Inhibition of the expression of ornithine decarboxylase and c-Myc by cell-permeant ceramide in difluoromethylornithine-resistant leukaemia cells

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Ceramide has emerged as a novel lipid mediator in cell growth and apoptosis. In difluoromethylornithine-resistant L1210 cells stimulated to growth from quiescence, the cell-permeant analogues of ceramide *N*-acetyl sphingosine (C_2 -ceramide) and *N*-hexanoyl sphingosine (C_6 -ceramide) inhibited the induction of ornithine decarboxylase (ODC) activity with IC_{50} of 8.3 and 1.5 μ M respectively. This effect was strictly related to the ability to inhibit cell growth and [³H]thymidine incorporation. The suppression of cell growth was also associated with apoptosis. The addition of bacterial sphingomyelinase resulted in a significant, but limited, reduction of ODC induction and [³H]thymidine incorporation. Bacterial lipopolysaccharide, which may act as a ceramide analogue, also inhibited the induction of the enzyme. Moreover, C_6 -ceramide largely

prevented the accumulation of ODC mRNA and its precursor, ODC heterogeneous nuclear RNA, that accompanied the induction of ODC activity. A slight increase in ODC turnover was also observed. The DNA-binding activity of some transcription factors known to bind and transactivate the ODC gene was investigated by gel mobility-shift assay under the same experimental conditions. However, only the binding of Myc/Max was negatively affected by the treatment with C_6 -ceramide. Furthermore, the amount of immunoreactive c-Myc, which increased after stimulation of the cells to growth, was strongly reduced by C_6 -ceramide. These results suggest that the inhibition of c-Myc and ODC expression may be early events in the response of leukaemia cells to ceramide.

INTRODUCTION

The sphingomyelin cycle is now recognized as an important signal-transduction pathway regulating cell growth and death [1,2]. The action of various cytokines, hormones and other extracellular agents results in early activation of a sphingomyelinase which cleaves membrane sphingomyelin resulting in the formation of phosphorylcholine and ceramide. In turn, ceramide has emerged as a novel intracellular messenger mediating at least some of the effects of these agents. The availability of short-chain cell-permeant analogues of ceramide, such as *N*-acetyl sphingosine (C_2 -ceramide) and *N*-hexanoyl sphingosine (C_6 -ceramide), has allowed examination of cellular activities and biochemical targets of ceramide. Short-chain ceramides exert potent and specific anti-proliferative effects by inducing G_0/G_1 arrest in the cell cycle, differentiation or apoptosis at micromolar concentrations [1,2]. Biochemical effects of exogenous ceramides include activation of specific Ser/Thr kinases and phosphatases [1–3], inhibition of phospholipase D [2,4], down-regulation of the expression of c-Myc [5] and Bcl-2 [6], dephosphorylation of retinoblastoma gene product (Rb) [7] and modulation of DNA-binding activity of the transcription factors NF- κ B [2,8] and AP-1 [9,10].

Ornithine decarboxylase (ODC), the first and key enzyme in polyamine biosynthesis, is essential for cell proliferation and is induced by growth stimuli [11–13]. The ODC gene is now recognized as an early response gene required for progression into the S-phase of the cell cycle and as a proto-oncogene [12,14].

Moreover some studies suggest additional roles for ODC in cellular differentiation [15] and apoptosis [16]. However, at the present no information is available about the relationship between ceramide and polyamine metabolism. In order to ascertain whether ceramide can affect ODC expression, we utilized a line of leukaemia L1210 cells selected for resistance to the ODC inhibitor difluoromethylornithine (L1210-DFMO^r cells). Because of gene amplification, these cells can express ODC at high levels, while maintaining the usual mechanisms of regulation of the enzyme [17]. The present paper reports the effects of exogenous short-chain ceramides on ODC induction and some transcription factors that may be involved in the control of ODC gene transcription.

