Ceramide induces a loss in cytosolic peroxide levels in mononuclear cells

Darren C. PHILLIPS¹ and Helen R. GRIFFITHS²

Molecular Biosciences Group, School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, U.K.

Ceramide (a sphingolipid) and reactive oxygen species are each partly responsible for intracellular signal transduction in response to a variety of agents. It has been reported that ceramide and reactive oxygen species are intimately linked and show reciprocal regulation [Liu, Andreieu-Abadie, Levade, Zhang, Obeid and Hannun (1998) *J. Biol. Chem.* **273**, 11313–11320]. Utilizing synthetic, short-chain ceramide to mimic the cellular responses to fluctuations in natural endogenous ceramide formation or using stimulation of CD95 to induce ceramide formation, we found that the principal redox-altering property of ceramide is to lower the [peroxide]_{cyt} (cytosolic peroxide concentration). Apoptosis of Jurkat T-cells, primary resting and phytohaemagglutinin-activated human peripheral blood T-lymphocytes was preceded by a loss in [peroxide]_{cyt}, as measured by the peroxide-sensitive probe 2',7'-dichlorofluorescein diacetate (also reflected in a lower rate of superoxide dismutase-inhibitable cytochrome *c* reduction), and this was not associated with a loss of membrane integrity.

Where growth arrest of U937 monocytes was observed without a loss of membrane integrity, the decrease in [peroxide]_{cyt} was of a lower magnitude when compared with that preceding the onset of apoptosis in T-cells. Furthermore, decreasing the cytosolic peroxide level in U937 monocytes before the application of synthetic ceramide by pretreatment with either of the antioxidants *N*-acetyl cysteine or glutathione conferred apoptosis. However, *N*-acetyl cysteine or glutathione did not affect the kinetics or magnitude of ceramide-induced apoptosis of Jurkat T-cells. Therefore the primary redox effect of cellular ceramide accumulation is to lower the [peroxide]_{cyt} of both primary and immortalized cells, the magnitude of which dictates the cellular response.

Key words: apoptosis, ceramide, cytosolic peroxide, growth arrest, reactive oxygen species, redox compartmentalization.

INTRODUCTION

The sphingolipid ceramide has been identified as an important, but not exclusive, signalling intermediate in the induction of cellular responses to a variety of agents. These include both physiological, e.g. tumour necrosis factor- α [1–4], IL-1 β (interleukin-1 β) [5], CD95 (Fas/APO-1) [1,6–8], and toxicological agents, e.g. H₂O₂, heat shock, UV light, ionizing radiation [4,9], anticancer drugs [10–12] or the bacterial endotoxin lipopolysaccharide [13]. Ceramide accumulation in response to extracellular agents appears to be driven either by the action of SMase (sphingomyelinases), catalysing the hydrolysis of sphingomyelin to ceramide [2,6,9], or through ceramide generation *de novo* via ceramide synthase [10,11]. Downstream events, which vary according to cell type and stimulus, include apoptosis and proliferation, after the activation of intracellular signalling cascades.

The application of cell-permeable synthetic ceramides or bacterial SMase to a variety of cell types is capable of inducing the apoptotic [2,4,12] and proliferative [14] responses supporting a cell-type-specific signalling role. Cell-cycle arrest by dephosphorylation of the retinoblastoma gene product [13,15,16], differentiation [17] and senescence [18] has also been observed on treatment of cells with synthetic ceramide.

Endogenous cellular ceramide and ROS (reactive oxygen species) levels are increased in response to overlapping external stimuli. The external application of synthetic ceramides or the ROS, H₂O₂, induces the activation of common downstream protein targets. Therefore evidence suggests that the effects of ROS

and ceramide are intimately related. Indeed, recent observations are suggestive of an association between ROS and ceramide at two levels: first, in the regulation of ceramide metabolism, where the intracellular antioxidant, glutathione, negatively regulates the activity of neutral SMase but not that of acid SMase [2,19,20], and secondly as a putative mediator of ceramide signalling after the disruption of the mitochondrial electron-transport chain [21–24].

However, the effects of antioxidants in detoxifying ceramide-induced apoptosis are inconsistent. U937 monocytes or MCF-7 breast carcinoma cell lines are not protected from short-chain ceramide-induced cell death by pretreatment with the antioxidants glutathione and NAC (*N*-acetyl cysteine) [2,25], whereas catalase was reported to antagonize the lethality of C₂-ceramide (*N*-acetyl-sphingosine) in WEHI 231 B-cells [26]. Additionally, in U937 monocytes, C₂-ceramide did not lead to the predicted enhancement of fluorescence by DCF (2',7'-dichlorofluorescein) [25], a cytosolic peroxide-sensitive dye.

We have described previously that the cellular responses of apoptosis or growth arrest induced by synthetic ceramides are associated with the magnitude of mitochondrial peroxide formation and the associated alteration in redox state [23]. However, we also observed that buthionine sulfoximine-mediated glutathione depletion in monocytes and T-cells ensured protection against ceramide-induced changes in cell cycle, indicating that complex compartmental differences in levels of oxidants/antioxidants may govern the outcome. Furthermore, given the relatively small perturbation in [peroxide]_m (mitochondrial peroxide concentration) observed, we questioned whether these

Abbreviations used: a.u., arbitrary units; C₂-ceramide, *N*-acetyl-sphingosine; C₆-ceramide, *N*-hexanoyl-sphingosine; CD95L, CD95 ligand; DAGK, diacylglycerol kinase; DCF, 2',7'-dichlorofluorescein; DCFH, non-fluorescent DCF; DCFH-DA, DCF diacetate; DPI, diphenylene iodonium; FCS, foetal calf serum; FS, forward scatter; IL, interleukin; MdX, median X; NAC, *N*-acetyl cysteine; PBL, peripheral blood lymphocyte; PE, phycoerythrin; PHA, phytohaemagglutinin; PI, propidium iodide; P/S, penicillin/streptomycin; ROS, reactive oxygen species; SMase, sphingomyelinase; SOD, superoxide dismutase; SS, side scatter.

¹ Present address: Division of Molecular Therapeutics, Department of Hematology–Oncology, St Jude Children's Research Hospital DT 5062, 332 North Lauderdale, Memphis, TN 38105, U.S.A.

² To whom correspondence should be addressed (e-mail h.r.griffiths@aston.ac.uk).

changes are capable of promoting extra-mitochondrial signalling. Consequently, we investigated the effects of ceramide and its generation by the physiological CD95L (CD95 ligand) on [peroxide]_{cyt} (cytosolic peroxide concentration) in the context of the ensuing cellular response. In the present study, we describe that the primary redox effect of cellular ceramide accumulation is to lower the [peroxide]_{cyt} of both primary and immortalized cells, the magnitude of which dictates the cellular response.

EXPERIMENTAL

Materials

All reagents were obtained from Sigma (Poole, Dorset, U.K.), solvents from Fisher (Loughborough, Leics., U.K.) and all gases from BOC Ltd (Guildford, U.K.) unless otherwise stated. RPMI 1640, foetal bovine serum and P/S [penicillin (1000 units/ml)/streptomycin (10 000 µg/ml)] were purchased from Gibco BRL (Paisley, Renfrewshire, U.K.). Human monoclonal anti-human CD95 (Fas/APO-1) antibody was from R & D Systems (Abingdon, Oxfordshire, U.K.). C₂-ceramide and C₆-ceramide (N-hexanoyl-sphingosine) were obtained from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). *Escherichia coli* DAGK (diacylglycerol kinase) and n-octyl-β-D-glycopyranoside were purchased from Calbiochem (Nottingham, U.K.). [γ -³²P]ATP was purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Fluorescently tagged monoclonal antibodies and isotype-negative controls were obtained from Diaclone (Besançon Cedex, France) or Serotec (Kidlington, Oxford, U.K.).

DCFH-DA (DCF diacetate) was dissolved in DMSO to stock solutions of 75 mM. Subsequent dilutions were made with serum-free RPMI 1640. The antioxidants NAC and glutathione were made up in serum-free RPMI 1640. In the [peroxide]_{cyt} assays, the final cellular concentration of DMSO employed did not exceed 0.1 %. Monoclonal anti-human CD95 (Fas/APO-1) antibody was reconstituted in sterile PBS (0.01 M Na₂HPO₄/0.002 M KH₂PO₄/0.003 M KCl/0.137 M NaCl; pH 7.4), containing 0.1 % fraction V BSA to a concentration of 500 µg/ml, and further dilutions were made in PBS/0.1 % BSA. C₂-/C₆-ceramide was dissolved in anhydrous DMSO to a stock solution of 20 mM. Subsequent dilutions were made in 1 mM fatty-acid-free BSA in PBS.

