCN, the effect of antimycin that they report may be independent of mitochondrial radical generation. The lack of protection by rotenone has also been reported by Matthews et al. [10]. Because of the lack of specificity, protection by TTFA does not indicate a role for SDH in TNFα signalling, particularly as three other inhibitors of this pathway could not prevent cell death. Both ethidium bromide and chloramphenicol were used by Schulze-Osthoff et al. [9] to generate L929-cell derivatives with reduced sensitivity to TNFα, but only in the absence of actinomycin D. As sensitivity of cells was not significantly altered in the presence of actinomycin D, this may indicate greater induction of protective factors in response to TNFα, rather than decreased sensitivity as suggested. Using chloramphenicol, we generated cells with greatly reduced levels of respiratory enzymes but TNFα sensitivity was unchanged. More recently, Grooten et al. [11] have suggested mitochondrial damage to be a relatively late, post-lethal event, following plasma-membrane damage by several hours.

Using several different assays we did not observe either H_2O_2 or $O_2^{-\cdot}$ generation in L929 cells in response to TNFα. Recently, TNFα-stimulated mitochondrial $O_2^{-\cdot}$ generation in L929 cells using lucigenin-enhanced chemiluminescence was suggested [64]. Detection of mitochondrial $O_2^{-\cdot}$ using this technique in intact cells is surprising, since both MnSOD (mitochondrial) and CuZnSOD (cytosolic) should effectively prevent its passage out through the plasma membrane to allow detection by extracellular probes (lucigenin and SOD). Also, as mitochondrial $O_2^{-\cdot}$ results from leakage of electrons at ubiquinone, inhibition by both antimycin A and KCN is unexpected. It is unlikely that H_2O_2 or $O_2^{-\cdot}$ are direct mediators of TNF-α-induced damage, as previous data have shown late increases in radical damage during lysis [10,14] and preventing removal of intracellular H_2O_2 by catalase inhibition does not increase the cytotoxic effects of TNFα. Small increases in intracellular hydroperoxides mediated by TNFα treatment were seen. However, as DCF can also utilize lipid hydroperoxides as oxidants [65], this increased fluorescence may represent generation of hydroperoxides, such as lipoxygenase products, and not H_2O_2. In addition to catalase, H_2O_2 can be detoxified using several cytosolic glutathione peroxidases. Therefore generation of H_2O_2 in catalase-deficient cellular compartments might result in oxidant generation which is not increased by aminotriazole treatment. However, an examination of the role of glutathione peroxidase in this context is complicated as these enzymes also regulate the lipoxygenase pathway by mediating GSH-dependent conversion of HPETEs into HETEs. Interestingly, a major protective role for glutathione peroxidase in prevention of HPETE-induced apoptosis of cells infected with human immunodeficiency virus has recently been shown [66]. As HPETE toxicity may involve lipid peroxidation [67] a role for lipid-derived oxygen radicals, such as peroxyl or alkoxyl radicals, might be envisaged during TNFα toxicity.

Lipoxygenase products, in particular HETES and HPETES, have been suggested to play a role in TNFα signalling in TA1 adipogenic cells [18,68]. In addition, there is considerable evidence for the involvement of an arachidonate-selective phospholipase A_2 and arachidonate mobilization duirng TNFα-mediated lysis [14,69–71] of L929 cells. However, no known products of arachidonate metabolism have been identified. Future work will involve an examination of which lipoxygenase pathway(s) might be activated in response to TNFα in this cell line.