EXPERIMENTAL

Materials

L1210-DFMO^r cells were generously given by Dr. Persson (University of Lund, Lund, Sweden). L-[1-¹⁴C]Ornithine was purchased from ICN biomedical (Irvine, CA, U.S.A.) and [³H]thymidine from New England Nuclear (Boston, MA, U.S.A.). DFMO was kindly provided by the Merrell Dow Research Institute (Strasbourg, France). *N*-Acetyl-D-sphingosine (C_2 -ceramide), *N*-hexanoyl-D-sphingosine (C_6 -ceramide), sphingomyelinase from *Staphylococcus aureus*, lipopolysaccharide (LPS) from *Escherichia coli*, phosphorylcholine and oleic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *N*-Hexanoyl-D-dihydrosphingosine (C_6 -dihydrocera-

Abbreviations used: ODC, ornithine decarboxylase; DFMO, difluoromethylornithine; LPS, lipopolysaccharide; C_2 -ceramide, *N*-acetyl-D-sphingosine; C_6 -ceramide, *N*-hexanoyl-D-sphingosine; C_6 -dihydroceramide, *N*-hexanoyl-D-dihydrosphingosine; EMSA, electrophoretic mobility-shift assay; Rb, retinoblastoma gene product.

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mide) was from Matreya Inc. (Pleasant Gap, PA, U.S.A.). Oligonucleotide primers for reverse transcriptase-PCR were synthesized with an ABI 391 DNA synthesizer (applied Biosystems, Cheshire, U.K.) and then purified by reverse-phase HPLC. PCR was carried out in a cetus DNA thermal cycler (Perkin-Elmer, Norwalk, CT, U.S.A.). Capillary electrophoresis was performed in a P/ACE System 2100 (Beckman Instruments, Fullerton, CA, U.S.A.). Anti-(mouse c-Myc protein) (rabbit polyclonal IgG) was from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Double-stranded oligonucleotides containing consensus binding sites for transcription factors were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). The oligonucleotide probes were labelled by polynucleotide kinase and [γ - 32 P]ATP (Amersham), and purified by separation on Sephadex G-25 before use.

Cell culture and treatment

Mouse L1210-DFMO^r cells were routinely grown as previously described [17,18]. For experiments, cells were shifted to, and maintained in, a DFMO-free medium for at least 15 days. Then quiescent cells (cell density $\geq 2 \times 10^6$ /ml) were seeded at 2×10^5 /ml in fresh RPMI 1640 medium containing 10 % fetal calf serum and 50 μ M 2-mercaptoethanol, and treated as indicated. Short-chain ceramides were added in ethanol. Control cells received equal amounts of the vehicle. Wild-type L1210, HL-60 and Jurkat cells were utilized under the same experimental conditions. Cell viability was checked by Trypan Blue exclusion. At the time indicated after seeding, cells were harvested and washed with PBS.

[3 H]Thymidine incorporation and DNA fragmentation

Cells were incubated with [3 H]thymidine (1 μ Ci/ml) for 4 h, rinsed twice with PBS, precipitated and washed once with cold 5 % trichloroacetic acid. Finally, the pellets were solubilized with 0.1 M NaOH and counted for radioactivity. In order to demonstrate internucleosomal digestion, DNA was extracted from cells incubated with C₆-ceramide; then aliquots of DNA (4 μ g) were analysed by agarose-gel electrophoresis and visualized after ethidium bromide staining.

Determination of ODC activity and polyamine content

Cell extracts were prepared and assayed for ODC activity as previously described [18]. ODC activity was determined in the presence of L-[1- 14 C]ornithine by measuring the amount of CO₂ released, and is expressed as units/mg of protein, where 1 unit corresponds to 1 nmol of CO₂/h of incubation. C₂-ceramide and C₆-ceramide did not affect ODC activity when added directly to the assay mixture at concentrations of 10–40 μ M. As for polyamine determination, about 3×10^6 cells were lysed in 0.4 ml of chilled 0.3 M HClO₄. Polyamines were analysed in acid extracts after derivatization with dansyl chloride [19].

Detection of ODC mRNA and ODC heterogeneous nuclear (hn)RNA

ODC mRNA and hnRNA were detected in L1210-DFMO^r cells by combined reverse transcriptase-PCR analysis [20]. Total RNA was isolated from about 10^7 cells using the guanidinium thiocyanate method [21]. Any contaminating DNA was removed by DNase treatment essentially as described by Hyttinen et al. [20]. This treatment was necessary because mouse genome contains many pseudogenes for ODC [11]. cDNA synthesis and PCR were performed as previously described [22] by using the same enzyme, *rTth* DNA polymerase (Perkin-Elmer), according to the manufacturer's instructions. The reaction mixture

