Ceramide inhibition of chondrocyte proliferation and bone growth is IGF-I independent

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Abstract

Proinflammatory cytokines inhibit growth plate development. However, their underlying mechanisms of action are unclear. These effects may be mediated by ceramide, a sphingosine-based lipid second messenger, which is elevated in a number of chronic inflammatory diseases. To test this hypothesis, we determined the effects of C2-ceramide, a cell permeable ceramide analogue, on the growth of the ATDC5 chondrogenic cell line and on cultured fetal mice metatarsals. In ATDC5 cells, C2-ceramide significantly induced apoptosis at both 40 (82%; $P < 0.05$) and 25 μM (53%; $P < 0.05$). At 40 μM , C2-ceramide significantly reduced proliferation ($[^3\text{H}]$ -thymidine uptake/mg protein) (62%; $P < 0.05$). C2-ceramide did not markedly alter the differentiation state of the cells as judged by the expression of markers of chondrogenesis and differentiation (sox 9, collagen II and collagen X). The IGF-I signalling pathway is the major autocrine/paracrine regulator of bone growth. Both in the presence and absence

of IGF-I, C2-ceramide (25 μM) induced an equivalent reduction in proliferation (60%; $P < 0.001$). Similarly, C2-ceramide (40 μM) induced a 31% reduction in fetal metatarsal growth both in the presence and absence of IGF-I (both $P < 0.001$). Furthermore, C2-ceramide reduced ADCT5 proliferation in the presence of AG1024, an IGF-I and insulin receptor blocker. Therefore, C2-ceramide-dependent inhibition appears to be independent of IGF-mediated stimulation of bone growth. Indeed, biochemical studies demonstrated that C2-ceramide (25 μM) pretreatment did not alter IGF-I-stimulated phosphorylation of insulin receptor substrate-1, Akt or P44/42 MAP kinase. In conclusion, C2-ceramide inhibits proliferation and induces apoptosis in growth plate chondrocytes through an IGF-I independent mechanism.

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Introduction

Chronic inflammatory diseases such as juvenile idiopathic arthritis and inflammatory bowel disease often lead to childhood growth retardation through a number of proposed mechanisms that includes nutritional deficiency, chronic inflammation, increased catabolism, defects in the growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis and use of glucocorticoids (MacRae *et al.* 2006a, Wong *et al.* 2006). Levels of proinflammatory cytokines, such as tumour necrosis factor (TNF)- α and interleukin (IL)-1 β are often raised in these diseases, and measures that block TNF- α action, such as anti-TNF therapy, lead to an improvement in growth. This effect has been reported to be independent of the concurrent reduction in growth associated with glucocorticoid therapy (Tynjala *et al.* 2006). TNF- α and IL-1 β may directly inhibit growth plate chondrocyte dynamics as well as longitudinal growth *in vitro* (Martensson *et al.* 2004, MacRae *et al.* 2006b), however, the underlying mechanisms that lead to these effects are unclear.

The IGF-I signalling pathway is the major autocrine/paracrine regulator of bone growth (Loveridge *et al.* 1990). Binding of IGF-I to its receptor utilises a family of soluble receptors, known as insulin receptor substrates (IRSs), to initiate a series of autophosphorylation events. This results in the activation of two distinct signalling pathways, phosphatidylinositol 3-kinase (PI-3K) and p44/p42 mitogen-activated protein kinase (MAPK), leading to pro-proliferative and anti-apoptotic effects.

In many cell types, signal transduction of IL-1 β and TNF- α involves the activation of neutral (N) and acidic (A) sphingomyelinase (SMase) pathways, which catalyse the degradation of the membrane phospholipid sphingomyelin into phosphocholine and ceramide (Mathias *et al.* 1993, Wiegmann *et al.* 1994, Rybakina *et al.* 2001). Ceramide levels are also elevated through a *de novo* synthesis pathway following activation of IL-1 β and TNF- α receptors (Memon *et al.* 1998, Xu *et al.* 1998). Ceramide has been shown to inhibit IGF-I-induced tyrosine phosphorylation of IRS-1 in myoblast and hepatic cells (Kanety *et al.* 1996, Strle *et al.* 2004). Ceramide has also been shown to induce apoptosis in a wide range of different

cell types, including pancreatic β -cells (Sjoholm 1995), cardiomyocytes (de Vries *et al.* 1997) and astrocytes (Oh *et al.* 2006). Furthermore, ceramide has been shown to induce apoptosis, proteoglycan degradation and matrix metalloproteinase expression in articular chondrocytes (Sabatini *et al.* 2000, Gilbert *et al.* 2004, 2006).

