Selective phosphotyrosine phosphatase inhibition and increased ceramide formation is associated with B-cell death by apoptosis

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Abstract Bis(maltolato)oxovanadium(IV) (BMOV), a protein phosphotyrosine phosphatase inhibitor, selectively induced apoptosis (as quantitated by TUNEL staining) in a B-cell line (Ramos) but not in a T-cell line (Jurkat). The pattern of BMOV-induced protein tyrosine phosphorylation was different in B-cells versus T-cells. Further, BMOV induced a 2-fold increase in ceramide levels in B-cells but not in T-cells and this resembled the ceramide increase following activation of the B-cell antigen receptor. A 2-fold increase in the ratio of ceramide to sphingomyelin in B-cells treated with BMOV suggested that sphingomyelinase activation was the result of the sustained tyrosine phosphorylation of specific proteins and activated the cell death pathway.

Key words: Phosphotyrosine phosphatase inhibitors; Sphingomyelinase activation; β-cells; Apoptosis

1. Introduction

Antigen receptor stimulation in immature B-cells and B-cell lines leads to cell death by apoptosis [1,2]. Phosphotyrosine phosphatase-2 (PTP2) is known to downregulate antigen receptor signalling [3,4], and mice deficient in the phosphatase PTP1C show increased tyrosine phosphorylation and enhanced death of self-reactive B-cells [5]. In addition, the radiation-induced cell death of B-lymphocytes involves activation of protein tyrosine kinases and can be augmented by the PTP inhibitor vanadate [6]. A selective PTP inhibitor bis(maltolato)oxovanadium(IV) (BMOV) induces cell death in B-cells but not in T-cells [7]. BMOV does not activate the phospholipase C (PLC) (PLCγ1), involved in T-cell receptor signalling [8], or any serine or threonine phosphate phosphatases [6,7] but increases tyrosine phosphorylation similar to that seen in signalling via the B-cell receptor [8]. The similarities in mechanism of action between BMOV and anti-IgM [7] are reinforced by the observation that B-cells can be rescued from either BMOV-induced death or B-cell receptor-induced apoptosis by interleukin-4 plus anti-CD40 antibody treatment [9,10]. However, little is known of the downstream targets of these B-cell specific tyrosine-phosphorylated proteins.

We have previously shown that B-cell receptor activation involves sphingomyelinase activation and increased ceramide (2-*N*-acetyl sphingosine) formation [11]. Similar increases in ceramide have been implicated in apoptosis induced by a variety of agents such as TNF-α, Fas, corticosteroids, ionizing radiation, as well as anti-IgM [11–17]. The apoptosis resulting from PLCγ2 activation has been suggested to involve IP_3/Ca^{2+} release and diacylglycerol (DAG) formation, leading to protein kinase C activation, and activation of c-Jun kinase (JNK) and p38MAPKinase [8], but the precise mechanism is not known. Since ceramide is known to activate both the stress kinases such as JNK [18–20] and p38MAPKinase [21], we investigated whether ceramide levels were elevated by the phosphatase inhibitor BMOV. We now report that BMOV induces formation of ceramide in Ramos B-cells but not in Jurkat T-cells and suggest that increased ceramide formation is essential for apoptosis in immature B-cells.

2. Materials and methods

2.1. Materials

[1-^{14}C]Palmitoyl CoA (59 mCi/mmol), [^{32}P]orthophosphate (1 Ci/mmol) were purchased from Amersham Life Science, Arlington Heights, IL, USA. Ceramide, C_2-ceramide and other lipids were purchased from Matreya, Inc. BMOV was synthesized as previously described [7].

2.2. Cell culture and FACS analysis

The human B-cell lymphoma line Ramos and the human T-cell leukemia line Jurkat were obtained from the American Type Culture Collection and were grown in RPMI 1640 media (Life Technologies, Inc.) with 10% fetal calf serum [6,7]. Cells were harvested and resuspended for cell viability assay by the propidium iodide (PI) exclusion test as previously described [7,15,16]. For quantitation, cells were run on a FACScan (Becton-Dickinson, San Jose, CA, USA) and the results analyzed using Lysis II software as previously described [7,22].

2.3. Detection of tyrosine-phosphorylated proteins

Cellular tyrosine phosphorylation was measured by immunoblotting with affinity-purified polyclonal rabbit anti-phosphotyrosine antibodies as described previously [7], and antibody binding was detected by enhanced chemiluminescence (Amersham) in accordance with the manufacturer's directions.