Methods

Cell culture and stimulation

The acute human T-cell leukaemia cell line, Jurkat, and the human monocyte cell line, U937, were maintained in RPMI 1640, supplemented with 10 % (v/v) heat-inactivated FCS (foetal calf serum) and 1 % P/S. Cells were incubated at 37 °C in a 95 % air/5 % CO₂ humidified atmosphere. The number of viable cells/ml was determined by Trypan Blue exclusion using an improved Neubauer haemocytometer (Weber Scientific International, Teddington, Middx., U.K.). Cells at a concentration of 2 × 10⁶/ml were serum-starved for 4 h under the described incubation conditions before treatment. Where indicated, cells were treated with CD95L or C₂-/C₆-ceramide for the times and concentrations mentioned above, and incubated at 37 °C in a 95 % air/5 % CO₂ humidified atmosphere. To investigate the role of ROS in the cellular responses to the above agents, cell suspensions were pretreated for 4 h with 10 mM NAC or glutathione. The role of surface γ glutamyl transpeptidase activity in providing the glutathione-mediated antioxidant protection was investigated by

preincubating the cells with 1 mM acivicin for 30 min before glutathione treatment. To prevent the cellular export of GSSG, cells were pretreated for 1 h with L-methionine or L-cystathionine [34]. Further inhibitory experiments with the NADPH oxidase inhibitor DPI (diphenylene iodonium; 2.5 µM) were performed to elucidate any role of this enzyme in the modulation of intracellular redox. Stimulation was discontinued by removing cell suspensions from culture vessels, centrifuging at 1000 g for 5 min (Eppendorf centrifuge 5415D; Eppendorf, Hamburg, Germany) and washing twice with 1 ml of ice-cold PBS before further experimental manipulation.

Individual additions to cell suspensions did not exceed 1 % of the total volume and were dispersed with gentle mixing by a pipette. Control experiments were conducted under identical conditions as tests, employing vehicle treatment.

T-cell isolation and cell culture

Briefly, 40 ml of venous blood was obtained from consenting adults and sodium citrate [0.4 % (w/v) final concentration] was added to prevent coagulation. Further manipulation of the blood was conducted under aseptic conditions. Blood was diluted with PBS containing 0.1 % (w/v) BSA in the ratio 2:5 in 50 ml conical tubes (Orange Scientific, Braine-l'Alleud, Belgium). Peripheral blood mononuclear cells were isolated by density-gradient centrifugation using Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) and the resting T-cells were purified by negative isolation employing magnetic beads (DynaL A/S, Oslo, Norway). For an examination *ex vivo*, T-cells were analysed for [peroxide]_{cyt} and lipid content immediately after isolation. Additionally, resting T-cells were cultured for 72 h in RPMI 1640, supplemented with 10 % FCS and 1 % P/S with or without 10 µg of PHA/(2 × 10⁶ T-cells) per ml (where PHA stands for phytohaemagglutinin) to elicit activation. Isolated T-cells were incubated at 37 °C in a 95 % air/5 % CO₂ humidified atmosphere. Before further experimental manipulation, T-cells in culture were washed twice, resuspended in serum-free RPMI 1640 and treated as described above for immortalized cell lines.

Phenotypic analysis of PBLs (peripheral blood T-lymphocytes) by immunofluorescence

The percentage of CD3⁺ PBL and the percentage of their activation [determined as the appearance of CD25 (IL-2α receptor)] were evaluated by flow cytometry after negative isolation from blood. The purity of extracted PBL was assessed based on the percentage of CD3⁺ PBL, and monocyte contamination was evaluated by the presence of CD14⁺ cells within the whole sample. PBS-washed PBL, *ex vivo* or after 72 h culturing, was treated with 10 µl of antibody/10⁶ cells and incubated on ice in the dark for 30 min. The antibodies used were the mouse monoclonal anti-human CD3 [phycoerythrin (PE)-conjugated], anti-human CD25 (FITC-conjugated) and anti-human CD14 (R-phycoerythrin-Cy5-conjugated) antigens. For each sample, isotype-negative controls of the monoclonal antibody were used to establish background fluorescence. PBL was fixed in 250 µl of 4 % (w/v) formaldehyde solution. Samples were then vortex-mixed vigorously and incubated in the dark for a further 15 min, followed by the addition of 200 µl of Isoton (Beckman Coulter, Miami, FL, U.S.A.). Samples were again vortex-mixed and incubated in the dark, at room temperature for a minimum of 10 min. Samples were stored in the dark at 4 °C for up to 24 h before analysis without significantly affecting the results. Samples were then analysed by flow cytometry (EPICS® XL-MCL;

Beckman Coulter) utilizing three-way colour compensation and corrected for background fluorescence detected with isotype-negative controls. A minimum of 10 000 PBLs were analysed per sample using the following gating strategy: the percentage of activation of CD3⁺ PBL was evaluated on a dual-parameter histogram of log FL2 (CD3 PE) versus log FL1 (CD25 FITC), where FL2 stands for fluorescence filter 2. The purity of PBL extracted was assessed as the percentage of CD3⁺ cells on an ungated histogram of SS (side scatter) versus log FL2 (CD3 PE). Monocyte contamination was determined on an ungated histogram of log FL4 (CD14 PE-Cy5) versus count.

Flow-cytometric DNA cell-cycle analysis

Briefly, PBS-washed cells were centrifuged at 100 g (Eppendorf centrifuge 5415D) for 5 min, the supernatant removed and the resulting cell pellet resuspended in 1 ml of hypo-osmotic fluorochrome solution [50 µg/ml PI (propidium iodide) in 0.1 % sodium citrate and 0.1 % Triton X-100] to extract and stain the nucleoids [27]. Samples were incubated in the dark at 4 °C for 14–24 h before flow-cytometric cell-cycle analysis. PI fluorescence of individual nuclei was measured using an EPICS[®] XL-MCL flow cytometer (Beckman Coulter), equipped with a 488 nm air-cooled argon laser. FS (forward scatter) and SS of the nuclei were simultaneously measured in addition to linear and log red fluorescence (FL3, bandwidth 605–635 nm). Clumps of nuclei were eliminated by appropriate gating. For each analysis, 20 000 events were recorded on the gated FL3 detector. The percentage of actual apoptosis was determined by quantifying the number of hypoploid (subdiploid) nuclei. Values were also expressed as a percentage of specific apoptosis according to the following equation:

$$\text{specific apoptosis} = [(T - C)/(100 - C)] \times 100 \quad (1)$$

where *T* is the percentage of apoptotic events from treated cells and *C* is the percentage of apoptotic events from control cells. The percentage of nuclei in the G₀/G₁ phase of the cell cycle was quantified using MultiCycle[™] for Windows (Phoenix Flow Systems, San Diego, CA, U.S.A.).

Evaluation of cytosolic peroxide levels

A procedure adapted from Bass et al. [28] was used to determine the cytosolic peroxide levels in cells, by flow cytometry, as the fluorescence generated from cells loaded with the peroxide-sensitive fluorescent probe, DCFH-DA. DCFH-DA rapidly diffuses into the cytosol of cells where it is hydrolysed to the oxidation-sensitive DCFH (non-fluorescent DCF). In the presence of cytosolic peroxide, DCFH is rapidly oxidized to the non-diffusible fluorescent DCF. Cells were loaded with 50 µM DCFH-DA/2 × 10⁶ cells for the final 40 min of treatment with different concentrations of CD95 or ceramide for different incubation periods. Immediately after agent/DCF-DA incubation, cells were analysed by flow cytometry (EPICS[®] XL-MCL). The first control population was always adjusted to the third log decade, giving an Mdx (median X) value of approx. 100. Measurements of FS, SS and log FL1 fluorescence (green light, bandwidth 505–545 nm) were recorded. The viable cell population, determined by FS and SS properties, was gated to exclude debris, clumped cells or machine noise. From each sample, 10 000 cells were examined on a histogram of log FL1 (DCF fluorescence) versus count. In control experiments, DCF was seen to have no quenching effect on H₂O₂-induced DCF fluorescence.