contained 0.25 μ g of total RNA extracted from cells, 5 units of *rTth* DNA polymerase, 0.2 mM each dNTP and 100 pmol of each primer in a final volume of 50 μ l. The PCR primers for mature ODC mRNA were designed so as to yield a PCR product of 224 nt. The 5' primer (5'-TCATAGCTGAGCCAGGCAG-ATA-3') was targeted to a sequence in exon 9 of mouse ODC mRNA and the 3' primer (5'-CTTGGGTCTCTTCTGCAGC-3') recognized a sequence at the junction of exons 10 and 11. For the detection of ODC hnRNA, another pair of primers was used, yielding a PCR product of 247 nt. The 5' primer (5'-ATGG-CTGCTCCCTTCTCTC-3') and the 3' primer (5'-ACCACC-CAGCATATCAAC-3') were targeted to sequences of intron 8 and 9 respectively. The reverse transcriptase-PCR consisted of a reverse transcriptase phase (60 °C for 30 min, 94 °C for 2 min) followed by amplification (94 °C for 45 s, 60 °C for 75 s). PCR products were visualized by ethidium bromide fluorescence after separation by 1.5 % agarose-gel electrophoresis or quantified by capillary electrophoresis coupled to laser-induced fluorescence [23]. The high sensitivity of this technique allowed detection of samples not visualized on agarose gels. For the semiquantitative determination of the relative amounts of the messenger, the number of PCR cycles and the amount of total RNA added were chosen in the range of proportionality of PCR amplification.

Western-blot analysis of c-Myc

After a wash with PBS, 6×10^6 cells were resuspended in 0.2 ml of lysis buffer (50 mM Tris/HCl, pH 7.4, 1 % Nonidet P40, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 μ g/ml aprotinin, leupeptin, pepstatin, 1 mM Na₃VO₄, 1 mM NaF), sonicated and centrifuged at 12000 g for 10 min. The supernatant was boiled in SDS-containing sample buffer and an aliquot containing 20 μ g of protein was subjected to SDS/PAGE (7.5 % gel). Separated proteins were transferred to nitrocellulose membrane at 4 °C overnight. The membrane was saturated with 3 % BSA for 1 h, then incubated with anti-(mouse c-Myc) antibody (1 μ g/ml) at room temperature for 2 h. Bands were revealed by the Amersham ECL detection system.

Nuclear extract preparation and electrophoretic mobility-shift assay (EMSA)

Cells (about 10^7) were washed in buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 5 mM KCl, 1 mM PMSF, 1 mM dithiothreitol), resuspended in buffer B (20 mM Hepes, 25 % glycerol, 450 mM KCl, 1 mM EDTA, 1 mM PMSF, 420 mM NaCl, 1.5 mM MgCl₂) and agitated gently at 4 °C for 45 min. The extract was centrifuged at 13000 g at 4 °C for 30 min, and the supernatant was collected, assayed for protein content [24] and adjusted to 1–2 μ g of protein/ μ l. Aliquots of extract were frozen at –80 °C or used immediately. Equal amounts (10 μ g) of nuclear extract protein were preincubated with binding buffer (60 mM final salt concentration; 20 mM Hepes, 2.5 mM MgCl₂, 1 mM dithiothreitol, 1 mM PMSF) and 1 μ g of poly(dI-dC) (and 50 ng of unlabelled competitor or uncompetitor oligonucleotide, where appropriate) for 2 min before labelled oligonucleotide (10000 c.p.m.) was added. After 20 min at room temperature, the mixture was loaded on a non-denaturing polyacrylamide gel made and run in 0.25 $\times$ Tris/borate/EDTA buffer. The gel was dried and subjected to autoradiography.

RESULTS

Effect of cell-permeant ceramides on cell growth and death

We have previously shown that dilution of quiescent L1210-DFMO^r cells in fresh medium containing serum leads to cell

Figure 1 Effect of cell-permeant ceramides on the growth curve and DNA synthesis of L1210-DFMO^r cells

(A) Quiescent cells were diluted in fresh medium containing serum and incubated in the absence (○) or presence of 10 μM C₂-ceramide (●), 5 μM C₆-ceramide (△) or 10 μM C₆-ceramide (▲). (B) Cells were seeded as above and treated with the indicated concentrations of C₂-ceramide (○) or C₆-ceramide (●). After 16 h, cells were incubated with [³H]thymidine for a further 4 h. Results represent means ± S.D. from three separate determinations. [³H]Thymidine incorporation for control cells was 25059 ± 474 d.p.m./well.