2.4. TdT-mediated dUTP-biotin nick end labelling (TUNEL) analysis of fragmented DNA

TUNEL staining for the in situ detection of fragmented DNA was carried out as previously described [22]. Following biotin-end terminal deoxynucleotidyl transferase treatment and detection with fluorescein-avidin, cells were washed two times in phosphate-buffered saline (PBS), resuspended in 400 ml of PBS and analyzed using a Becton-Dickinson FACScan flow cytometer and Lysis analysis software.

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Abbreviations: PTP, phosphotyrosine phosphatase; ceramide, 2-*N*-acetyl sphingosine; BMOV, bis(maltolato)oxovanadium(IV); TdT, terminal deoxytransferase; TUNEL, TdT-mediated dUTP-biotin nick end labelling; DAG, diacylglycerol

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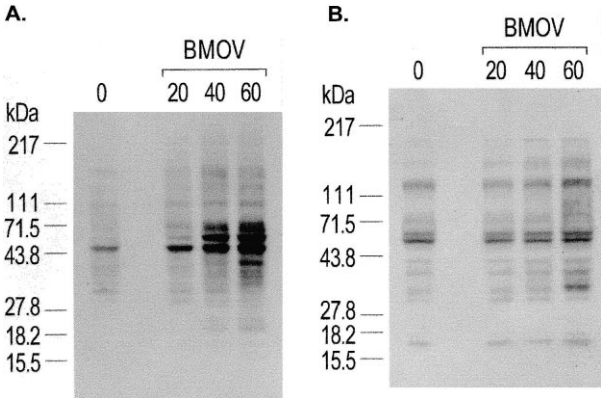

Fig. 1. Induction of cellular tyrosine phosphorylation by BMOV. Ramos B-cells (A) and Jurkat T-cells (B) were treated 16 h with BMOV at concentration of 20, 40 and 60 μM. Cellular tyrosine phosphorylation was detected by anti-phosphotyrosine immunoblots of whole cell lysate following SDS gel electrophoresis as described in the text.

2.5. Measurement of ceramide

Ceramide and sphingomyelin were labelled by culturing 2×10^6 cells/flask for 24 h in media containing 10 μCi [^{3}H]palmitate, the radioisotope removed by washing and the cells then exposed to BMOV for 24 or 48 h [11,15]. The extracted lipids [11] were applied to LHP-K thin layer chromatography (TLC) plates (Whatman) and developed in chloroform:methanol:glacial acetic acid:water (85:4.5:5.0:0.5, v/v) to resolve the ceramide doublet from other lipids. Sphingomyelin was resolved from phosphoglycerides in chloroform:methanol:glacial acetic acid:water (65:25:8.8:4.5, v/v). Plates were sprayed with En^{3}Hance (Dupont) and developed overnight with Xomat-AR film (Kodak) to identify the bands by comparison with authentic standards. Bands were then scraped for liquid scintillation counting.

Ceramide levels were quantified by conversion to ceramide [1-^{32}P]phosphate by *Escherichia coli* DAG kinase as described previously [11,13].

3. Results

3.1. The PTP inhibitor BMOV produces different tyrosine phosphorylation patterns in B-cells and T-cells

BMOV treatment for 16 h induced a striking increase in protein tyrosine phosphorylation in Ramos B-cells (Fig. 1A), with a maximum response between 40 and 60 μM. Some of the proteins showing increased tyrosine phosphorylation have previously been identified as syk (60 kDa) Zap70 kinase (70 kDa) and PLCγ2 (150 kDa). In contrast, treatment of Jurkat T-cells with 40 μM BMOV induced no increase in protein tyrosine phosphorylation (Fig. 1B). At 60 μM BMOV, some increase in phosphorylation was seen in the Jurkat cells (but not at 60, 70 or 150 kDa) and the pattern was still distinct from that seen in the Ramos B-cells (Fig. 1A).

3.2. The PTP inhibitor BMOV induces apoptosis in B-cells but not in T-cells

The increased protein tyrosine phosphorylation induced by BMOV in Ramos B-cells correlated well with increased cellular apoptosis in these cells as determined using TUNEL staining, which recognizes the multiple 3-hydroxy ends of apoptotic fragments of DNA. Thus treatment of Ramos B-cells with 25 μM BMOV for 48 h resulted in 70% death and 50 μM BMOV resulted in 80% cell death (Fig. 2A), whereas a similar treatment of Jurkat T-cells produced 5% and 25% cell death, respectively (Fig. 2B).