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REFERENCES

1 Elias, E. and Berry, C. O. A. (1991) Leukaemia **5**, 879–885
2 Kyprianou, N., Alexander, R. B. and Isaacs, J. T. (1991) J. Natl. Cancer Inst. **83**, 346–350
3 Fesel, K., Kolb-Bachofen, V. and Kolb, H. (1991) Am. J. Pathol. **139**, 251–254
4 Gromkowski, S. H., Mama, K., Yagi, J., Sen, R. and Rath, S. (1990) Int. Immunol. **2**, 903–908
5 Fiers, W. (1991) FEBS Lett. **285**, 199–212
6 Wong, G. H. W. and Goeddel, D. V. (1988) Science **242**, 941–944
7 Wong, G. H. W., Elwell, J. H., Oberly, L. W. and Goeddel, D. V. (1989) Cell **58**, 928–931
8 Schulze-Osthoff, K., Bakker, A. C., Vanhaesebroeck, B., Beyaert, R., Jakob, W. A. and Fiers, W. (1992) J. Biol. Chem. **267**, 5317–5323
9 Schulze-Osthoff, K., Beyaert, R., Vandevoorde, V., Haegeman, G. and Fiers, W. (1993) EMBO J. **12**, 3095–3104
10 Matthews, N., Neale, M. L., Jackson, S. K. and Stark, J. M. (1987) Immunology **62**, 153–155
11 Grooten, J., Goossens, V., Vanhaesebroeck, B. and Fiers, W. (1993) Cytokine **5**, 546–555
12 Lancaster, J. R., Laster, S. M. and Gooding, L. R. (1989) FEBS Lett. **248**, 169–174
13 Granger, D. L. and Lehninger, A. L. (1982) J. Cell Biol. **95**, 527–535
14 Brekke, O., Shalaby, M. R., Sundan, A., Espevik, T. and Bjerve, K. S. (1992) Cytokine **4**, 269–280
15 Pogrebniak, H., Matthews, W., Mitchell, J., Russo, A., Samuni, A. and Pass, H. (1991) J. Surg. Res. **50**, 469–474
16 Yamauchi, N., Kuriyama, H., Watanabe, N., Neda, H., Maeda, M. and Niitsu, Y. (1989) Cancer Res. **49**, 1671–1675
17 Warren, S., Torti, S. V. and Torti, F. M. (1993) Lymphokine Cytokine Res. **12**, 75–80
18 Chang, D. J., Ringold, D. M. and Heller, R. A. (1992) Biochem. Biophys. Res. Commun. **188**, 538–546
19 Buttke, T. and Sandstrom, P. A. (1994) Immunol. Today **15**, 7–9
20 Larrick, J. W. and Wright, S. C. (1990) FASEB J. **4**, 3215–3223
21 Cross, A. R. and Jones, O. T. G. (1991) Biochim. Biophys. Acta **1057**, 281–298
22 Murrell, G. A. C., Francis, M. J. O. and Bromley, L. (1990) Biochem. J. **265**, 659–665
23 Meier, B., Radeke, H. H., Selle, S. et al. (1989) Biochem. J. **263**, 539–545
24 Radeke, H. H., Meier, B., Topley, N., Flöge, J., Habermehl, G. G. and Resch, K. (1990) Kidney Int. **37**, 767–776
25 Murphy, H. S., Shayman, J. A., Till, G. O. et al. (1992) Am. J. Physiol. **263**, L51–L59
26 Meier, B. M., Cross, A. R., Hancock, J. T., Kaup, F. J. and Jones, O. T. G. (1991) Biochem. J. **275**, 241–245
27 Jones, S. A., Hancock, J. T., Jones, O. T. G., Neubauer, A. and Topley, N. (1994) J. Am. Soc. Nephrol. **5**, 1483–1491
28 Tada, H., Shiho, O., Kuroshima, K., Koyama, M. and Tsukamoto, K. (1986) J. Immunol. Methods **93**, 157–165
29 Margoliash, E. and Frohwirt, M. (1959) Biochem. J. **71**, 570–572
30 Jones, O. T. G. and Hancock, J. T. (1994) Methods Enzymol. **233**, 222–229
31 Spolsky, C. M. and Eisenstadt, J. M. (1972) FEBS Lett. **25**, 319–324
32 King, M. P. and Attardi, G. (1989) Science **246**, 500–503
33 Rickwood, D., Wilson, M. T. and Darley-Usmar, V. M. (1987) in Mitochondria: A Practical Approach (Darley-Usmar, V. M., Rickwood, D. and Wilson, M. T., eds.), pp. 1–16, IRL Press, Oxford
34 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
35 Hatefi, Y. (1978) Methods Enzymol. **53d**, 5–10