Figure 2 Effect of C₆-ceramide on viability (A) and internucleosomal DNA fragmentation (B) of L1210-DFMO^r cells

(A) Quiescent cells were diluted in fresh medium containing serum in the absence (○) or presence of 5 μM C₆-ceramide (●) or 10 μM C₆-ceramide (△). Cell viability was checked by Trypan Blue exclusion. Results are means ± S.D. from at least three determinations. (B) Electrophoretic analysis of DNA extracted from cells incubated in the absence (lane 1) or presence of 10 μM C₆-ceramide for 24 h (lane 2).

growth and to the induction of ODC activity and mRNA [18,22,23]. In order to ascertain whether ceramide can affect the proliferation of L1210 cell lines, growth curves and [³H]thymidine incorporation of L1210-DFMO^r cells stimulated by serum were analysed in the presence of exogenous ceramides. Figure 1(A) shows that C₆-ceramide (10 μM) blocked cell growth completely, whereas C₂-ceramide was much less effective. This is reflected in a different ability to inhibit [³H]thymidine incorporation (Figure 1B); both short-chain ceramides caused a dose-dependent inhibition of DNA synthesis, but IC₅₀ was about 15 μM for C₂-ceramide and 2.5 μM for C₆-ceramide. Bacterial sphingomyelinase (100 m-units/ml) was a modest inhibitor of thymidine incor-

Table 1 Effect of cell-permeant ceramides and related compounds on ODC induction

Quiescent cells were diluted in fresh medium containing serum and treated as indicated. After 16 h cells were harvested and assayed for ODC activity. Results are means ± S.D. from at least three separate determinations. Average ODC activity of control was 246 and 14.5 units/mg of protein for L1210-DFMO^r and wild-type L1210 cells respectively.

Addition	Concentration	ODC activity (% of control)
L1210-DFMO ^r cells		
C ₂ -Ceramide	10 μM	44.4 ± 2.5
	20 μM	25.5 ± 1.4
C ₆ -Ceramide	5 μM	12.1 ± 6.8
	10 μM	2.3 ± 2.6
Sphingomyelinase	10 mU/ml	82.1 ± 1.8
	100 mU/ml	80.1 ± 2.1
LPS	1 μg/ml	74.7 ± 3.0
	5 μg/ml	43.7 ± 1.2
Oleic acid	10 μM	103.1 ± 1.1
Phosphorylcholine	10 μM	100.6 ± 1.7
Wild-type L1210 cells		
C ₂ -Ceramide	10 μM	57.3 ± 4.4
	20 μM	42.9 ± 3.1
C ₆ -Ceramide	5 μM	49.8 ± 1.4
	10 μM	6.0 ± 0.7

poration, which was reduced to 79.0 ± 1.2% of control. Finally, C₆-ceramide was able to reduce the growth of wild-type L1210 cells (not shown).

Since it has been shown that ceramide can elicit apoptosis of several types of cell [2], we considered the possibility that the inhibition of the growth of L1210-DFMO^r cells was associated with a cytotoxic effect. Figure 2(A) shows that C₆-ceramide caused a marked and dose-dependent reduction of cell viability when the treatment was prolonged for more than 16 h. Furthermore this toxic effect was associated with internucleosomal DNA fragmentation (Figure 2B), considered one of the hallmarks of apoptosis.

Figure 3 Effect of increasing concentrations of cell-permeant ceramides on ODC induction in L1210-DFMO^r cells

Quiescent cells were diluted in fresh medium containing serum and incubated in the presence of the indicated concentrations of C₂-ceramide (○) and C₆-ceramide (●). Cells were harvested 16 h after seeding. Results are means from three separate determinations. S.D. is shown where large enough.