Whilst previous studies have shown that IL-1 β and TNF α inhibit growth plate chondrocyte differentiation and induce cell death (Martensson *et al.* 2004, MacRae *et al.* 2006b), the direct effects of ceramide on growth plate chondrocytes have yet to be reported. In this study, the ATDC5 chondrogenic cell line was used to characterise and compare the effects of ceramide on cell proliferation, differentiation and apoptosis. Subsequently, the effect of ceramide on IGF-I signalling was examined using ATDC5 cells and the fetal murine metatarsal model.

Materials and Methods

Chondrocyte cell culture

The ATDC5 chondrocyte cell line was sourced from the RIKEN cell bank (Ibaraki, Japan) and maintained as described by Atsumi *et al.* (1990). Cells were maintained in T175 tissue culture flasks (Greiner Bio-One GmbH, Frickenhausen, Baden-Württemberg, Germany) at a density of 250 000 cells/flask in a maintenance medium of Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 (Invitrogen) supplemented with 5% FCS (Invitrogen), 10 μ g/ml human transferrin, 3×10^{-8} M sodium selenite (Sigma), sodium pyruvate (1 mM; Invitrogen) and gentamycin (50 μ g/ml, Invitrogen). For individual experiments semi-confluent cultures were passaged with trypsin-EDTA (Sigma) and cultured (day 0) at a density of 6000 cells/cm² in multi-well plates (Costar, High Wycombe, Bucks, UK) in a differentiation medium that consisted of maintenance medium supplemented with insulin (10 μ g/ml; Sigma). Incubation was at 37 °C in a humidified atmosphere of 95% air/5% CO₂ and the medium was changed every second or third day. In all experiments, unless otherwise stated, C2-ceramide (Sigma), a cell permeable ceramide analogue, was added to chondrocyte cultures on day 6, at a final concentration of 40, 25 and 10 μ M. The diluent for C2-ceramide was ethanol (final concentration 0.1%). All control cultures received 0.1% ethanol only.

ATDC5 cells were deprived of serum and insulin/transferrin/selenium for 18 h before the initiation of treatments in the presence of IGF-I (100 ng/ml; Bachem, UK Ltd, St Helens, Merseyside, UK), AG1024 (Sigma) and before the initiation of the cell-signalling studies.

Ceramide mechanisms associated with proinflammatory cytokine exposure

D609 (10 μ g/ml; Sigma), an inhibitor of A-SMase activity, was added to cells cultured in 48 well plates, in the presence of IL-1 β and TNF- α (both 10 ng/ml). The rate of chondrocyte

proliferation was assessed over a 24 h period starting on day 6. On day 7, the chondrocytes were incubated with 0.2 μ Ci/ml [³H]thymidine (37 MBq/ml; Amersham Pharmacia Biotech) for the last 2 h of the culture period. The amount of radioactivity incorporated into trichloroacetic acid-insoluble precipitates was measured (Farquharson *et al.* 1999).

Chondrocyte number, proliferation and apoptosis

Proliferation was determined over a 24 h period starting on day 6, as described above. Protein content of the trichloroacetic acid-insoluble precipitates was determined as a measure of cell number. Protein content was measured using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc.) based on the Bradford dye-binding procedure and γ -globulin as standard (Farquharson *et al.* 1995). Apoptosis of the cells was measured using the APO Percentage Apoptosis assay (Biocolor Ltd, Belfast, Northern Ireland), which quantifies dye uptake by apoptotic cells only after the translocation of phosphatidylserine to the outer surface of the cell membrane (Fadok *et al.* 1992). Apoptosis was assessed following the manufacturer's protocol in cells cultured in 48-well plates over a 24 h period starting on day 6.