36 Darley-Usmar, V. M., Capaldi, R. A., Takamiya, S. et al. (1987) in Mitochondria: A Practical Approach (Darley-Usmar, V. M., Rickwood, D. and Wilson, M. T., eds.), pp. 113–152, IRL Press, Oxford
37 Estornell, E., Romano, F., Pallotti, F. and Lenaz, G. (1993) FEBS Lett. **332**, 127–131
38 Ragan, C. I., Wilson, M. T., Darley-Usmar, V. M. and Lowe, P. N. (1987) in Mitochondria: A Practical Approach (Darley-Usmar, V. M., Rickwood, D. and Wilson, M. T., eds.), IRL Press, Oxford
39 Haughland, R. P. (ed.) (1992) Molecular Probes. Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes Inc., Eugene, OR
40 Tzagoloff, A. (ed.) (1982) Mitochondria, Plenum Press, New York
41 Dileepan, K. N. and Kennedy, J. (1985) Biochem. J. **225**, 189–194
42 Gutman, M. and Hartstein, E. (1974) FEBS Lett. **49**, 170–173
43 Staron, K. and Kaniuga, Z. (1974) FEBS Lett. **45**, 1–2
44 O'Donnell, V. B., Smith, G. C. M. and Jones, O. T. G. (1994) Mol. Pharmacol. **46**, 778–785
45 Salari, H., Braquet, P. and Borgeat, P. (1984) Prostaglandins Leukotrienes Med. **13**, 53–60
46 Flynn, D. L., Rafferty, M. F. and Boctor, A. M. (1986) Prostaglandins Leukotrienes Med. **22**, 357–360
47 Tang, D. G. and Honn, K. V. (1994) Ann. N.Y. Acad. Sci. **744**, 199–215
48 Hepburn, A., Boeynaems, J.-M., Fiers, W. and Dumont, J. E. (1987) Biochem. Biophys. Res. Commun. **149**, 815–822
49 Suffys, P., Beyaert, R., Van Roy, F. and Fiers, W. (1987) Biochem. Biophys. Res. Commun. **149**, 735–743
50 Watanabe, N., Niitsu, Y., Neda, H. et al. (1988) Immunopharmacol. Immunotoxicol. **10**, 109–116
51 Kühn, H., Schewe, T. and Rapoport, S. M. (1994) Adv. Enzymol. Relat. Areas Mol. Biol. **58**, 273–311
52 Chamulitrat, W., Hughes, M. F., Eling, T. E. and Mason, R. P. (1991) Arch. Biochem. Biophys. **290**, 153–159
53 Roy, P., Roy, S. K., Mitra, A. and Kulkarni, A. P. (1994) Biochim. Biophys. Acta **1214**, 171–179
54 Nelson, M. J., Seitz, S. P. and Cowling, R. A. (1990) Biochemistry **29**, 6897–6903
55 Nelson, M. J. and Cowling, R. A. (1990) J. Am. Chem. Soc. **112**, 2820–2821
56 Matthews, W., Mitchell, J., Russo, A., Samuni, A. and Pass, H. (1991) J. Surg. Res. **50**, 469–474
57 Richter, C., Wendel, A., Weser, U. and Azzi, A. (1975) FEBS Lett. **51**, 300–303
58 Kaur, H. and Halliwell, B. (1994) Methods Enzymol. **233**, 67–82
59 Schutze, S., Berkovic, D., Tomsing, O., Unger, C. & Krönke, M. (1991) J. Exp. Med. **174**, 975–988
60 Ford-Hutchinson, A. W., Gresser, M. and Young, R. N. (1994) Annu. Rev. Biochem. **63**, 383–417
61 Holtzman, M. J., Pentland, A., Baenziger, N. L. and Hansbrough, J. R. (1989) Biochim. Biophys. Acta **1003**, 204–208
62 Barradas, M. A., Jeremy, J. Y., Kontogiorghes, G. J., Mikhailidis, D. P., Hoffbrand, A. V. and Dandona, P. (1989) FEBS Lett. **245**, 105–109
63 Hirschelmann, R., Kuhn, C., Zarnack, S. et al. (1988) Biomed. Biochim. Acta **47**, S256–S259
64 Hennet, T., Richter, C. and Peterhans, E. (1993) Biochem. J. **289**, 587–592
65 Cathcart, R., Schwiers, E. and Ames, B. N. (1983) Anal. Biochem. **134**, 111–116
66 Sandstrom, P. A., Tebbey, P. W., Van Cleave, S. and Buttke, T. M. (1994) J. Biol. Chem. **269**, 798–801
67 Halliwell, B. and Gutteridge, J. M. C. (1984) Biochem. J. **218**, 1–14
68 Haliday, E. M., Ramesha, C. S. and Ringold, G. (1991) EMBO. J. **10**, 109–115
69 Knauer, M. F., Longmuir, K. J., Yamamoto, R. S., Fitzgerald, T. P. and Granger, G. A. (1990) J. Cell. Physiol. **142**, 469–479
70 Hayakawa, M., Ishida, N., Takeuchi, K. et al. (1993) J. Biol. Chem. **268**, 11290–11295
71 Neale, M. L., Fiera, R. A. and Matthews, N. (1988) Immunology **64**, 81–85

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