Effect of cell-permeant ceramides on the induction of ODC activity

The effect of cell treatment with short-chain ceramides or other related compounds on the induction of ODC activity is shown in Table 1. Both C₂-ceramide and C₆-ceramide inhibited the induction of the enzyme, but C₆-ceramide proved to be more potent, with a reduction of about 90% at a concentration of 5 μM. The addition of bacterial sphingomyelinase caused a significant, but limited, reduction of ODC induction (by about 29%). LPS was an effective inhibitor, whereas oleic acid, another amphiphilic lipid, and phosphorylcholine, the other product of sphingomyelin hydrolysis, were without effect. Table 1 also shows that exogenous ceramides provoked inhibition of ODC induction in wild-type L1210 cells, although the response was less pronounced than in the DFMO-resistant cells. Thus the ODC-inhibiting effect of ceramides is not limited to a particular ODC-overproducing cell line. In fact, even in HL-60 and Jurkat cells, C₆-ceramide (10 μM) provoked early inhibition of ODC induction, which preceded the appearance of cell toxicity and DNA fragmentation (not shown).

Figure 5 Effect of C₂-ceramide and bacterial sphingomyelinase on the time course of ODC activity after changing to a serum-free medium

Quiescent cells were seeded in medium containing serum and, after 16 h, shifted to fresh serum-free RPMI 1640. After a further 1 h incubation (time 0), cells were treated as follows: ○, Control; ●, 5 μM C₂-ceramide; ▲, 100 mU/ml sphingomyelinase.

The effect of exogenous ceramides on ODC induction in L1210-DFMO^r cells was investigated in more detail. Figure 3 shows that C₂-ceramide and C₆-ceramide exerted dose-dependent inhibition with IC₅₀ of 8.3 and 1.5 μM respectively. A virtually complete suppression of ODC induction by C₂-ceramide was observed at a concentration of 40 μM (2.1 ± 0.7% of control, not shown). The time course of ODC induction in the presence or absence of short-chain ceramides is shown in Figure 4(A). ODC induction was reduced by over 50% after 4 h of treatment with C₆-ceramide (5–10 μM) and remained very low thereafter. C₆-ceramide was effective even at decreasing preinduced ODC activity, i.e. when added to cells containing high levels of the enzyme (Figure 4B). A significant reduction in ODC activity was observed as soon as 2 h after treatment with C₆-ceramide. C₂-ceramide was a less effective inhibitor than C₆-ceramide at any time investigated. Moving cells containing preinduced ODC to a serum-free medium resulted in a further and marked increase in ODC activity, which was almost completely prevented by the addition of C₂-ceramide at a concentration as low as 5 μM (Figure 5). After 8 h of treatment, ODC activity was only 13%

Figure 4 Effect of cell-permeant ceramides on the time course of ODC activity in L1210-DFMO^r cells

(A) Quiescent cells were seeded in fresh medium containing serum and immediately treated with ceramides. (B) Cells were seeded as above, but treated with ceramides after 16 h (time 0). ○, Control; ●, 10 μM C₂-ceramide; △, 5 μM C₆-ceramide; ▲, 10 μM C₆-ceramide. Results represent means ± S.D. from three separate determinations.

Table 2 Comparison of the effects of C₆-dihydroceramide and C₆-ceramide on ODC induction in L1210-DFMO⁺ cells

Quiescent cells were seeded in fresh medium containing serum and immediately treated with 10 μ M C₆-dihydroceramide or C₆-ceramide. Alternatively, in order to examine the effect on preinduced ODC, cells were seeded as above, but treated with 10 μ M C₆-dihydroceramide or C₆-ceramide after 16 h. In both cases, ODC activity was measured 4 h after the treatment with ceramides. Results represent means $\pm$ S.D. from at least three determinations.

	ODC activity (% of control)	
	Treatment at seeding	Treatment 16 h after seeding
C ₆ -Dihydroceramide	109.8 $\pm$ 5.5	85.9 $\pm$ 13.2
C ₆ -Ceramide	27.4 $\pm$ 3.5	11.0 $\pm$ 6.9

Table 3 Effect of C₆-ceramide on polyamine content of L1210-DFMO⁺ cells

Quiescent cells were diluted in fresh medium containing serum and incubated in the absence (control cells) or presence of 5 μ M C₆-ceramide for 16 h. Results are means $\pm$ S.D. from three determinations.