Evaluation of SOD (superoxide dismutase)-inhibitable cytochrome *c* reduction

Cells (200 µl, 10⁷/ml) in Hanks balanced salt solution were dispensed into an optically clear flat-bottomed 96-well plate containing 160 µM cytochrome *c*, in the presence or absence of C₂-ceramide (20 µM) and either SOD (3000 units/ml) or DPI (2.5 µM). Cytochrome *c* reduction was monitored at 550 nm after incubation with the aforementioned agents at 37 °C for 1 or 16 h. Superoxide release was calculated in terms of nmol/10⁷ cells using a molar absorption coefficient of 21 × 10³ · M⁻¹ · cm⁻¹ at 550 nm for cytochrome *c*.

Analysis of cytosolic peroxide levels of mononuclear cells from peripheral whole blood

Blood (200 µl) was treated with C₂-/C₆-ceramide for 1 h in a water bath in the dark at 37 °C with gentle horizontal shaking. Optimal concentrations were predetermined from the responses achieved from cell lines *in vitro*. At total treatment time minus 20 min, T_{end-20 min}, DCFH-DA was added to give a final concentration of 50 µM, and samples were returned to the water bath. At the end of agent treatment period, blood was stained with antibodies at a saturating concentration of at least 10 µl of antibody/100 µl of blood. Samples were then incubated on ice for a further 30 min. To identify monocytes and T-cells, PBLs were stained with mouse monoclonal anti-human CD3 (PE-conjugated) and anti-human CD14 (RPE-Cy5-conjugated) antigens respectively. To account for overlapping emission spectra of the fluorescent-tagged antibodies and DCF, three-way colour compensation was applied. Red blood cells were lysed and the mononuclear cell population was fixed with Optilysate C (Beckman Coulter) as described above. Cell suspensions were immediately analysed by flow cytometry. Histograms of log FL2 (CD3 PE) versus SS and log FL4 (CD14 RPE-Cy5) versus SS were used to gate CD3⁺ lymphocytes and CD14⁺ monocytes respectively. The DCF fluorescence of CD3⁺ T-lymphocytes and CD14⁺ monocytes were analysed on separate single-parameter histograms of log FL1 (DCF) versus count. The median fluorescence (Mdx) intensity of each sample was recorded.

Determination of intracellular glutathione content

Intracellular glutathione content was determined by the recycling assay described by Tietze [29]. Briefly, at the end of each treatment period, samples were washed with ice-cold PBS and centrifuged at 250 g for 5 min. An aliquot was removed for estimating the protein content and cells were then resuspended in 3.33 % (w/v) 5-sulphosalicylic acid dihydrate, vortex-mixed vigorously and centrifuged at 13 000 g to precipitate the interfering proteins. Supernatant (25 µl) was added to 50 µl of 6 mM DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] and 150 µl of NADPH (0.3 mg/ml) in triplicate, and incubated at 37 °C for 3 min, before the addition of 25 µl of glutathione reductase (20 units/ml). The plate was immediately read at 420 nm and then read again at +1 and +5 min to determine DTNB reduction. Values of the test sample were calibrated against a standard curve of glutathione from 0 to 60 nmol and expressed per mg of cellular protein.

Protein determination

Protein concentration was determined in quadruplicate using the bicinchoninic acid assay by the method of Smith et al. [30], using BSA as the standard.

Quantification of endogenous T-cell ceramide content

Lipids were extracted from Jurkat T-cells ($2 \times 10^6/\text{ml}$) by the method of Bligh and Dyer [31] and the concentration of ceramide quantified by the DAGK assay as described in [32,33]. The mass of ceramide and diacylglycerol extracted from each cell sample was obtained from the standard curve constructed from known concentrations of type III ceramide (from bovine brain; Sigma, Poole, Dorset, U.K.) and standardized as the lipid concentration/ 10^6 cells. The efficiency of the assay was evaluated from the standard curve and a known concentration of C_6 -ceramide. Cellular ceramide levels were adjusted according to the percentage efficiency of conversion and were always $> 90\%$.

Statistical analysis

Statistical analysis was performed by one-way ANOVA, followed by Tukey's or Dunnett's *post hoc* test analysis or by Student's *t* test. $P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered to be significant.

RESULTS

As reported previously [23], the treatment of Jurkat T-cells with $20 \mu\text{M } C_2/C_6$ -ceramide induced a rapid, time-dependent increase in the percentage of specific apoptosis, which was significant at 3 h, but was not observed at the lower synthetic ceramide concentration of $1\text{--}10 \mu\text{M}$ (see Figure 1a). Maximal specific apoptosis (55%) was observed after 8 h treatment with $20 \mu\text{M } C_2$ -ceramide. Treatment of Jurkat T-cells for longer periods of up to 36 h with $20 \mu\text{M } C_2$ -ceramide did not further increase the percentage of specific apoptosis ($P > 0.05$; see Figure 1a). Comparable kinetics of apoptosis in Jurkat T-cells was observed with $0\text{--}20 \mu\text{M } C_6$ -ceramide (results not shown) [23].

During the first 16 h of treatment of Jurkat T-cells with C_2 -ceramide, $[\text{peroxide}]_{\text{cyt}}$ was monitored by flow cytometry using the fluorescence emission of DCF from cells preloaded with the peroxide-sensitive dye, DCFH-DA. The median fluorescence (Mdx) of the DCFH-DA-treated viable cell population, determined by FS and SS properties, decreased immediately after treatment with $20 \mu\text{M } C_2$ -ceramide. The first significant decreases in DCF Mdx were observed after 15 min treatment with $20 \mu\text{M } C_2$ -ceramide ($P < 0.05$) when compared with vehicle-treated control Jurkat T-cells. Basal peroxide levels were fixed to approx. $\text{Mdx} = 100$, where a maximum decrease in $\Delta\text{DCF Mdx}$ (where ΔDCF denotes the change in DCF) of approx. -60 arbitrary units (a.u.) was observed after 4 h treatment with $20 \mu\text{M } C_2$ -ceramide (see Figure 1b). DCF fluorescence of cells treated with $20 \mu\text{M } C_2$ -ceramide for more than 8 h was not measured, as no further alterations in specific apoptosis were observed at these longer incubation periods. $\Delta\text{DCF Mdx}$ induced by 16 h treatment of Jurkat T-cells with $10 \mu\text{M } C_2$ -ceramide decreased significantly to approx. -35 a.u. ($P < 0.01$) (see Figure 1b) without the appearance of apoptosis ($P > 0.05$; see Figures 1a and 1b). Similar alterations in $\Delta\text{DCF Mdx}$ of Jurkat T-cells treated with $20 \mu\text{M } C_2$ -ceramide were observed after 45 min (see Figure 1b) and this was not associated with the appearance of apoptosis at this time point ($P > 0.05$; see Figure 1a). Similar effects on the DCF fluorescence were observed in Jurkat T-cells after treatment with $0\text{--}20 \mu\text{M } C_6$ -ceramide (results not shown).

