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New insights on the use of desipramine as an inhibitor for acid ceramidase

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Abstract Treatment of different cancer cell lines with desipramine induced a time- and dose-dependent downregulation of acid ceramidase. Desipramine's effect on acid ceramidase appeared specific for amphiphilic agents (desipramine, chlorpromazine, and chloroquine) but not other lysomotropic agents such as ammonium chloride and bafilomycin A1, and was not transcriptionally regulated. The cathepsin B/L inhibitor, CA074ME, but not the cathepsin D inhibitor, pepstatin A, blocked desipramine's effect on acid ceramidase. Desipramine led to a more pronounced downregulation of sphingosine compared to ceramide suggesting acid ceramidase inhibition is important to desipramine's mechanism of action. This study reveals a new mechanism of action for desipramine.

Keywords: Desipramine; Ceramide; Sphingomyelin; Acid ceramidase; Acid sphingomyelinase; Cationic amphiphilic drugs

1. Introduction

Studies have shown that the tricyclic anti-depressant desipramine induces intracellular proteolytic degradation of mature acid sphingomyelinase but not other lysosomal enzymes, and accordingly it has been widely used in the literature as a specific acid sphingomyelinase inhibitor [1,2]. Desipramine effect on acid sphingomyelinase has been also observed to be induced by other cationic amphiphilic drugs, but not by neutral or anionic ones [3]. Acid sphingomyelinase is normally localized in the lysosome and hydrolyses sphingomyelin generating ceramide, a major tumor suppressor lipid [4]. Ceramide is an intracellular mediator for a wide variety of extracellular agents known to induce apoptosis including ionizing radiation, chemotherapy, Fas ligand and tumor necrosis factor α [4]. In most cases, desipramine was able to block cell killing induced by such cytotoxic agents, which was attributed to blockade of ceramide generation through inhibition of acid sphingomyelinase [4–8].

In 1995, another ceramide metabolizing enzyme, human acid ceramidase was purified and characterized [9]. Similar to acid sphingomyelinase, acid ceramidase is found predominantly in the lysosomal compartment. However, unlike acid sphingomyelinase which generates ceramide, acid ceramidase deacylates ceramide to generate sphingosine and a fatty acid [4,10]. Sphingosine is an important cell signaling sphingolipid molecule that is also implicated in apoptosis [4]. Moreover, sphingosine can be further metabolized to sphingosine-1-phosphate, another bioactive sphingolipid that often exerts anti-apoptotic functions [4].

In this report, we show that the widely used inhibitor of acid sphingomyelinase desipramine is also an inhibitor of sphingosine generation acid ceramidase. We believe that this information is highly valuable in re-evaluating some of the conclusions that were based on the assumption that desipramine effects are solely due to inhibition of acid sphingomyelinase.

2. Materials and methods

2.1. Cell lines

The human prostate cancer cell line DU145, bladder cancer cell line 5637, and Hela cervical cancer cell line were all purchased from ATCC (Manassas, VA, USA), and cultured in RPMI 1640 (Mediatech Inc., Herndon, VA, USA) supplemented with 10% heat-inactivated bovine growth serum (Hyclone, Logan, UT, USA). The head and neck cancer cell line SCC-14A, was acquired from Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI) and maintained in DMEM 1× MOD medium with L-glutamine and 4.5 g/L glucose (Media-tech, Herndon, VA), supplemented with 10% heat-inactivated bovine growth serum. Cells were maintained in 5% CO_2 at 37 °C.

2.2. Reagents

Desipramine, pepstatin A, and leupeptin were purchased from Sigma (St. Louis, MO, USA), CA074Me was purchased from Calbiochem (San Diego, CA, USA), and bafilomycin A_1 was obtained from BIOMOL. LysoTracker Red was obtained from Molecular Probes (Eugene, OR, USA). Antibodies used for immunoblotting were anti-acid ceramidase (Pharmingen, San Diego, CA, USA), rabbit anti-cathepsin L (Calbiochem), mouse anti-cathepsin B (Oncogene), mouse anti-LAMP-1 (BD Pharmingen), goat anti-mouse IgG-HRP conjugate (Sigma), and goat anti-rabbit IgG-HRP conjugate (Santa Cruz).