	Polyamine content (nmol/mg of protein)		
	Putrescine	Spermidine	Spermine
Quiescent cells	8.56 $\pm$ 0.45	28.31 $\pm$ 0.74	8.25 $\pm$ 0.27
Control cells	23.04 $\pm$ 1.32	25.19 $\pm$ 1.90	8.51 $\pm$ 0.77
C ₆ -Ceramide-treated cells	4.12 $\pm$ 0.13	16.63 $\pm$ 0.18	7.39 $\pm$ 0.15

of control. In contrast, the change to a serum-free medium had little effect on the ability of exogenous sphingomyelinase to affect ODC activity.

Structural specificity of ceramide action

The structural specificity of ceramide action in L1210-DFMO⁺ cells was tested by comparison with C₆-dihydroceramide, a close structural analogue of C₆-ceramide. Table 2 shows that the induction of ODC activity was not significantly affected after a 4 h treatment with 10 μ M C₆-dihydroceramide, whereas it was markedly reduced by C₆-ceramide. Even the ability of C₆-dihydroceramide to decrease preinduced ODC activity was much less than that of C₆-ceramide. In agreement with this, C₆-dihydroceramide (10 μ M) hardly affected cell growth at all after 43 h of treatment (88 $\pm$ 7.9 % of control).

Effect of C₆-ceramide on polyamine content

The inhibition of ODC induction after treatment of L1210-DFMO⁺ cells with C₆-ceramide resulted in the prevention of putrescine accumulation (Table 3). The spermidine level was also reduced, whereas spermine content did not change significantly after 16 h with 5 μ M C₆-ceramide. However, the addition of either putrescine (0.1–1 mM) or spermidine (0.01–1 mM) to the cell medium did not reverse the negative effects on cell growth and viability induced by C₆-ceramide at 5 or 10 μ M (not shown). Exogenous spermine (0.01–1 mM) was also ineffective.

Effect of C₆-ceramide on the expression of ODC mRNA and ODC turnover

In order to investigate the possibility that the ceramide-induced changes in ODC activity are mediated at the level of gene

Figure 6 Effect of C₆-ceramide on the expression of mature ODC mRNA (A, B) and ODC hnRNA (C)

(A, B) L1210-DFMO⁺ cells were analysed for mature ODC mRNA by reverse transcriptase–PCR (21 cycles). PCR products (224 bp) were visualized by ethidium bromide fluorescence after separation by agarose-gel electrophoresis. (A) Lane 1, molecular-size markers (*Bgl*I and *Hin*II pBR328 DNA fragments of 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220 and 154 bp); lane 2, quiescent cells; lane 3, control cells (8 h after dilution); lane 4, cells diluted and treated with 5 μ M C₆-ceramide for 8 h; lane 5, cells diluted and treated with 10 μ M C₆-ceramide for 8 h. (B) Lane 1, molecular-size markers; lane 2, control cells (16 h after dilution); lane 3, cells diluted and treated with 5 μ M C₆-ceramide for 16 h; lane 4, cells diluted and treated with 10 μ M C₆-ceramide for 16 h. (C) L1210-DFMO⁺ cells were analysed for ODC hnRNA by reverse transcriptase–PCR (27 cycles). PCR products (247 bp) were visualized by ethidium bromide fluorescence after separation by agarose-gel electrophoresis. Lane 1, molecular-size markers; lane 2, quiescent cells; lane 3, control cells (8 h after dilution); lane 4, cells diluted and treated with 10 μ M C₆-ceramide for 8 h.

Figure 7 Effect of C₆-ceramide on the rate of ODC decay

Cycloheximide (0.2 mM) was added 16 h after cell seeding (time 0) and cell samples were removed at 30 min intervals. $\circ$, Control cells; $\bullet$, cells treated with 5 μ M C₆-ceramide at seeding, 16 h before cycloheximide; $\triangle$, cells treated with 10 μ M C₆-ceramide 1.5 h before cycloheximide.

expression, the messenger for ODC was detected by combined reverse transcriptase–PCR analysis. The level of mature ODC mRNA, very low in quiescent cells, increased after dilution of the cells, and the treatment with C₆-ceramide for 8 h (Figure 6A) or 16 h (Figure 6B) reduced this increase markedly. Quantification of PCR products (18 cycles) by capillary electrophoresis showed that the content of the messenger was 24 and 8 % of control after 16 h treatment with 5 and 10 μ M C₆-ceramide respectively. C₆-ceramide (10 μ M) also inhibited the increase in the precursor, ODC hnRNA, which followed stimulation to cell growth (Figure 6C). According to analysis by capillary electrophoresis, the content of ODC hnRNA in C₆-ceramide-treated cells was 12 %