3.3. BMOV induces cell death and increased ceramide formation in B-cells

PI stains DNA fragments in cells that have undergone apoptosis and lost membrane integrity. Ramos B-cells showed a dose-dependent increase in both cell death (30% at 50 μM BMOV for 24 h) (Fig. 3A) and ceramide labelling by [^{3}H]palmitate (3-fold at 50 μM BMOV for 24 h) (Fig. 3B). A similar correspondence between cell death and ceramide formation was observed after 48 h exposure to BMOV, where the 75% cell death at 40 μM BMOV (Fig. 3C) corresponded to an average 2-fold increase in ceramide labelling (Fig. 3D).

Quantitation of ceramide levels by the DAG kinase assay revealed a 1.6-fold increase in ceramide content over basal levels (80 pmol/mg protein) after treatment of Ramos B-cells for 24 h with 40 μM BMOV. In contrast, Jurkat T-cells showed no increase in ceramide over basal at concentrations up to 60 μM of BMOV for 24 h (data not shown). When Jurkat T-cells were exposed to the same (50 μM) concentrations of BMOV for 24 h or 48 h, there was little evidence of cell death by PI staining (Fig. 3A,C) or any significant increase in the formation of [^{3}H]ceramide (Fig. 3B,D).

3.4. Induction of apoptosis with BMOV increases ceramide, decreases sphingomyelin and the increase is not blocked by inhibitors of sphingosine synthesis

Treatment of Ramos B-cells with increasing concentrations of BMOV led to increased labelling of ceramide relative to other lipids such as cholesterol (Fig. 4A), corresponding to a

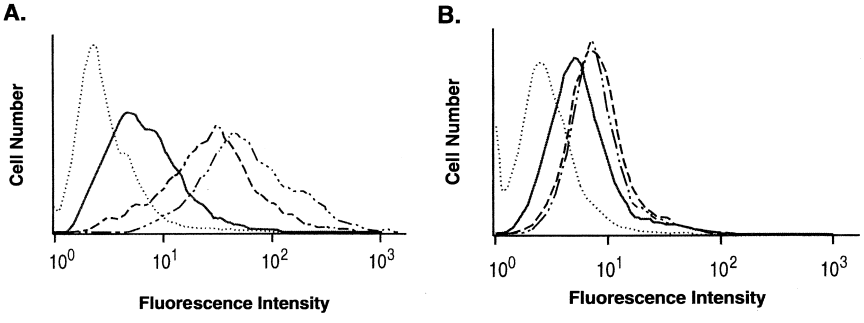

Fig. 2. TUNEL analysis of BMOV-treated B-cells and T-cells. Cells were treated with BMOV for 48 h, stained by the TUNEL method and analyzed by flow cytometry as described in the text. TUNEL analysis was performed without terminal deoxytransferase (TdT) as control (···) and with TdT on cells treated with 0 μM (—), 25 μM (- - - -) or 50μM (-·-·-) BMOV. A, Ramos B-cells; B, Jurkat T-cells.

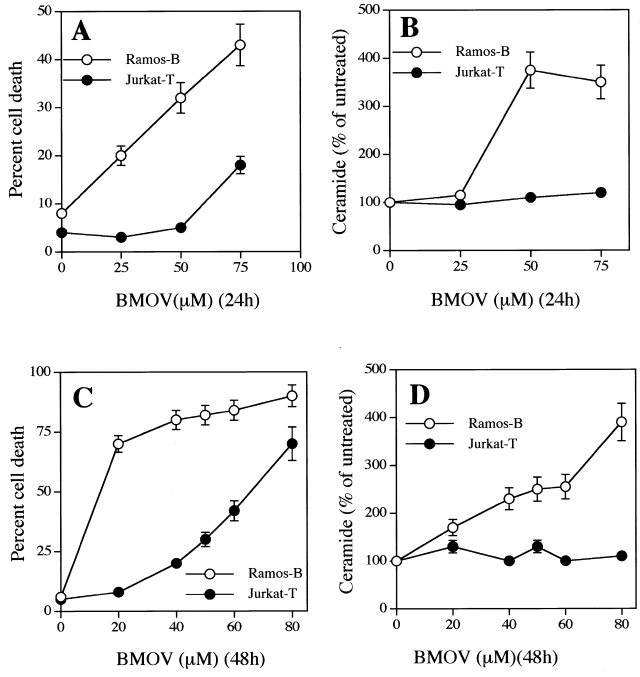

Fig. 3. Correlation of cell death with ceramide production. Cells were treated with BMOV for either 24 h (A,B) or 48 h (C,D). A,C: Induction of cell death by BMOV. Cell death was quantitated by PI staining as described in the text. B,D: Ceramide was quantitated by labelling cells with [^{3}H]palmitate, determining the level of ceramide labelling relative to protein content and expressing this as a percent of untreated cell ceramide levels.