Analysis of chondrogenic gene expression

On days 13 and 15, C2-ceramide was added to cells cultured in six-well plates. During this period of cell maturation, the cells express established markers of the chondrocyte differentiated phenotype (Mushtaq *et al.* 2002). The experiment was stopped on day 17 and total RNA was extracted from chondrocytes by repeated aspiration through a 25-gauge syringe needle in 0.5 ml Ultraspec (Biotechx, Houston, TX, USA). Following extraction with chloroform, RNA in the aqueous phase was precipitated with isopropanol and bound to RNA Tack resin (Biotechx) following the manufacturer's protocol. After washing with 75% ethanol, the RNA was eluted in 100 μ l ribonuclease-free water (Houston *et al.* 1999). For each sample, total RNA content was assessed by absorbance at 260 nm and purity by A260/A280 ratios, which were 1.9–2.0 in all cases. All preparations were diluted to a concentration of 50 ng/ μ l and stored at –70 °C. Gene expression was analysed by semi-quantitative RT-PCR (Jefferies *et al.* 1998, 2000, Farquharson *et al.* 1999, Houston *et al.* 1999). Aliquots of 500 ng RNA (or an equivalent volume of water as a control) were reverse transcribed in 20 μ l reactions containing cDNA equivalent to 10 ng RNA and 200 nM gene-specific primers (Table 1) in 11.1 $\times$ PCR buffer (Jefferies *et al.* 2000). Primers were designed to span introns. The cycling profile was 1 min at 92 °C (first cycle, 2 min), 1 min at 55 °C and 1 min at 70 °C. The number of cycles performed was carefully titrated to ensure that the reactions were in the exponential phase. Reaction products were analysed on 1.5% agarose gels in the presence of ethidium bromide (250 μ g/l), and a digital image of each gel was captured using a gel documentation system (Bio-Rad Laboratories).

Table 1 Primer pairs used for semi-quantitative RT-PCR analysis

		Primer sequence	Cycles	Product size (bp)
Gene				
18S		Unknown, purchased commercially from Ambion ^a	20	488
Collagen Type II	Forward	5' CACACTGGTAAGTGGGGCAAGACC 3'	30	172
	Reverse	5' GGATTGTGTTGTTTCAGGGTTCGGG 3'		
Collagen Type X	Forward	5' AGGCAAGCCAGGCTATGGAA 3'	35	583
	Reverse	5' GCTGTCCTGGAAAGCCGTTT 3'		
Sox 9	Forward	5' ATCTGAAGAAGGAGAGCGAG 3'	35	263
	Reverse	5' TCAGAAGTCTCCAGAGCTTG3'		

^aAmbion, Huntingdon, Cambs, UK.

IGF-I studies in ATDC5 cells

The cells were treated with C2-ceramide (25 µM) for 24 h in the presence of IGF-I. In further studies, the cells were treated with C2-ceramide (25 µM) in the presence of IGF-I or AG1024, an IGF-I and insulin receptor blocker, (10 µM; Sigma) for 24 h (Lee *et al.* 2005, Sutter *et al.* 2006). The diluent for AG1024 was dimethylsulfoxide (final concentration 0.1%). Control cultures received 0.1% dimethylsulfoxide only. The minimum concentration at which ceramide could inhibit IGF-I-induced proliferation in ATDC5 cells was examined using C2-ceramide concentrations between 10 and 40 µM at 5 µM intervals using serum-free medium.

Western blotting analysis

On day 6, ATDC5 cells were treated with C2-ceramide (25 µM) for 24 h in serum-free medium, and then either lysed immediately (control cultures received 0.1% ethanol only) or stimulated with IGF-I (100 ng/ml) for 10 min before lysis (control cultures received 0.1% ethanol and were not stimulated with IGF-I).

Cells were lysed in PhosphoSafe extraction buffer (Merck Biosciences Ltd) containing 'Complete' protease inhibitor cocktail (Roche) according to the manufacturer's instructions. Lysates (corresponding to 25 µg protein) were run on 10% Bis-Tris gels (Invitrogen) and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline/Tween-20 (TBST; 50 mM Tris-HCl, 300 mM NaCl, 0.1% Tween-20, pH 7.6) at 4 °C overnight with gentle shaking. The membranes were probed for 1 h at room temperature with primary antibodies raised in rabbit (all 1:1000 dilution in 5% milk) against phospho-Akt (ser 473), total Akt, phospho-P44/42 MAP kinase (Thr202-/Tyr204), total P44/42 MAP kinase, Phospho-SAPK/Jun N-terminal kinase (JNK) (Thr183/Tyr185), total stress-activated protein kinase (SAPK)/JNK, phospho-p38 MAP kinase (Thr180/Tyr182) and total p38 MAP kinase (Cell Signaling Technology, Beverly, MA, USA). The blots were washed 4×15 min in TBST. The membranes were then incubated with anti-rabbit IgG-peroxidase (Cell Signaling Technology) for 1 h (1:1000 dilution in 5% milk). The membranes were washed, as