As reported previously by us [23], treatment of the human monocytic cell line U937 with either C_2 - or C_6 -ceramide ($0\text{--}20 \mu\text{M}$) for up to 36 h induced an enhancement in the percentage of nuclei in the G_0/G_1 phase of the cell cycle when compared with

Figure 1 Loss in endogenous $[\text{peroxide}]_{\text{cyt}}$ from Jurkat T-cells precedes apoptosis

Jurkat T-cells ($2 \times 10^6/\text{ml}$) were serum-starved for 4 h before the addition of C_2 -ceramide for $0\text{--}36$ h. Incubations were performed at 37°C in a 95% air/ 5% CO_2 humidified atmosphere. For DNA cell-cycle analysis by flow cytometry (a), treatments were terminated by washing the cells twice with ice-cold PBS, cell pellets resuspended in 1 ml of hypo-osmotic fluorochrome solution and incubated in the dark at 4°C overnight. The subdiploid DNA content of 20 000 nucleoids from each sample was analysed on a gated histogram of FL3 (PI) versus count. Results are presented as the means $\pm$ S.E.M. from at least seven individual experiments, expressed as the percentage of specific apoptosis according to eqn (1). For the analysis of cytosolic peroxide levels (b), cell samples were loaded with $50 \mu\text{M}$ DCFH-DA 40 min before the termination of the incubation period. At the end of the treatment period, samples were analysed immediately for DCF fluorescence by flow cytometry as described in the Experimental section and the Mdx value of 10 000 cells was recorded. Results are presented as the means $\pm$ S.E.M. from at least three individual experiments.

vehicle-treated controls. This result was first evident after 16 h treatment of U937 cells with $20 \mu\text{M } C_2/C_6$ -ceramide ($P < 0.05$; results not shown). This was also observed at longer incubation periods with $10 \mu\text{M } C_6$ -ceramide ($P < 0.05$; see Figure 2a). The increase in U937 monocyte nucleoids in the G_0/G_1 phase of the cell cycle was associated with the prevention of apoptosis induced by serum withdrawal. Low-dose C_6 -ceramide ($1 \mu\text{M}$) treatment for 36 h did not significantly affect either the percentage of nucleoids in the G_0/G_1 phase of the cell cycle ($P > 0.05$) or the percentage of actual apoptosis compared with that observed in vehicle-treated control U937 monocytes ($P > 0.05$; see Figure 2a). Monocytes treated with either 10 or $20 \mu\text{M } C_6$ -ceramide for 36 h showed significantly high G_0/G_1 DNA content compared with vehicle controls ($P < 0.01$). At 36 h, the G_0/G_1 content remained high at the same level as for U937 monocytes treated with 10 or $20 \mu\text{M } C_6$ -ceramide for 24 h (results not shown; $P < 0.05$). Correspondingly, the synthetic ceramide treatment of U937 monocytes induced a dose-dependent inhibition of serum-deprivation-induced actual apoptosis. C_6 -ceramide ($10 \mu\text{M}$) treatment for 36 h significantly inhibited the appearance of actual apoptosis due to serum starvation by approx. 12% ($P < 0.05$). Greater protection against actual apoptosis by 18% ($P < 0.01$) was seen after $20 \mu\text{M } C_6$ -ceramide treatment (see Figure 2a). Identical results were obtained after treatment of U937 monocytes with C_2 -ceramide (results not shown).

Figure 2 C₆-ceramide-induced growth arrest in U937 monocytes preceded by a loss in [peroxide]_{cyt}

U937 cells (2×10^6 /ml) were serum-starved in RPMI 1640 for 4 h before the addition of 0–20 μ M C₆-ceramide and incubated in a 95% air/5% CO₂ humidified atmosphere at 37 °C for 8–36 h. For DNA cell-cycle analysis by flow cytometry (a), treatments were terminated by washing the cells twice with ice-cold PBS and cell pellets were resuspended in 1 ml of hypo-osmotic fluorochrome solution and then incubated in the dark at 4 °C overnight. The subdiploid DNA content (% actual apoptosis) of 20 000 nucleoids from each sample was analysed on a gated histogram of FL3 (PI) versus count. The percentage of G₀/G₁ content of DNA cell cycles were quantified using MultiCycle™ for Windows (Phoenix Flow Systems). Results are presented as the arithmetic mean percentages $\pm$ S.E.M. from at least three individual experiments. * $P < 0.05$ or ** $P < 0.01$ compared with vehicle treatment by one-way ANOVA, followed by Dunnett's multiple comparison test. For the analysis of cytosolic peroxide levels (b), U937 monocytes were treated as described in the legend to Figure 1. Results are presented as the means $\pm$ S.E.M. from at least three individual experiments.

In U937 cells, no alteration in the intracellular H₂O₂ concentration (as DCF fluorescence) was observed for the first 30 min of 20 μ M C₆-ceramide treatment when compared with vehicle-treated control cells (see Figure 2b). Δ DCF MdX then rapidly decreased to a minimum of –40 in C₆-ceramide-treated U937 cells within 4 h, which then increased to –25 a.u. on increasing the treatment period to 8 h (see Figure 2b). Δ DCF MdX remained at –25 a.u. at 16 h incubation with 20 μ M C₆-ceramide. At a lower concentration (10 μ M), C₆-ceramide decreased DCF fluorescence compared with control, with slower kinetics than that observed with the higher dose (20 μ M), and with less potency at all time points examined (2, 4 and 16 h, $P < 0.05$; see Figure 2b). The maximal decreases in Δ DCF MdX of U937 monocytes treated with 20 μ M ceramide were significantly less than those achieved in Jurkat T-cells ($P < 0.01$; Figures 1b and 2b).

Flow-cytometric evaluation of the cellular uptake of the membrane-impermeable dye PI and cell size revealed no evidence of necrosis in U937 monocytes treated with C₂-/C₆-ceramide (0–20 μ M) for up to 16 h. This was confirmed in Jurkat T-cells treated with 0–10 μ M C₂-/C₆-ceramide for 16 h and 20 μ M C₂-/C₆-ceramide for up to 8 h. However, after 16 h treatment of Jurkat T-cells with 20 μ M C₂-/C₆-ceramide, a subpopulation of Jurkat T-cells had reduced cell size with high PI uptake, indicating late apoptosis or secondary necrosis (results not shown). To confirm the specificity of the effect on DCF fluorescence, the ceramide

metabolite sphingosine-1-phosphate was also examined. Sphingosine-1-phosphate had no effect on DCF fluorescence (results not shown).

To establish the contribution of NADPH oxidase activity to any change in [peroxide]_{cyt} that was measured as Δ DCF MdX, we treated cells with the NADPH oxidase inhibitor DPI, and analysed for ceramide-induced changes in DCF fluorescence and apoptosis. After 1 h, DPI alone or in combination with 20 μ M C₂-ceramide did not significantly alter the DCF fluorescence observed in either Jurkat T-cells or U937 monocytes treated with 20 μ M C₂-ceramide alone (results not shown). By 16 h, DPI significantly decreased the DCF MdX in U937 cells, with a trend to further decrease when co-incubated with ceramide (see Figure 3a). Indeed, examination of the effects of DPI on apoptosis showed that, while inducing slight apoptosis on its own ($7.8 \pm 0.9\%$), co-incubation with C₂-ceramide significantly enhanced apoptosis in U937 cells to $18.6 \pm 2.3\%$ ($P < 0.001$) compared with C₂-ceramide alone (see Figure 3c). In addition, DPI significantly promoted C₂-ceramide-induced apoptosis in Jurkat T-cells ($P < 0.001$; see Figure 3d). Figure 3(b) shows that this was associated with a significant decrease in DCF MdX (16 h; ceramide alone = 72 a.u., ceramide + DPI = 25.7 a.u.; $P < 0.001$).

An alteration in DCF fluorescence is probably a representation of changing availability of catalyst or hydrolysis of DCFH-DA in ceramide-treated cells. Therefore we examined whether ceramide could modulate cellular superoxide anion production using a specific cytochrome *c* reduction method. Figure 3(f) confirms that ceramide inhibits basal superoxide production by Jurkat T-cells after 16 h treatment ($P < 0.05$). DPI alone or DPI in combination with C₂-ceramide was able to decrease superoxide production further in Jurkat T-cells. Similarly, Figure 3(e) illustrates that ceramide significantly decreased superoxide production by U937 monocytes, but that this was not further enhanced by co-incubation with DPI. As a positive control, the application of exogenous SOD to either U937 monocytes or Jurkat T-cells resulted in total loss of cytochrome *c* reduction, demonstrating absolute dependence on superoxide for the reaction (see Figures 3e and 3f).