2.3. Lysosomal stability assay

Lysosomal stability was measured using the fluorescent dye LysoTracker Red (LTR). Cells were seeded overnight in 60 mm plates. The next day, medium was removed and replaced with medium containing 200 nM LTR. Cells were loaded with LTR for 30 min at

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37 °C. Media were then aspirated and cells were washed once with PBS, followed by addition of fresh medium containing the treatment (10 μm desipramine, 10 mM NH_4Cl or 100 μM bafilomycin A_1) and incubated for 3 h at 37 °C. After treatment, cells were lifted with trypsin, washed once in PBS, and resuspended in 0.5 ml growth medium. LTR fluorescence was measured by FACS analysis (564–606 nm). A decrease in fluorescence intensity corresponds to an increase in lysosomal pH, and a minimum of 10 000 events were scored for each sample.

2.4. Ceramide measurement

2.1×10^6 cells were seeded in 100 mm plates overnight. The next day, media were removed and replaced with media containing vehicle control or desipramine (10 μM) for the indicated time. Following treatment, cells were harvested by gentle scraping and immediate centrifugation at 4 °C for 5 min at 400 × g. Cell pellets were resuspended in ice cold PBS and stored at −80 °C. For sphingolipid analysis, lipid extracts were examined by mass spectrometry as previously described [11].

2.5. Immunoblot analysis

Cells were seeded in 60 mm plates as described above and treated accordingly. Cells were lifted by gently scraping the plates, washed once with ice cold PBS and lysed in lysis buffer (PBS, 1% Triton X-100, 10% glycerol) containing protease inhibitors pepstatin A (0.5 μg/ml), leupeptin (0.5 μg/ml), aprotinin (5 μg/ml), and PMSF (100 μg/ml) for 10 min on ice. Insoluble material was removed by centrifugation at 14 000 rpm for 15 min at 4 °C. The supernatants were then supplemented with SDS to a final concentration of 2% and stored at −80 °C. Protein concentrations were determined using the DC Protein Assay (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Fifty micrograms of protein per sample was separated on NuPAGE 4–12% bis–tris gels (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membranes (BioRad). Following transfer, membranes were blocked for 1 h at room temperature in tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% non-fat dry milk and incubated overnight at 4 °C with primary antibody at a dilution of 1:2000 (actin), 1:250 (acid ceramidase), 1:300 (LAMP–1), 1: 400 (cathepsin B), 1:2000 (cathepsin L). Overnight incubations were performed in 5% milk in TBS-Tween. Following overnight incubation, membranes were washed three times for 10 min each in TBS-Tween and incubated for 1 h at room temperature with secondary antibody in 5% milk TBS-Tween at a dilution 1:5000 (goat anti-mouse IgG) or 1:50 000 (goat anti-rabbit). Membranes were then washed three times more and incubated for 5 min at room temperature with Super Signal HRP substrate (Pierce Biotechnology Inc., Rockford, IL, USA).

2.6. Reverse transcriptase PCR

DU145 cells were seeded in six-well plates as described above. The next day media were gently removed and replaced with media containing 2% BGS and desipramine (10 μM) or ethanol control. Cells were collected at the indicated time points, and total RNA was extracted using RNAqueous-4PCR kit (Ambion Inc., Austin, TX), including the DNase I treatment step to remove DNA contamination. The level of acid ceramidase mRNA was assayed by a two-step RT-PCR protocol (Ambion), and Rig/S15 was used as an internal control. The sequence of the primer for amplification of acid ceramidase was as follows: F – tgtggatagggttcctcactaga, R – ttgtgtatacggtcagcttgttg 375 bp. All reactions were performed in a programmable thermal cycler (reverse transcription at 55 °C for 1 h; PCR at 95 °C, 3 min; 95 °C, 30 s; 52 °C, 1 min and extension at 72 °C for 1 min; final extension at 72 °C, 10 min). The PCR product was separated on a 2% agarose gel.

3. Results

3.1. Desipramine induces the downregulation of acid ceramidase

We first determined the effects of 10 μM desipramine on acid ceramidase in DU145 cancer cells. As seen in the western blot (Fig. 1A), desipramine-induced downregulation of acid ceramidase within 15 min and the enzyme was below detection level by 1 h. The levels of other lysosomal proteins such as LAMP-1, cathepsin B and cathepsin L did not decrease following treatment with desipramine or increased (Fig. 1B). Acid ceramidase downregulation was also observed to be dose dependent (Fig. 1C). Furthermore, the action of desipramine was not cell line dependent since we also observed acid ceramidase downregulation in three additional cell lines, SCC-14A, 5637 and HeLa cells (Fig. 1D). Similarly, desipramine-induced acid ceramidase downregulation has been also described recently in the mouse fibroblast cell line L929 [12]. Finally, we show that similar to desipramine, other cationic amphiphilic drugs that previously have been shown in the literature to downregulate acid sphingomyelinase (chloroquine and chlorpromazine) [13] were able to downregulate acid ceramidase (Fig. 1E). These results demonstrate that desipramine (and related amphiphilic molecules) induces a time- and dose-dependent downregulation of acid ceramidase, at the same concentration that is used in the literature to inhibit acid sphingomyelinase.