described above, and developed using the electrogenerated chemiluminescent labeling (ECL)-plus Western Blotting Detection System (Amersham Biosciences).

Immunoprecipitation of IRS-1

ATDC5 cells were treated with C2-ceramide (25 µM) for 24 h in serum-free medium and stimulated with IGF-I (100 ng/ml) for 10 min (control cultures received 0.1% ethanol and were not stimulated with IGF-I). Cells were then lysed in lysis buffer (150 mM NaCl; 10 mM Tris-HCl pH 7.4; 0.5% NP40; 1 mM sodium vanadate; 0.5 M EDTA; 0.1 M phenylmethylsulphonyl fluoride; 4 mg/l aprotinin). IRS-1 was immunoprecipitated by incubating cell lysates overnight at 4 °C with 2 µg/ml anti-IRS-1 antibody and 30 µl protein A-Sepharose beads. Protein bound to the beads was washed four times with lysis buffer, and 10 µl aliquots were run on 3–8% Tris-Acetate gels (Invitrogen) and transferred onto a nitrocellulose membrane. The membranes were blocked as described above and probed for 1 h at room temperature with antiphosphotyrosine clone 4G10 and total IRS-1 antibodies (Upstate, Lake Placid, New York, USA), raised in mouse and rabbit respectively. The blots were washed 4×15 min in TBST. The membranes were then incubated with anti-mouse IgG-peroxidase (Cell Signalling Technology) for 1 h (1:1000 dilution in 5% milk). The membranes were washed and developed as described above.

Organ culture

The middle three metatarsals were aseptically dissected from 19-day-old embryonic Swiss mice. Bones were cultured at 37 °C in a humidified atmosphere of 95% air/5% CO₂ in 24 well plates. Each culture well contained 300 µl α-minimum essential medium (MEM) (Invitrogen) supplemented with 0.2% BSA, Fraction V (Sigma); 1 mmol/l β-glycerophosphate (Sigma); 0.05 mg/ml L-ascorbic acid phosphate (Wako Pure Chemicals Ltd, Neuss, North Rhine-Westphalia, Germany); 0.05 mg/ml gentamycin and 1.25 µg/ml fungizone (Invitrogen; Mushtaq *et al.* 2004). C2-ceramide was added at a final concentration of 40, 30, 20 and 10 µM for an 8-day period. Controls were treated with BSA

carrier. In further studies, C2-ceramide was added at a final concentration of 40 μ M for an 8-day period, in the presence of IGF-I (100 ng/ml). The minimum concentration at which ceramide could affect metatarsal growth was examined using C2-ceramide concentrations between 0 and 40 μ M at 10 μ M intervals. In all experiments, the medium was changed every second or third day. The experimental protocol was approved by Roslin Institute's Animal Users Committee and the animals were maintained in accordance with Home Office guidelines for the care and use of laboratory animals.

Morphometric analysis

Digital images of the metatarsals were captured every second day of culture and viewed on a Nikon Eclipse TE3000 microscope (Nikon, Kingston upon Thames, Surrey, UK), using a digital camera (DS Camera Head DS-5M; Nikon). The total length of the bone through the centre of the mineralising zone was determined using image analysis software (DS Camera Control Unit DS-L1; Nikon). All results are expressed as a percentage change from harvesting length which was regarded as baseline.

Statistical analysis

All experiments were performed at least twice. General Linear Model analysis was used to assess the data. All data are expressed as the mean $\pm$ S.E.M. of six observations within each experiment. Statistical analysis was performed using Minitab 14 (State College, PA, USA). $P < 0.05$ was considered to be significant.