To determine the importance of [peroxide]_{cyt} on the outcome, U937 monocytes were pretreated with either of the antioxidants NAC or glutathione. Cellular glutathione increased by 28 and 21% from a basal level of 34.8 nmol/mg of protein after pretreatment with glutathione and NAC respectively, whereas acivicin pretreatment significantly inhibited the decrease induced by exogenous glutathione ($P < 0.01$). The antioxidants prevented significantly the accumulation of nucleoids in the G₀/G₁ phase of the cell cycle mediated by 16 h treatment with 20 μ M C₆-ceramide ($P < 0.01$), and this was accompanied by the appearance of subdiploid DNA. C₆-ceramide (20 μ M) significantly increased the appearance of subdiploid DNA of U937 monocytes pretreated with either NAC or glutathione compared with no pretreatment ($P < 0.05$). The percentage of nucleoids in the G₀/G₁ phase of vehicle-treated controls was not affected by pretreatment with NAC ($P > 0.05$) or glutathione ($P > 0.05$; see Figure 4b). Further, there was no significant difference in the inhibitory effects of either NAC or glutathione on synthetic ceramide-mediated G₀/G₁ nucleoid accumulation ($P > 0.05$; see Figure 4b) or the percentage of actual apoptosis induced by either species in the presence of either antioxidant ($P > 0.05$; see Figure 4a). Pretreatment of U937 monocytes with either of the antioxidants glutathione or NAC significantly decreased Δ DCF to approx. –35 a.u. ($P < 0.001$) after 16 h vehicle treatment. On addition of 20 μ M C₆-ceramide to NAC- or glutathione-pretreated U937 monocytes, Δ DCF decreased further to approx. –55 a.u., and was significantly lower than U937 cells pretreated with NAC or

Figure 3 Ceramide-induced decrease in $[\text{peroxide}]_{\text{cyt}}$ is not directly related to NADPH oxidase inhibition of superoxide generation

U937 or Jurkat T-cells ($2 \times 10^6/\text{ml}$) were serum-starved in RPMI 1640 for 4 h with or without $10 \mu\text{M}$ DPI before the addition of $20 \mu\text{M}$ C₂-ceramide for 16 h at 37°C . Before the termination of the incubation period, cell samples were loaded with $50 \mu\text{M}$ DCFH-DA for 40 min as described in the legend to Figure 1. Samples were analysed immediately for DCF fluorescence by flow cytometry and the Mdx of 10 000 cells was recorded (a), (b). The same samples were also analysed for evidence of apoptosis (c), (d) by DNA cell-cycle analysis as described in the legend to Figure 2. U937 or Jurkat T-cells ($10^7/\text{ml}$) were resuspended in phenol-red-free Hanks balanced salt solution containing glucose with or without $10 \mu\text{M}$ DPI or 30 units/ml SOD in the presence of cytochrome *c* ($160 \mu\text{M}$) before the addition of $20 \mu\text{M}$ C₂-ceramide for 16 h at 37°C . Cytochrome *c* reduction was monitored at 550 nm and the superoxide released was calculated as described in the Experimental section (e), (f). Results are presented as the means $\pm$ S.E.M. from three individual experiments and $*P < 0.05$ or $**P < 0.01$ or $***P < 0.001$ represents significant difference from vehicle-treated control samples. $+P < 0.05$, $++P < 0.01$ or $+++P < 0.001$ indicates significant difference from C₂-ceramide-treated cells.

glutathione alone ($P < 0.01$; see Figure 4c). The pretreatment of Jurkat T-cells with either of the antioxidants NAC or glutathione, did not increase the kinetics or the magnitude of apoptosis induced by $20 \mu\text{M}$ C₂-ceramide (see Figure 5a). Treatment of U937 monocytes or Jurkat T-cells with concentrations of either NAC or glutathione in excess of $10 \mu\text{M}$ was by itself cytotoxic (results not shown). Previously, we have reported that efflux of GSSG from the cell precedes synthetic ceramide-mediated Jurkat T-cell apoptosis and may be a contributory mechanism to the apoptosis process [23]. However, pretreatment with the glutathione efflux inhibitors L-cystathionine or L-methionine [34] did not affect Jurkat T-cell apoptosis (0 – $20 \mu\text{M}$; $P > 0.05$ for each concentration and species; see Figure 5b) or G₀/G₁ growth arrest in U937 monocytes (results not shown) induced by C₂-C₆-ceramide, despite protecting cellular glutathione status (U937 cells: 29.83 ± 1.27 nmol of glutathione/mg of protein; U937 + $20 \mu\text{M}$ ceramide for 1 h: 25.95 ± 1.61 nmol of glutathione/mg of protein; U937 +

methionine + $20 \mu\text{M}$ ceramide for 1 h: 28.7 ± 1.7 nmol of glutathione/mg of protein, $n = 9$).

To determine whether the effects observed in immortalized cells were reproduced in a mixed population of primary cells, T-lymphocytes and monocytes (identified by expression of the antigens CD3 and CD14 respectively) from peripheral whole blood were individually gated and the DCF fluorescence of CD3⁺ and CD14⁺ populations analysed on a single-parameter histogram of FL1 versus count (see Figures 6a and 6b). The DCF fluorescence of CD3⁺ T-cells and CD14⁺ monocytes displayed decreased $[\text{peroxide}]_{\text{cyt}}$ after the treatment of whole blood with $20 \mu\text{M}$ C₆-ceramide when compared with vehicle-treated whole-blood T-cells or monocytes (see Figures 6c and 6d).

To substantiate further the observations of decreased $[\text{peroxide}]_{\text{cyt}}$ in response to short-chain ceramides obtained in immortalized cell lines and whole blood, we extracted primary resting T-cells from venous whole blood. Purity (as the percentage

Figure 4 Antioxidants drive synthetic ceramide-treated U937 monocytes into apoptosis rather than growth arrest

U937 cells (2×10^6 /ml) were serum-starved in RPMI 1640 for 4 h with or without 10 mM NAC or 10 mM glutathione before the addition of 20 μ M C₂-C₆-ceramide for 16 h at 37 °C. Samples were prepared for DNA cell-cycle analysis as described in the legend to Figure 2. The sub-diploid DNA content was recorded to represent the percentage of actual apoptosis (a). The percentage of G₀/G₁ content of DNA (b) was quantified using MultiCycle™ for Windows (Phoenix Flow Systems). Results are presented as mean percentages $\pm$ S.E.M. from three individual experiments. * $P < 0.05$ or ** $P < 0.01$ or *** $P < 0.001$ indicates significant difference of samples pretreated with NAC or glutathione compared with no pretreatment by one-way ANOVA, followed by Tukey's multiple comparison test. For the analysis of cytosolic peroxide levels (c), 50 μ M DCFH-DA was added to each sample 40 min before the end of the incubation period. At the end of 16 h, cell samples were analysed immediately for DCF fluorescence by flow cytometry as described in the legend to Figure 1. Results are presented as mean percentages $\pm$ S.E.M. ($n = 3$). ** $P < 0.01$ indicates significant difference between samples treated with 20 μ M C₆-ceramide with glutathione or NAC pretreatment compared with no pretreatment. xxx $P < 0.001$ represents significant difference of test samples from control (Ctrl) cells with no pretreatment and ++ $P < 0.01$ indicates significant difference between test and control samples both pretreated with NAC or glutathione compared with one-way ANOVA followed by Dunnett's multiple comparison test.

of CD3⁺ T-cells) was always > 94%. Monocytic contamination was quantified simultaneously (CD14 expression) and was always < 1.5% (results not shown). T-cells were then cultured in RPMI 1640 in the presence or absence of 10 μ g/ml PHA to induce activation. T-cell activation was assessed as the percentage of CD3⁺CD25⁺ T-cells. Flow cytometry histograms of SS versus FS showed an increase in the size of T-cells, which was coupled with a significant increase in CD3⁺CD25⁺ levels to $43.62 \pm 15.39\%$ positive T-cells (mean $\pm$ S.D., $n = 12$) after activation with PHA for 3 days compared with $2.605 \pm 2.082\%$ for resting T-cells (mean $\pm$ S.D., $n = 12$, $P < 0.001$).

Figure 5 Antioxidants do not protect Jurkat T-cells from ceramide-mediated apoptosis

Jurkat T-cells (2×10^6 /ml) were serum-starved in RPMI 1640 for 4 h with or without 10 mM NAC or 10 mM glutathione before the addition of 20 μ M C₂-ceramide for 1–12 h (a). Alternatively, cell suspensions were treated with 10 mM L-cystathionine or L-methionine before treatment with 0–20 μ M C₂-C₆-ceramide for 16 h (b). All incubations were performed in a 95% air/5% CO₂ humidified atmosphere at 37 °C for 36 h. Experiments were terminated by washing with ice-cold PBS. Cell pellets were resuspended in 1 ml of hypo-osmotic fluorochrome solution as described in the Experimental section before DNA cell-cycle analysis by flow cytometry: 20 000 nucleoids were counted for each sample. Results are presented as mean percentages $\pm$ S.E.M. from three to seven individual experiments. Results were analysed for significant difference between samples pretreated with NAC, glutathione, L-cystathionine or L-methionine compared with no pretreatment by one-way ANOVA followed by Tukey's multiple comparison test.