3.2. Cysteine proteases are involved in acid ceramidase degradation

Next, we wanted to determine the mechanism of downregulation of acid ceramidase. Desipramine treatment did not change acid ceramidase mRNA steady-state levels as determined by RT-PCR, demonstrating that downregulation was not due to decreased transcription or changes in message stability (Fig. 2A).

Next, the role of lysosomal pH in regulating the levels of acid ceramidase was investigated since desipramine is known to affect lysosomal pH. Treatment of cells with desipramine and with two other lysomotropic drugs NH_4Cl and bafilomycin A_1 resulted in loss of lysosomal pH as judged by loss of lysotracker red intensity (Fig. 2B). However, unlike desipramine, NH_4Cl and bafilomycin A_1 did not downregulate acid ceramidase (Fig. 2B), suggesting that neutralizing lysosomal pH was not sufficient to produce desipramine-induced acid ceramidase downregulation.

Since acid ceramidase resides in the protease-rich lysosomes, lysosomal proteases seemed to be likely candidates for mediating the downregulation of acid ceramidase. To investigate this we tested several protease inhibitors including the broad spectrum cysteine protease inhibitor leupeptin (25 μM), the cathepsin B/L specific inhibitor CA074ME (10 μM) [14,15], and the cathepsin D inhibitor pepstatin A (1 μg/ml). Although pepstatin A had no effect on acid ceramidase downregulation, leupeptin and CA074ME almost completely reversed acid ceramidase downregulation in desipramine treated cells (Fig. 2C). These data strongly suggest that downregulation of acid ceramidase by desipramine is mediated through a cathepsin B/L dependent pathway.

3.3. Sphingolipid modulation following treatment with desipramine

Desipramine has been widely used as an agent to inhibit ceramide generation through acid sphingomyelinase. Since we have now demonstrated above that desipramine degrades acid ceramidase, which deacylates ceramide generating sphingosine, we quantified by mass spectrometry the amount of sphingomyelin, ceramide and sphingosine in DU145 cells following treatment with desipramine. We observed that 10 μM desipramine treatment resulted in an accumulation of sphingomyelin (Fig. 3A), consistent with its inhibitory effect on acid sphingomyelinase. Total ceramide levels did not decrease and were in-