4-fold increase at 80 μM. The ceramide in Ramos B-cells is predominantly the longer chain form, with $C_{16:0}$, $C_{22:0}$, $C_{24:1}$ and $C_{24:0}$ fatty acids in the ratio 4:1:4:5. In contrast, in Jurkat T-cells, the ratio was 6:1:1:1, with palmitate ($C_{16:0}$) predominating (data not shown). The ratio of [^{3}H]palmitate incorporation into ceramide versus that into sphingomyelin showed a dose-dependent 2.5-fold increase (from 0.08 to 0.22) in Ramos B-cells but not in Jurkat T-cells exposed to increasing concentrations of BMOV (Fig. 4B).

4. Discussion

Protein PTP inhibitor, BMOV, induced protein phosphorylation changes in Ramos B-cells which were very similar to those seen after immature B-cell receptor cross-linking [7] and both resulted in apoptosis. We have previously shown that B-cell receptor cross-linking is associated with a 4-fold increase in ceramide and that ceramide was a cause of apoptosis [11,15]. Further evidence implicating ceramide in apoptosis is the fact that both exogenous C_2- and C_6-ceramide induced apoptosis in B-cells [15] and overexpression of anti-apoptotic proteins Bcl_{xL} [11] or Bcl_2 [25] conferred resistance to apoptosis induced by ceramide. Since the tyrosine phosphorylation pattern induced by BMOV was different in B-cells from T-cells and the increase in ceramide and resulting apoptosis was only seen in B-cells, it seems likely that this specific tyrosine phosphorylation is involved in regulating the formation of ceramide in B-cells.

Ceramide is both a biosynthetic precursor of sphingomyelin and a product of the action of sphingomyelinase on sphingomyelin [17], but several studies have implicated a neutral sphingomyelinase activity in pro-apoptotic ceramide formation [12,13,24]. One cloned sphingomyelinase [23] appears to function as a lyso-PAF-PLC [26] and is localized in Golgi not plasma membrane [27] so this is probably not the pro-apoptotic sphingomyelinase. A second protein, with PLC-type activity towards sphingomyelin at neutral pH [24], contains a consensus sequence site for protein tyrosine phosphorylation (^{238}KNRIEHQY) as well as sites for serine and threonine kinases, suggesting that it could be a candidate for the pro-apoptotic sphingomyelinase, but this remains to be verified. Despite the absence of a clear candidate neutral sphingomyelinase, there is considerable evidence for the activation of several other PLC-type enzymes by tyrosine phosphorylation [28], including the PLCγ2 phosphorylated by BMOV, as well as indirect evidence for the activation of phospholipase D by tyrosine phosphorylation [29]. Thus, phosphorylation may be a general mechanism for activating enzymes of this type.

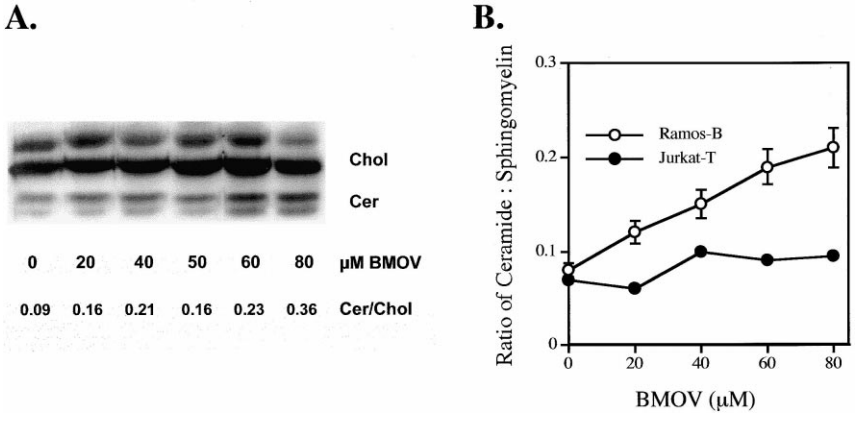

Fig. 4. Ceramide formation is associated with decreased sphingomyelin. (A) HPTLC separation of [3H]palmitate-labelled lipids. This shows the increased formation of ceramide (a doublet) compared to other lipids such as cholesterol (Chol) (an increase from 0.09 to 0.36) following treatment of Ramos B-cells with increasing concentrations of BMOV (0–80 μM) for 48 h. (B) The ceramide:sphingomyelin ratio increases with increasing BMOV. Ramos B-cells and Jurkat T-cells were labelled for 24 h with [^{3}H]palmitate and treated with increasing concentrations of BMOV for 48 h. Lipids were isolated, separated by HPTLC as described in the text, radioactivity determined and the ratio of ceramide to sphingomyelin calculated.

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