DNA cell-cycle analysis of PHA-activated T-cells revealed the appearance of S-phase and G₂M phase nucleoids. As expected, resting T-cells had no evidence of DNA in the S-phase or G₂M phase of the cell cycle (results not shown). A limiting factor in performing the necessary experiments was the number of T-cells obtained from 40 ml of blood, typically 10×10^6 . Consequently, for experimental purposes, half the concentration of cells, 1×10^6 cells/ml, was used. After 6 h treatment with 0–20 μ M C₂-ceramide, DNA cell-cycle analysis by flow cytometry revealed no significant accumulation of subdiploid DNA in either resting ($P > 0.05$; see Figure 7a) or activated ($P > 0.05$) primary T-cells (see Figures 8a–8d). Furthermore, at this time point, C₂-ceramide induced a concentration-dependent decrease in DCF fluorescence in both resting and activated T-cells (see Figures 7c and 8e), which was initially significant at a concentration of 5 μ M C₂-ceramide in both cell types ($P < 0.05$). After 24 h treatment of resting or activated T-cells with 5 μ M C₂-ceramide, DCF fluorescence was significantly decreased to approx. –10 a.u. from vehicle-treated controls ($P < 0.05$; results not shown), with no significant evidence of subdiploid DNA ($P > 0.05$) at this time point (see Figures 7b and 8g). On extending the incubation to 24 h, C₂-ceramide induced a dose-dependent increase in the appearance of subdiploid DNA in resting T-cells, which was first significant at a concentration of 10 μ M, inducing approx. 30% DNA

Figure 6 Treatment of whole blood with C_6 -ceramide decreases the $[peroxide]_{cyt}$ of $CD3^+$ T-lymphocytes and $CD14^+$ monocytes

Venous blood was obtained from healthy consenting adult humans and treated with $20 \mu M$ C_6 -ceramide for 2 h at $37^\circ C$ with rotation. Reactions were terminated by incubation on ice with simultaneous treatment with the fluorescently tagged monoclonal antibodies CD3-PE and CD14-CY5PE and $50 \mu M$ DCFH-DA for 30 min. Red blood cells were lysed and leucocytes fixed as described in the Methods section. Samples were analysed by flow cytometry and the DCF fluorescence of the $CD3^+$ and $CD14^+$ populations quantified. Typical flow-cytometric histograms of (a) CD3-PE (FL2) versus SS, the highly fluorescent $CD3^+$ cell population isolated and gated 'Y', (b) CD14-CY5PE (FL4) versus SS, the highly fluorescent $CD14^+$ cell population isolated and gated 'Z', (c) DCF (FL1) versus count of the gated $CD3^+$ population 'Y' and (d) DCF (FL1) versus count of the gated $CD14^+$ population 'Z' are shown. Grey shading represents the DCF fluorescence of $CD3^+$ (c) or $CD14^+$ (d) populations respectively from vehicle-treated whole blood. Black outlines with no shading represent the DCF fluorescence obtained from whole blood treated with $20 \mu M$ C_6 -ceramide. Results are representative of three independent experiments. Histogram overlay was performed using WinMDI 2.8.

fragmentation ($P < 0.05$), and at $20 \mu M$, inducing 32% ($P < 0.01$; see Figure 7b). Likewise, in activated T-cells, at 24 h post-treatment, the extent of DNA fragmentation was identical with that observed in resting T-cells for each concentration of C_2 -ceramide ($P > 0.05$ for each dose; see Figures 8f–8i).

Treatment of Jurkat T-cells with CD95L induced a time- and dose-dependent increase in apoptosis. Specific apoptosis was initially observed after 4 h treatment with $1 \mu g/ml$ ($P < 0.05$) and $5 \mu g/ml$ ($P < 0.05$) CD95L. These concentrations of CD95L mediated an increase in the percentage of specific apoptosis to a maximum of approx. 70% after 12 h ($P < 0.01$) and 16 h ($P < 0.01$) respectively, after which a plateau was observed. Incubations of Jurkat T-cells with these concentrations of CD95L did not induce any further alteration in the percentage of specific

Figure 7 Synthetic short-chain ceramides induce loss in $[peroxide]_{cyt}$ in resting human T-lymphocytes before the appearance of DNA fragmentation

Primary human PBLs were extracted by density-gradient centrifugation and purified by negative isolation. T-cells were cultured for 3 days in RPMI 1640 (10% FCS and 1% P/S) at a concentration of $2 \times 10^6/ml$. T-cells ($1 \times 10^6/ml$) were then serum-starved in RPMI 1640 for 4 h before treatment with C_2 -ceramide (0– $20 \mu M$) for 6 h (a, c) or 24 h (b). For the quantification of apoptosis (a, b), samples were resuspended in hypo-osmotic fluorochrome solution and incubated overnight in the dark at $4^\circ C$ before DNA cell-cycle analysis by flow cytometry as described in the Experimental section. The percentage of actual apoptosis was calculated from the subdiploid content of 20 000 nucleoids/sample. For the analysis of cytosolic peroxide levels (c), cell samples were loaded with $50 \mu M$ DCFH-DA 40 min before the termination of the incubation period. At the end of the treatment period, samples were analysed immediately for DCF fluorescence by flow cytometry as described in the Experimental section, and the Mdx of 10 000 cells was recorded. ΔDCF represents the change in DCF Mdx of test samples from vehicle controls. The results are presented as the arithmetic means $\pm$ S.D. from at least three individual experiments. All cell cultures and treatments were performed at $37^\circ C$ in a 95% air/5% CO_2 humidified atmosphere. Statistical analysis was performed by one-way ANOVA followed by Dunnett's *post hoc* test analysis, where $**P < 0.01$ represents significant difference from controls. NS, no significant difference between vehicle-treated controls and tests.

Figure 8 Synthetic short-chain ceramides induce loss in [peroxide]_{cyt} in activated human T-lymphocytes before the appearance of DNA fragmentation

Primary human PBLs were extracted by density-gradient centrifugation and purified by negative isolation (DynaL A/S). T-lymphocytes were cultured for 3 days in RPMI 1640 (10 % FCS and 1 % P/S) containing 10 μ g of PHA/(2 $\times$ 10⁶ T-cells) per ml. T-cells were then serum-starved in RPMI 1640 for 4 h before treatment with C₂-ceramide (0–20 μ M) for 6 h (a)–(e) or 24 h (f)–(i). For DNA cell-cycle analysis by flow cytometry, samples were washed twice with ice-cold PBS, resuspended in hypo-osmotic fluorochrome solution and incubated overnight in the dark at 4 °C. The subdiploid region of 20 000 nucleoids from each sample was evaluated based on the count of gated histograms versus FL3 (PI). Shown are DNA cell-cycle histograms obtained from samples treated with vehicle (a), (f) or 5 μ M (b), (g), 10 μ M (c), (h) or 20 μ M (d), (i) C₂-ceramide. Numbers represent the mean percentage of subdiploid DNA $\pm$ S.D. from at least three individual experiments. Data were analysed for statistical difference by one-way ANOVA followed by Dunnett's multiple comparison test, where * P < 0.05 and ** P < 0.01 represent significant differences from controls. For the analysis of cytosolic peroxide levels (e), cell samples were loaded with 50 μ M DCFH-DA 40 min before the termination of the incubation period. At the end of the treatment period, samples were analysed immediately for DCF fluorescence by flow cytometry as described in the Experimental section, and the Mdx of 10 000 cells was recorded. Shown is an overlay of DCF histograms from T-cells treated with vehicle or 5 or 10 or 20 μ M C₂-ceramide. Histogram overlay was performed using WinMDI 2.8. Results are representative of those obtained from at least three individual experiments. All cell cultures and treatments were performed at 37 °C in a 95 % air/5 % CO₂ humidified atmosphere.

apoptosis after 16 h treatment (P > 0.05). There was no significant difference in the maximal percentage of specific apoptosis induced by 1 and 5 μ g/ml CD95L (P > 0.05). Treatment of Jurkat T-cells with 100 ng/ml CD95L failed to induce any significant increase in the percentage of specific apoptosis (P < 0.05) for up to 16 h. Intermediate concentrations of CD95L (250 and 500 ng/ml) also induced significant time-dependent increases in the specific apoptosis, which were maximal after 16 h (20 %) and 36 h (55 %) respectively and significantly different from the maximal apoptotic response of 70 % induced by higher concentrations of CD95L (P < 0.001; see Figure 9a).