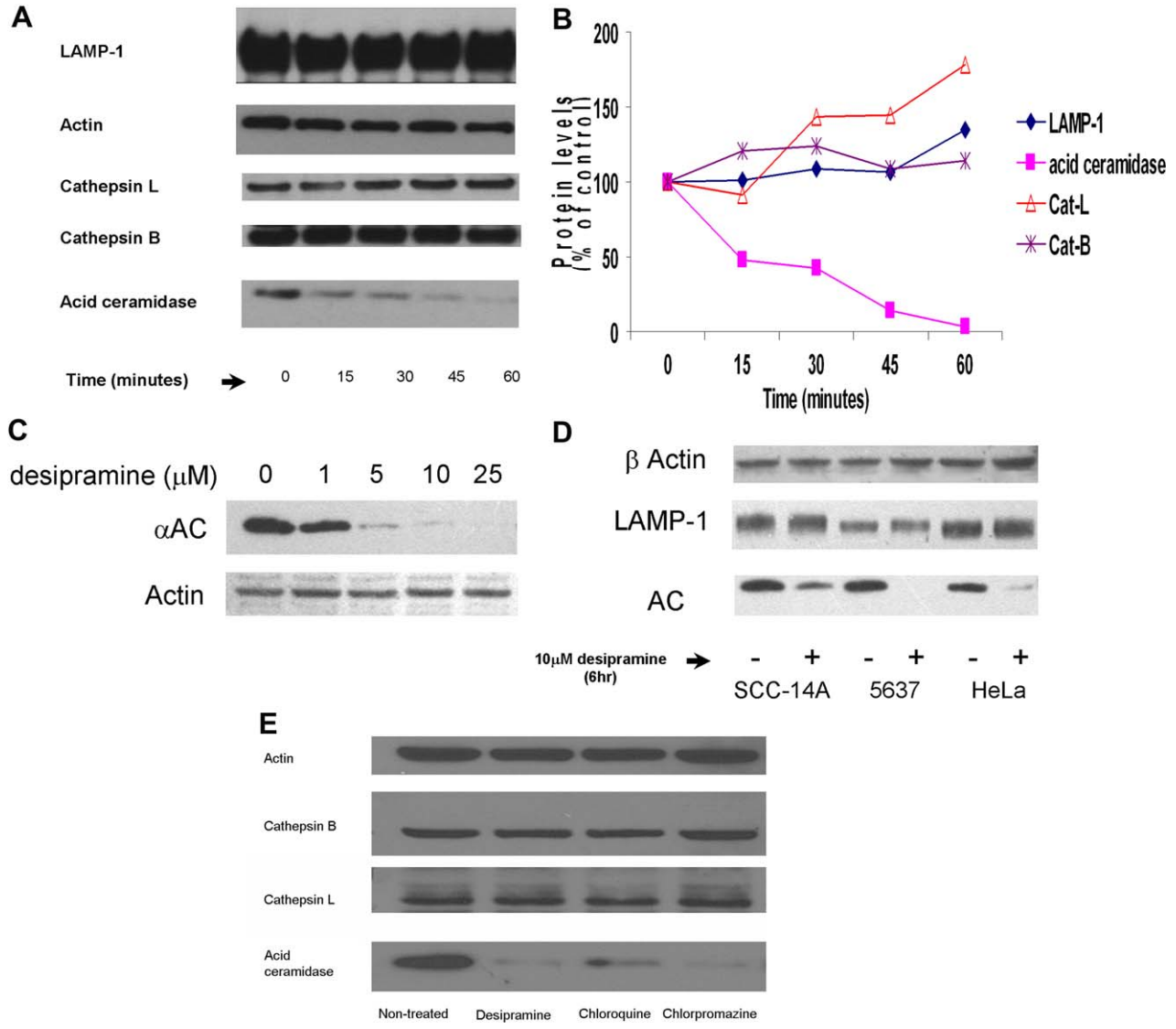

Fig. 1. Downregulation of acid ceramidase by desipramine and other cationic amphiphilic drugs. DU 145 cells were plated overnight in 10% DMEM and treated with 10 μM desipramine for the indicated time points (A) or for 6 h with the indicated desipramine concentrations (C). Following treatment, cells were collected, lysed, and proteins were analyzed by Western blotting as described in Section 2. B represents densitometry analysis of the blot in A normalized to actin. In D, SCC-14A, 5637 and HeLa cells were treated with 10 μM desipramine for 6 h then subjected to Western blotting. E is a Western blot of Du145 treated for 6 h with 5 μM desipramine, chloroquine or chlorpromazine. All Western blots have been performed at least twice with the same results.

creased following treatment with desipramine (Fig. 3B) which was surprising in view of its projected mechanism of action and use as an inhibitor of acid sphingomyelinase. However, since acid ceramidase is a key enzyme in ceramide metabolism and we have demonstrated its degradation by desipramine (Figs. 1 and 2), we would expect that ceramide might increase with a concomitant decrease in sphingosine levels following drug treatment which was what we observed (Fig. 3C). These results support the dual effects of desipramine on both acid sphingomyelinase and acid ceramidase.

4. Discussion

In this report, we investigated the effect of the amphiphilic drug desipramine on acid ceramidase, an enzyme that metabolizes ceramide to sphingosine. We have demonstrated that desipramine produces a dose- and time-dependent downregulation of acid ceramidase at the protein level at the relatively low dose of 5 μM, a dose that is commonly used in the literature to inhibit acid sphingomyelinase. Other cationic amphiphilic drugs (chlorpromazine, chloroquine) previously shown at similar doses to inhibit acid sphingomyelinase were also observed to produce the same effect on acid ceramidase suggesting that acid ceramidase and sphingomyelinase are both targets for this group of compounds [13].