Results

Proinflammatory cytokine exposure inhibits proliferation via a ceramide mechanism

TNF- α (10 ng/ml) treatment of the ATDC5 chondrocyte cell line significantly reduced cell proliferation compared with control cultures (Fig. 1; 93%; $P < 0.001$). To determine whether this inhibition might be mediated via an A-SMase ceramide mechanism, we assayed the effects of the A-SMase inhibitor D609 in conjunction with TNF- α treatment. D609 (10 μ g/ml) significantly increased proliferation over tenfold compared with cytokine treatment alone (Fig. 1; $P < 0.001$), and in the absence of TNF- α (Fig. 1; 84%; $P < 0.001$). These results suggest that TNF- α reduces proliferation through an A-SMase ceramide mechanism.

Characterisation of the effect of C2-ceramide on ATDC5 cells

In order to examine the effect of ceramide on cell growth, the relative contribution of altered cell proliferation, apoptosis or differentiation were assessed. In the ATDC5 cells, 40 μ M C2-ceramide significantly reduced cell

Figure 1 Effect of TNF- α (10 ng/ml), D609 (10 μ g/ml) and TNF- α + D609 on [³H] thymidine uptake (d.p.m.), mean $\pm$ 1 S.E.M., $n = 6$; * $P < 0.001$ compared with control.

proliferation over a 24 h period (Fig. 2A). This was confirmed in further studies when thymidine uptake was corrected for protein content (Fig. 2B). Apoptosis was increased at both 40 and 25 μ M C2-ceramide following exposure for 24 h (Fig. 2C). There was no significant alteration in C2-ceramide-induced mRNA expression of markers of chondrogenesis or differentiation (sox 9, collagen II and collagen X) at all concentrations studied (Fig. 3).

IGF-I studies

The IGF-I signalling pathway is the major autocrine/paracrine regulator of bone growth. Therefore, we undertook a series of studies to examine the effects of C2-ceramide on IGF-I-induced proliferation and bone growth. The lowest concentration of C2-ceramide that inhibited ATDC5 cell proliferation following 18 h serum deprivation was 25 μ M in the presence of IGF-I (Fig. 4A). Cells exposed to C2-ceramide (25 μ M) in the presence or absence of IGF-I for 24 h indicated that IGF-I alone significantly increased proliferation (Fig. 4B; $P < 0.001$). In the presence of IGF-I, C2-ceramide induced a 68% reduction in proliferation (Fig. 4B; $P < 0.001$). However, in the absence of IGF-I, ceramide induced a comparable 61% decrease (Fig. 4B; $P < 0.001$).

To confirm that these effects are physiologically relevant, we tested the effects of C2-ceramide on bone growth using a fetal metatarsal model. The lowest concentration of C2-ceramide that significantly reduced fetal metatarsal growth was 40 μ M in the presence of IGF-I (Fig. 5A). A 31% reduction in metatarsal growth was observed at 40 μ M C2-ceramide both in the presence and absence of IGF-I (Fig. 5B; both $P < 0.001$). In conclusion, C2-ceramide does not appear to inhibit exogenous IGF-I-induced growth plate chondrocyte proliferation or bone growth.

The effect of C2-ceramide on endogenous IGF-I-induced ATDC5 cell proliferation was examined (Fig. 4C). AG1024

Figure 2 Effect of C2-ceramide (10, 25 and 40 μ M) on (A) $[^3\text{H}]$ thymidine uptake (d.p.m.) following 24 h exposure; (B) $[^3\text{H}]$ thymidine uptake (d.p.m./mg protein) following 24 h exposure and (C) apoptosis (optical density (OD) at 540 nm) following 24 h in ATDC5 cell culture; mean $\pm$ 1 s.e.m., $n=6$; * $P<0.01$; † $P<0.001$ compared with control.

(10 μ M) reduced proliferation by 28% compared with control cells ($P<0.001$). C2-ceramide (25 μ M) significantly reduced proliferation compared with AG1024 treatment (55%, $P<0.001$). C2-ceramide and AG1024 in combination further reduced proliferation compared with C2-ceramide alone (46%; $P<0.01$). Therefore, C2-ceramide does not inhibit endogenous IGF-I-induced ATDC5 proliferation.