Figure 8 Effect of C_6 -ceramide on the induction of c-Myc protein (A) and DNA-binding activity of Myc/Max (B)

(A) Western-blot analysis of c-Myc induction in L1210-DFMO^r cells. Lane 1, quiescent cells; lane 2, control cells (1 h after dilution); lane 3, cells diluted and treated with $10 \mu\text{M}$ C_6 -ceramide for 1 h; lane 4, control cells (4 h after dilution); lane 5, cells diluted and treated with $10 \mu\text{M}$ C_6 -ceramide for 4 h; lane 6, control cells (7 h after dilution); lane 7, cells diluted and treated with $10 \mu\text{M}$ C_6 -ceramide for 7 h. (B) EMSA using nuclear extracts of L1210-DFMO^r cells and labelled double-stranded oligonucleotide containing the Myc/Max binding site. Lane 1, quiescent cells; lane 2, control cells (4 h after dilution); lane 3, cells diluted and treated with $10 \mu\text{M}$ C_6 -ceramide for 4 h; lane 4, the nuclear extract from control cells was incubated in presence of an excess of unlabelled competing oligonucleotide; lane 5, the nuclear extract from control cells was incubated in the presence of an excess of unlabelled non-competing oligonucleotide.

of that of control cells. The effect of C_6 -ceramide treatment on ODC turnover was also examined (Figure 7). A slight increase (about 2-fold) in the ODC decay after cycloheximide treatment was observed in cells incubated with C_6 -ceramide for 16 h. However, a shorter treatment did not affect ODC turnover significantly.

Effect of C_6 -ceramide on the induction of c-Myc protein and DNA-binding activity of Myc/Max

Since it has been shown that mouse ODC gene is a transcriptional target for c-Myc [25,26] and that, on the other hand, ceramide can down-regulate c-Myc mRNA in HL-60 cells [5], Western-blot analysis of c-Myc was performed in L1210-DFMO^r cells stimulated to growth (Figure 8A). The content of c-Myc protein, very low in quiescent cells, progressively increased after cell dilution, and the treatment with $10 \mu\text{M}$ C_6 -ceramide strongly reduced this induction within a few hours. Then we examined whether this inhibition resulted in a reduction of Myc/Max DNA-binding activity. As judged by EMSA (Figure 8B, lanes 1–3), stimulation to cell growth increased the binding of nuclear extracts to a synthetic oligonucleotide containing the Myc/Max consensus sequence, and C_6 -ceramide prevented this increase. Competition studies confirmed the specificity of the binding complexes (Figure 8B, lanes 4 and 5). EMSA was also performed with oligonucleotides containing consensus sequences for other transcription factors that can modulate ODC gene transcription, i.e. Sp1 [27,28], cAMP-response-element-binding proteins [29,30] and Fos/Jun [31]. However, the treatment with $10 \mu\text{M}$ C_6 -ceramide for 1 or 4 h did not affect the DNA-binding activity of these transcription factors significantly (not shown).

DISCUSSION

The present study shows that cell-permeant ceramides can inhibit ODC induction in the low micromolar range. C_6 -Ceramide proved to be about 5-fold more potent than C_2 -ceramide, in accord with reports showing a greater effect of C_6 -ceramide on protein phosphatase 2A activity [32] or phosphatidate-induced stimulation of DNA synthesis and phospholipase D activity [4]. However, the efficacy of C_2 -ceramide in inhibiting ODC induction was remarkably increased by incubating cells in a serum-free medium. Bielawska et al. [33] have observed that the presence of serum or albumin in the cell medium increased the IC_{50} of C_2 -ceramide for HL60 cell growth by a factor of 10.

C_6 -Ceramide action showed some structural specificity in that C_6 -dihydroceramide, which lacks the double bond on the sphingoid backbone, did not reproduce the early effects of C_6 -ceramide on ODC induction and cell growth. Previous reports generally show the specificity of biological activities of ceramide compared with dihydroceramide [1,2]. For instance, Sawai et al. [9] found that C_2 -dihydroceramide failed to induce apoptosis of HL-60 cells and provoked an increase in c-Jun expression that was only one-third of that with C_2 -ceramide. LPS was also able to inhibit ODC induction. LPS has been shown to possess structural similarity to ceramide and to stimulate ceramide-activated protein kinase in myeloid cells [34]. In fact, Joseph et al. [34] have proposed that LPS acts by mimicking the second-messenger function of ceramide.