CD95L induced a transient increase in [ceramide]_i (endogenous ceramide concentration) in a concentration-dependent fashion.

The [ceramide]_i increased significantly to approx. 150 % (P < 0.05) and 180 % (P < 0.05) of control levels after 30 min treatment with 0.5 and 1 μ g/ml CD95L respectively and to approx. 240 % (P < 0.05) and 320 % (P < 0.01) of control levels after 1 h treatment with 0.5 and 1 μ g/ml CD95L respectively. After 4 h treatment with either 0.5 or 1 μ g/ml CD95L, [ceramide]_i returned to that of control levels (P > 0.05 for both concentrations; see Figure 9b).

Since CD95L mediated an increase in the level of the intracellular signal-transduction molecule ceramide, and considering our observations of increased [peroxide]_{cyt} in Jurkat T-cells in response to synthetic ceramide, we examined the effects of CD95L on [peroxide]_{cyt} in Jurkat T-cells. Treatment of Jurkat T-cells

Figure 9 CD95-induced apoptosis in Jurkat T-cells preceded by endogenous ceramide production and loss in $[\text{peroxide}]_{\text{cyt}}$

Jurkat T-cells ($2 \times 10^6/\text{ml}$) were serum-starved for 4 h before the addition of CD95L (0–5 $\mu\text{g}/\text{ml}$) for 0–24 h. Incubations were performed at 37 °C in a 95% air/5% CO_2 humidified atmosphere and terminated by washing the cells twice with ice-cold PBS before further experimental manipulation. (a) For quantification of apoptosis, samples were prepared and analysed as described in the legend to Figure 1. (b) Lipids were extracted and endogenous ceramide quantified against a standard curve of known ceramide concentrations by the DAGK assay as described. (c) For the analysis of cytosolic peroxide levels, cell samples were loaded with 50 μM DCFH-DA 40 min before the termination of the incubation period. At the end of the treatment period, samples were analysed immediately for DCF fluorescence by flow cytometry as described in the Experimental section, and the MdX of 10 000 cells was recorded. $\Delta\text{DCF MdX}$ represents the difference in MdX DCF of CD95-treated cells from that of vehicle-treated cells for each time point. Results are presented as the means $\pm$ S.E.M. from three to five individual experiments.

with CD95L mediated a decrease in DCF fluorescence. $\Delta\text{DCF MdX}$ was significantly decreased after 2 h treatment with 1 $\mu\text{g}/\text{ml}$ CD95L ($P < 0.01$) and 8 h treatment with 500 ng/ml CD95L ($P < 0.05$; see Figure 9c), which was before any significant observation of apoptosis ($P > 0.05$ for both concentrations; see Figure 9a) and after the accumulation of intracellular ceramide in response to either concentration of CD95L (see Figure 9b). The decrease in $\Delta\text{DCF MdX}$ occurred with faster kinetics and was of greater magnitude after treatment of Jurkat T-cells with 1 $\mu\text{g}/\text{ml}$ CD95L when compared with that observed after treatment with 500 ng/ml

CD95L. The decrease in $\Delta\text{DCF MdX}$ on treatment with CD95L reached a plateau after 4 and 8 h treatments with 1 $\mu\text{g}/\text{ml}$ and 500 ng/ml CD95L respectively (see Figure 9c).

DISCUSSION

It is reported in the present study that the application of short-chain ceramides to T-cells or monocytes induces a loss in cytosolic peroxide levels. This is in contrast with the direct effects of synthetic short-chain ceramides on the mitochondria that mediate an increase in mitochondrial peroxide production, as described previously by us [23] and by others [21,22]. Collectively, this supports the notion that ceramide may differentially modulate the redox state at discrete subcellular compartments [35]. Indeed, several authors have suggested recently that ROS may inhibit caspase-3 activity, where a decrease in ROS is associated with increased apoptosis [36,37]. On the basis of our evidence to support that antioxidant treatment of cells before the addition of ceramide enhanced the apoptotic response, we hypothesize that, in the cellular models utilized here, it is the decrease in $[\text{peroxide}]_{\text{cyt}}$ that is of primary importance in dictating the cellular response to ceramide rather than its mitochondrial effects. However, a mediator role for the loss in $[\text{peroxide}]_{\text{cyt}}$ cannot be clearly established from these results.

Jurkat T-cells treated with synthetic, short-chain ceramides show a time-dependent increase in the appearance of fragmented DNA, a marker for apoptosis, as described by others [2,3,6–8,12], which was maximal at 8 h post-treatment with C_2 -ceramide (20 μM). In a previous study, we reported that no significant difference between the short-chain C_2 - and C_6 -ceramides was observed in the induction of apoptosis in T-cells [23]. At 16 h post-treatment, cell death induced by 20 μM C_2 -ceramide was also associated with the formation of a subpopulation of Jurkat T-cells, displaying a decrease in cell size coupled with the loss of membrane permeability, as evaluated by flow-cytometric analysis of the cellular uptake of the membrane-impermeable dye PI when the cells enter late apoptosis or secondary necrosis. Unlike Jurkat T-cells, treatment of U937 cells with synthetic ceramide induced an accumulation of nucleoids in the G_0/G_1 phase of the cell cycle, which is indicative of cell-cycle arrest [15–17], but was not as pronounced as described previously [17]. Growth arrest was observed after 16 h incubation with 20 μM C_2 -ceramide, consequent to the onset of apoptosis in Jurkat T-cells, and it was persistent for up to 36 h. Furthermore, cell size and membrane permeability remained unaltered after ceramide treatment, indicating no induction of necrosis. U937 monocytes are reported to undergo apoptosis after treatment with concentrations of ceramide in excess of 20 μM [12,22].

Contrary to the observation of ceramide-mediated transient increases in $[\text{peroxide}]_{\text{m}}$ [23], use of the cytosolic peroxide-sensitive dye DCFH-DA revealed an overall loss in $[\text{peroxide}]_{\text{cyt}}$ before the appearance of DNA fragmentation or G_0/G_1 growth arrest in Jurkat T-cells and U937 monocytes respectively. An apoptotic response in Jurkat T-cells to C_2 -ceramide was preceded by an immediate, almost exponential-like decrease in $[\text{peroxide}]_{\text{cyt}}$. In contrast, where growth arrest was observed in U937 monocytes, the decrease in $[\text{peroxide}]_{\text{cyt}}$ was initially delayed, and decreased substantially approx. 40 min post-treatment. Additionally, the maximal loss in $[\text{peroxide}]_{\text{cyt}}$ in U937 cells was smaller when compared with that observed in Jurkat T-cells where it was associated with apoptosis. The apparent loss in $[\text{peroxide}]_{\text{cyt}}$ was not due to the leakage of DCFH-DA from the cytosolic environment, since the cells remained viable, as indicated by the minimal uptake of the membrane-impermeable dye PI throughout

the DCF analysis period. Although 20 μM C_2 -ceramide induced a population of Jurkat T-cells with high PI uptake and decreased cell size after 16 h treatment, these cells were excluded from $[\text{peroxide}]_{\text{cyt}}$ determination, as they had undergone secondary necrosis. Lower concentrations of synthetic ceramide (10 μM), which did not compromise Jurkat T-cell membrane permeability after 16 h treatment, showed decreased $[\text{peroxide}]_{\text{cyt}}$ which was not at a level that was associated with apoptosis. Therefore the magnitude and kinetics of $[\text{peroxide}]_{\text{cyt}}$ loss are probably dictated by the cellular responses to synthetic ceramides.