Cell signalling

The IGF-I-induced phosphorylation of IRS-1, Akt or P44/42 MAP kinase was not inhibited by 24 h C2-ceramide exposure (Fig. 6). Further, studies determined the direct effects of C2-ceramide on MAPK subfamilies, which are associated with cell survival and stress stimuli. The phospho-SAPK/JNK, phospho-p38 MAP kinase or P44/42 MAP kinase expression was not altered by 24 h C2-ceramide exposure (data not shown). Therefore, C2-ceramide does not inhibit signalling of the MAPK subfamilies or IGF-I in ATDC5 chondrogenic cells.

Discussion

Ceramide is an intracellular second messenger, whose signalling plays an important role in the regulation of cell proliferation, differentiation and survival (Kolesnick 2002, Ruvolo *et al.* 2002, Menaldino *et al.* 2003). This is the first study to demonstrate that C2-ceramide inhibits proliferation and induces apoptosis in growth plate chondrocytes. Ceramide has been shown to induce apoptosis in a wide

range of different cell types, including pancreatic β -cells (Sjoholm 1995), cardiomyocytes (de Vries *et al.* 1997), astrocytes (Oh *et al.* 2006) and articular chondrocytes (Sabatini *et al.* 2000, Gilbert *et al.* 2004, 2006).

The partial attenuation of TNF- α -induced inhibition of proliferation by D609, an inhibitor of ceramide synthesis, suggests that ceramide may mediate the effects of pro-inflammatory cytokines in growth plate chondrocytes. An increase in proliferation was also observed in the presence of

Figure 3 Semi-quantitative RT-PCR analysis of collagen II, collagen X and sox 9 expression in ATDC5 cells following exposure to C2-ceramide at 10, 25 and 40 μ M compared with control (C) samples; B, blank.

Figure 4 Effect of (A) C2-ceramide (10, 15, 20, 25, 30, 35 and 40 μM); (B) C2-ceramide +/– IGF (100 ng/ml) and (C) C2-ceramide (25 μM), AG1024 (10 μM) and C2-ceramide + AG1024 on $[^3\text{H}]$ thymidine uptake (d.p.m.) in ATDC5 cells; mean $\pm$ 1 S.E.M., $n=6$; * $P<0.001$ compared with control.

D609 in the control cells, without exogenous cytokines, suggesting that growth plate chondrocytes have an endogenous production of ceramide. Blocking this endogenous production may act as an internal regulator of proliferation.

Standardisation of $[^3\text{H}]$ thymidine uptake to protein content (Phornphutkul *et al.* 2006) confirms that an actual reduction in proliferation was observed following ceramide exposure, rather than solely a reduced uptake due to fewer cells through increased apoptosis. Both TNF- α and IL-1 β exposure have also been reported to inhibit proliferation and induce apoptosis in ATDC5 cells (MacRae *et al.* 2006b), and the results of this present study now suggest that ceramide may be acting as a second messenger in both processes. Both IL-1 β and TNF α have also been shown to markedly reduce the gene expression of collagen II, collagen X and aggrecan (MacRae *et al.* 2006b), but in our present study ceramide did not alter the gene expression of markers of chondrogenesis and differentiation (sox 9, collagen II and collagen X). This suggests that ceramide generation does not affect differentiation in growth plate chondrocytes. Therefore, ceramide production may be just one of many different pathways through which the pro-inflammatory cytokines act.

Having established that C2-ceramide inhibits proliferation and induces apoptosis, we went on to demonstrate that these effects are not mediated through the inhibition of the IGF-I signalling pathway, which is the major autocrine/paracrine regulator of bone growth (Loveridge *et al.* 1990). Strle *et al.* (2004) demonstrated that ceramide inhibits IGF-I-induced protein synthesis and differentiation in myoblasts. However, it has also been reported that ceramide can inhibit myoblast

growth and differentiation in the absence of exogenous IGF-I (Meadows *et al.* 2000, Strle *et al.* 2004). This apparent discrepancy is likely to be a consequence of experimental design, and in particular the presence or absence of serum and

Figure 5 Effect of (A) C2-ceramide (10, 20, 30 and 40 μM) in the presence of IGF-I (100 ng/ml) and (B) C2-ceramide (40 μM) +/– IGF (100 ng/ml) on fetal metatarsal bone growth (percentage change in bone length from baseline percentage); mean $\pm$ 1 S.E.M., $n=6$; * $P<0.001$ compared with control.