Bacterial sphingomyelinase exerted a significant, but limited, effect on ODC induction and DNA synthesis of L1210-DFMO^r cells. Exogenous sphingomyelinase has been reported to mimic some of the effects of short-chain ceramides, but failed to reproduce the C_2 -ceramide-induced changes in the cell cycle of NIH 3T3 cells [35] and in DNA synthesis of T-lymphocytes [36]. Furthermore Linardic and Hannun [37] have recently demonstrated that the sphingomyelin pool involved in signalling is distinct from the plasma-membrane outer-leaflet pool and is characterized by resistance to bacterial sphingomyelinase.

ODC is subjected to a complex array of cell-specific regulatory mechanisms that govern transcription, mRNA stability and translation, and enzyme degradation [11–13]. Inhibition of the expression of ODC mRNA and its precursor appears to be a major mechanism of ceramide action on ODC induction, suggesting an effect at the level of gene transcription. Mammalian ODC gene transcription can be regulated by a number of transcription factors [25–31], and, in particular, c-Myc/Max complexes can transactivate mouse ODC gene through interaction with two CACGTG motifs located in the first intron [25,26]. In addition, c-Myc and Max can play a role in growth-associated expression of ODC [16,38], which, in turn, is a mediator of at least some c-Myc functions [16,39]. In agreement with the reported down-regulation of c-Myc mRNA by C_2 -ceramide in HL-60 cells [5], C_6 -ceramide inhibited the induction of c-Myc protein in L1210-DFMO^r cells and the binding activity of nuclear extracts to oligonucleotide containing the Myc/Max consensus sequence, whereas the binding of other transcription factors tested was not affected significantly, as judged by EMSA. Thus these results may be consistent with the involvement of c-Myc in the ceramide-induced inhibition of ODC expression.

Another important point of ODC regulation is at the level of protein degradation [13]. ODC turns over very rapidly and its degradation rate is a function of the antizyme/ODC ratio. Antizyme is a regulatory protein that binds to ODC and makes it susceptible to degradation by the 26S proteasome [13]. A 16 h treatment with C_6 -ceramide provoked some acceleration of ODC

turnover, which might be due to a higher antizyme/ODC ratio secondary to inhibition of ODC synthesis.

The ODC-inhibiting effect appears to be closely related to an anti-proliferative action, with IC_{50} for ODC induction similar to the IC_{50} for thymidine incorporation. However, the growth suppression was not due to a simple cytostatic effect, but was associated with the induction of apoptosis, as shown for other leukaemia cell lines [2]. The impairment of ODC induction was accompanied by an alteration in polyamine levels. However, it may not be surprising that exogenous polyamines could not reverse the anti-proliferative and cytotoxic effects of ceramide, since polyamine accumulation can actually favour apoptosis in some experimental systems, including DFMO-resistant L1210 cells [39–41]. On the other hand, it should be observed that ablation of either ODC or c-Myc can arrest the cell cycle in G_1 [16], and ODC is required downstream of c-Myc for myeloid cell cycle progression [39]. Furthermore Rb dephosphorylation, which can play an important role in ceramide-induced G_1 cell cycle arrest [7], leads to inactivation of the transcription factor E2F, which in turn may control c-Myc transcription [42]. Quite recently, Zhang et al. [43] have found that ceramide can provoke cell cycle arrest and apoptosis in ALL-697 leukaemia cells, apparently through two distinct pathways, one involving Rb and the other involving Bcl-2. Thus at present it cannot be excluded that the down-regulation of ODC expression may be an event related to the pathway leading to cell cycle arrest, and not to the pathway leading to apoptosis.

Although the role of ODC in ceramide action remains to be completely defined, the present report shows that down-regulation of this enzyme represents an early event largely preceding growth suppression and cytotoxicity. In fact, ODC inhibition may be a general aspect of ceramide action in leukaemia cells, since it was found not only in wild-type L1210 and DFMO-resistant cells, but also in HL-60 and Jurkat cells, which represent well-established models for studying the anti-proliferative effects of ceramide [1,2,44].

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