In the light of the evidence suggesting that cytoplasmic peroxide levels may play only a minor role in the oxidation of DCF [38,39], where the major catalysts are ferryl-containing molecules, it is important to adopt a further analytical method that allows direct determination of ROS. Cytochrome *c* reduction by superoxide anion radical is widely used and accepted as a specific measure of oxidant formation. Hence, this methodology was adopted to substantiate our findings with DCF. Whereas no change in superoxide production in response to ceramide was recorded at 1 h, both U937 cells and Jurkat T-cells showed a lower capacity to reduce cytochrome at 16 h, and the effect seen was greater in T-cells. The overall effects of NADPH oxidase inhibition with DPI and ceramide suggest that modulation of NADPH oxidase may be a contributor to the loss in $[\text{peroxide}]_{\text{cyt}}$ observed. However, the results suggest that other unknown mechanisms may also contribute to this process. Furthermore, the correlation between DPI-enhanced ceramide-induced apoptosis at 16 h and loss in $[\text{peroxide}]_{\text{cyt}}$ provides further evidence for the hypothesis that loss in $[\text{peroxide}]_{\text{cyt}}$ is the determining factor in cellular outcome. Clearly, more detailed examination of the potential interaction of ceramide with the NADPH oxidase system is required.

If the excessive production of mitochondrial peroxide was the sole mediator of apoptosis or growth arrest, then a beneficial effect might be expected by pretreatment of Jurkat T-cells or U937 monocytes respectively with antioxidants. However, we observed that either of the antioxidants NAC or glutathione failed to abrogate synthetic ceramide-mediated apoptosis at any time point. Furthermore, the inhibition of GSSG efflux (which was confirmed by demonstrating increased intracellular total glutathione) with L-cystathionine or L-methionine did not protect Jurkat T-cells from C_2 -ceramide-induced apoptosis, despite offering protection against methotrexate (D. C. Phillips and H. R. Griffiths, unpublished work) or puromycin [34] treatment. This again suggests that the effects of ceramide are not due to an increase in ROS. Surprisingly, in U937 monocytes, NAC and glutathione inhibited synthetic ceramide-induced G_0/G_1 growth arrest by driving ceramide-treated cells into apoptosis as indicated by the presence of fragmented DNA. In effect, antioxidants transformed the cellular response of U937 monocytes to ceramide treatment from a cytostatic to a cytotoxic response. Reducing the intracellular peroxide levels of U937 monocytes by pretreatment with antioxidants, which were not toxic by themselves, permits $[\text{peroxide}]_{\text{cyt}}$ to decrease after ceramide treatment to a level which confers apoptosis. Whereas NAC and glutathione treatment alone did cause a decrease in basal DCF fluorescence (-30 a.u.), this was insufficient to induce apoptosis (where -55 a.u. was associated with apoptosis in T-cells), although higher concentrations of either antioxidant were toxic (results not shown). Indeed, several authors have recently suggested that ROS may inhibit caspase-3 activity, where a decrease in ROS below a minimum threshold is associated with increased apoptosis [36,37].

Mansat-de Mas et al. [12] observed increased fluorescence of a DCFH-DA analogue, C2938, within 20 min of treatment with C_6 -ceramide that returned to basal level after 30 min, whereas Lee and Um [25] described no alteration in DCF fluorescence

after treatment of U937 cells with C_2 -ceramide. However, in our experiments, the concentration of C_2/C_6 -ceramide applied to whole cells encompasses the fluctuation range achieved by endogenous natural ceramides in response to external stimuli (reviewed in [40]). Where other authors have reported massive increases in ROS production at the mitochondria [22], the concentrations of C_2/C_6 -ceramide used were in excess of 5-fold the concentration (per 10^6 cells) that we have used to elicit small transient increases in $[\text{peroxide}]_{\text{m}}$ [23] or the loss in $[\text{peroxide}]_{\text{cyt}}$ reported here. Therefore enhanced production of cytosolic peroxide described in response to high doses of ceramides/ 10^6 cells is probably a consequence of ROS leakage from the mitochondria into the cytosol with loss of compartmental redox effects.

The biochemical and molecular processes that lead to cellular responses to various agents analysed in immortalized cell lines are often criticized, as they are continuously cycled and possess higher metabolic rates. It was therefore of importance to study the cellular and redox effects of synthetic short-chain ceramides on normal, primary human cells. The observation that decreased $[\text{peroxide}]_{\text{cyt}}$ precedes apoptosis in human $\text{CD}3^+$ T-cells and $\text{CD}14^+$ monocytes in peripheral whole blood treated with C_2/C_6 -ceramide and in purified resting T-cells or PHA-activated T-cells *in vitro* supports those results obtained from cell lines. The ability of C_2 -ceramide to induce apoptosis, which was preceded by loss in $[\text{peroxide}]_{\text{cyt}}$, was independent of the activation state and phase of the cell cycle, where identical levels of $[\text{peroxide}]_{\text{cyt}}$ loss and DNA fragmentation were observed in resting and PHA-activated T-cells. Resting T-cells possessed only DNA that was in the G_0 phase of the cell cycle, whereas PHA-activated T-cells displayed the appearance of a small number of S-phase and G_2M phase nucleoids, indicating progression through the cell cycle. In contrast, under identical culture conditions and treatments, Mengubas et al. [41] reported that C_2 -ceramide killed normal human T-lymphocytes via a non-apoptotic mechanism that was prevented by PHA activation. In the results described herein, the membrane permeability of resting and activated primary human T-cells treated with C_2 -ceramide (0–20 μM) was not compromised 6 h post-treatment when analysed for uptake of the membrane-impermeable dye PI by flow cytometry, indicating that necrosis did not contribute to the death process (results not shown).

In an attempt to analyse the effects of intrinsic endogenous ceramide generation on $[\text{peroxide}]_{\text{cyt}}$, Jurkat T-cells were treated with a known inducer of ceramide accumulation, CD95L [6–8]. In the present study, we have shown that Jurkat T-cell treatment with CD95L leads to an accumulation of ceramide within 30 min, which increased further at 1 h. This was followed by a decrease in $[\text{peroxide}]_{\text{cyt}}$ before the appearance of apoptosis. Whereas the extent of apoptosis that we observed after treatment with 1 or 0.5 $\mu\text{g}/\text{ml}$ CD95L was similar to that mediated by 20 μM C_2 -ceramide, the kinetics of the appearance of fragmented DNA was slower. Correspondingly, the magnitude of decrease in $[\text{peroxide}]_{\text{cyt}}$ generation was less and occurred more slowly. In contrast, other authors [20] have shown that, whereas CD95 receptor stimulation induced ceramide formation in human glioma cells via the activation of acid SMase, no ROS formation was observed and the ensuing apoptosis was unaffected by antioxidants. Similarly, L929 cells expressing the CD95 receptor undergo CD95-induced apoptosis, which is not inhibitable by antioxidants [42]. These observations are in contrast with the increase in DCF fluorescence reported in IL-1 β /tumour necrosis factor- α -activated peripheral blood monocytes before the apoptosis induced by CD95L treatment, both of which were inhibitable by antioxidants [43], and other studies have suggested that CD95 induces ROS generation via stimulation of the NADPH oxidase

system [44,45]. Other studies have also shown that neuroglioma cells overexpressing Cu or ZnMn SOD showed significant attenuation of CD95-induced apoptosis, that catalase treatment inhibited CD95-induced apoptosis of normal neurogliomas [46], and that NAC or glutathione inhibited CD95L-induced apoptosis in the HL60 variant HL-525, in the absence of an increase in endogenous ceramides [47]. These discrepancies may exist due to differences in cell lineage or the degree of saturation and length of the fatty acid chain between endogenous ceramides generated by ligands such as FAS and synthetic, exogenously applied short-chain ceramides. However, during the preparation of this paper, we found that Aronis et al. [48] have also reported the observation of a decrease in ROS production in response to CD95 receptor stimulation in Jurkat T-cells that correlates with enhanced cell death, further supporting the conclusions of the observations reported in the present study.

We have observed that short-chain ceramide manipulates the intracellular redox state through mitochondrial and non-mitochondrial pathways. Mitochondrial peroxide generation may be important in parallel, but acting within a different subcellular compartment, with ceramide-induced loss in [peroxide]_{cyt}. The loss in [peroxide]_{cyt} in response to short-chain ceramides in the immortalized cell lines of U937 monocytes and Jurkat T-cells has been reproduced in primary human cells of identical lineage, indicating that the effects are not related to cellular immortality. Furthermore, the results presented here, taken together with our previous observations [23], indicate the existence of discrete redox-sensitive entities which may be targeted and manipulated by ceramide to produce independent responses.

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