Figure 6 Effect of C2-ceramide (25 μ M) on IGF-I-stimulated phosphorylation of (a) IRS-1, compared with total IRS-1, (b) Akt, compared with total Akt and (c) P44/42, compared with total P44/42 in ATDC5 cells.

the concentration of ceramide used (Strle *et al.* 2004). In our experiments, the lowest concentration of C2-ceramide that inhibited ATDC5 cell proliferation following 18 h serum deprivation was 25 μ M in the presence of IGF-I, compared with 40 μ M under standard serum-containing culture conditions. This indicates that the ATDC5 cells are more susceptible to the actions of ceramide when serum deprived, and under stress.

C2-ceramide induced a comparable reduction in ATDC5 proliferation and fetal metatarsal growth both in the absence and presence of exogenous IGF-I. Furthermore, in the presence of AG1024, an IGF-I and insulin receptor blocker, ceramide was still able to inhibit proliferation. These data suggest that C2-ceramide is able to inhibit bone proliferation by mechanisms that are independent of IGF-I signalling, which are in agreement with the previous studies (Meadows *et al.* 2000, Strle *et al.* 2004).

Cell signalling studies confirmed that ceramide did not inhibit the IGF-I-induced phosphorylation of IRS-1, or the major downstream pathways of IRS-1; P44/42 MAP kinase and PI3 kinase (as determined by Akt phosphorylation). Previous studies have reported that ceramide inhibits IGF-I-induced IRS-1 phosphorylation in myoblasts (Strle *et al.* 2004) and inhibits insulin-induced IRS-1 phosphorylation in hepatic cells (Kanety *et al.* 1996). However, studies in other

cell types, such as adipocytes (Summers *et al.* 1998) and motor neuron cells (Zhou *et al.* 1998) have shown that ceramide does not inhibit insulin-induced IRS-1 phosphorylation. Ceramide has been shown to inhibit IGF-I-induced Akt phosphorylation in myoblasts (Strle *et al.* 2004) and to inhibit insulin-induced Akt phosphorylation in adipocytes (Stratford *et al.* 2004) and motor neuron cells (Zhou *et al.* 1998). Therefore, the effects of ceramide on the IGF-I signalling pathway may depend on cell type, as well as whether stimulation is through insulin or IGF-I.

Involvement of MAPK subfamilies (P44/42 MAP kinase, c-JNK, and p38 kinase) in ceramide-induced apoptosis have been reported in various cell types, including astrocytes (Blazquez *et al.* 2000, Oh *et al.* 2006), neuronal cells (Verheij *et al.* 1996, Willaime *et al.* 2001, Willaime-Morawek *et al.* 2003), lung cancer-derived cells (Kurinna *et al.* 2004) and vascular smooth muscle cells (Loidl *et al.* 2004). The P44/42 MAP kinase pathway plays a major role in regulating cell growth, survival and differentiation. In contrast, JNK and p38 pathways are activated in response to chemical and environmental stress (Xia *et al.* 1995, Cobb 1999). However, our studies showed that in growth plate chondrocytes, whilst ceramide induced apoptosis, the phosphorylation of P44/42 MAP kinase, JNK and p38 kinase was not altered.

Our previous studies have shown that IGF-I plays a major role in promoting longitudinal growth in the mouse metatarsal model (Mushtaq *et al.* 2004). Furthermore, the growth inhibitory effect of dexamethasone was reversed in this model following exposure to IGF-I (Mushtaq *et al.* 2004). However, the growth inhibitory effects of TNF- α and IL-1 β are only partially reversed following treatment with IGF-I (Martensson *et al.* 2004), suggesting that some of the effects of these proinflammatory cytokines may be IGF-I independent. Furthermore, recombinant human growth hormone therapy in children with growth retardation and chronic inflammatory disease may result in a rise in IGF-I and a cessation in further deterioration in growth (Davies *et al.* 1997, Bechtold *et al.* 2003).

However, the failure to observe a clear normalisation of growth may be explained by the possibility that the growth retardation in these children may be due to a combination of a defect in growth regulatory pathways that are IGF-I dependent and independent.

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