A number of studies have been published lately on the potential use of cationic amphiphilic drugs as chemo-sensitizers, and chemotherapeutic agents, though the mechanism of action is still not well defined [13,16,17]. From our data, it may be as well that inhibition of acid ceramidase by these drugs plays an important role in their chemo-sensitizing ability, especially

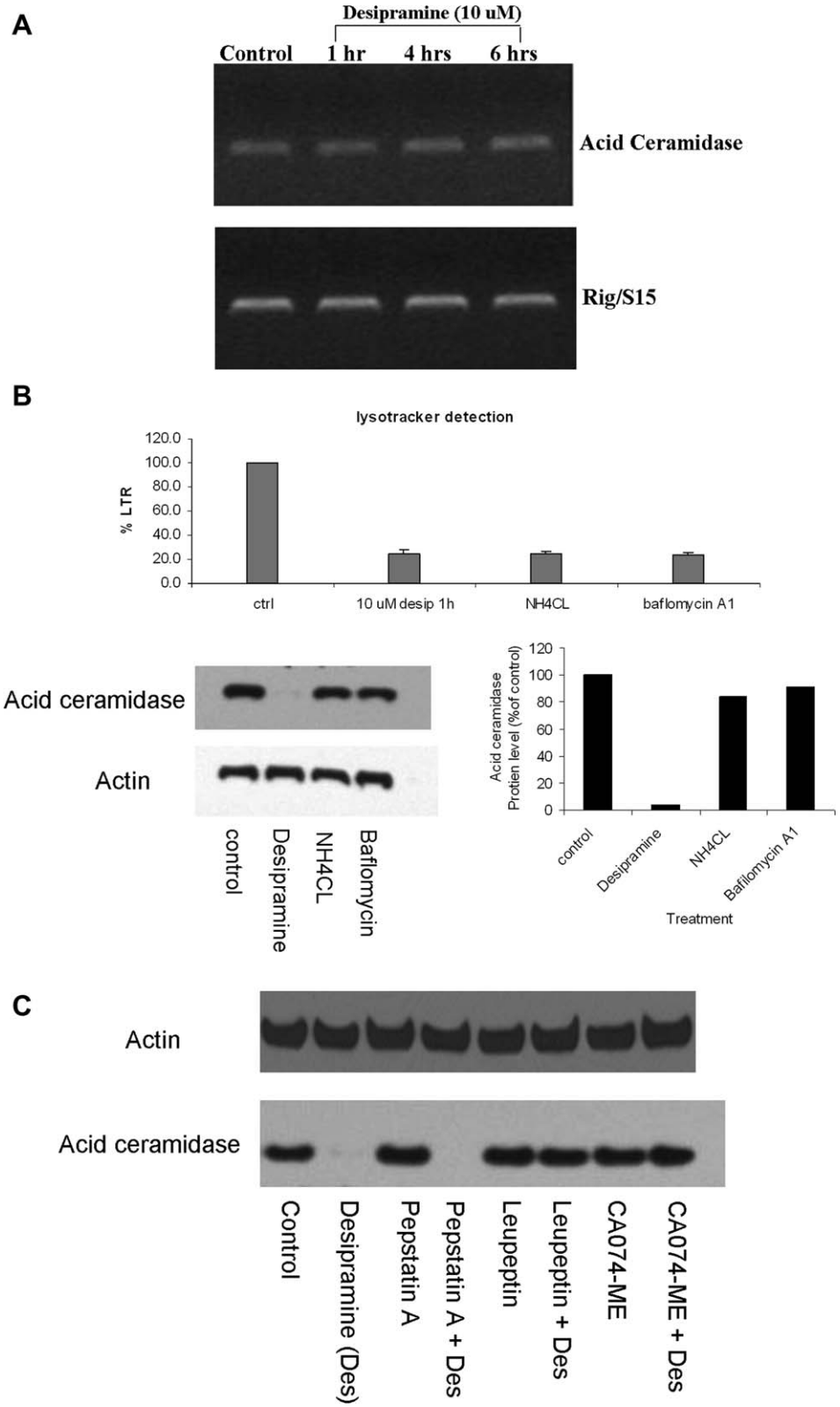

Fig. 2. Downregulation of acid ceramidase by desipramine is mediated through Ca074ME sensitive proteases. (A) RT-PCR of acid ceramidase in DU 145 cells following treatment with 10 μM desipramine for the indicated time range. (B) Comparison of the effect of three lysomotropic agents on lysosomal pH. Cells were loaded with Lysotracker red for 30 min at 37 °C. Lysotracker red was removed and cells were treated with 10 μm desipramine, 10 mM NH_4CL or 100 μM Bafilomycin A_1 for 3 h followed by FACS analysis to measure Lysotracker red fluorescence as described in Section 2. A decrease in fluorescence intensity corresponds to an increase in lysosomal pH. Results are expressed as mean + S.D. for at least two different experiments. We also measured by Western blotting the effect of the same drugs on acid ceramidase levels which were also quantified by densitometry. (C) Effect of the protease inhibitors leupeptin, CA074, and pepstatin A on acid ceramidase protein levels following a 6 h treatment with 10 μM desipramine.

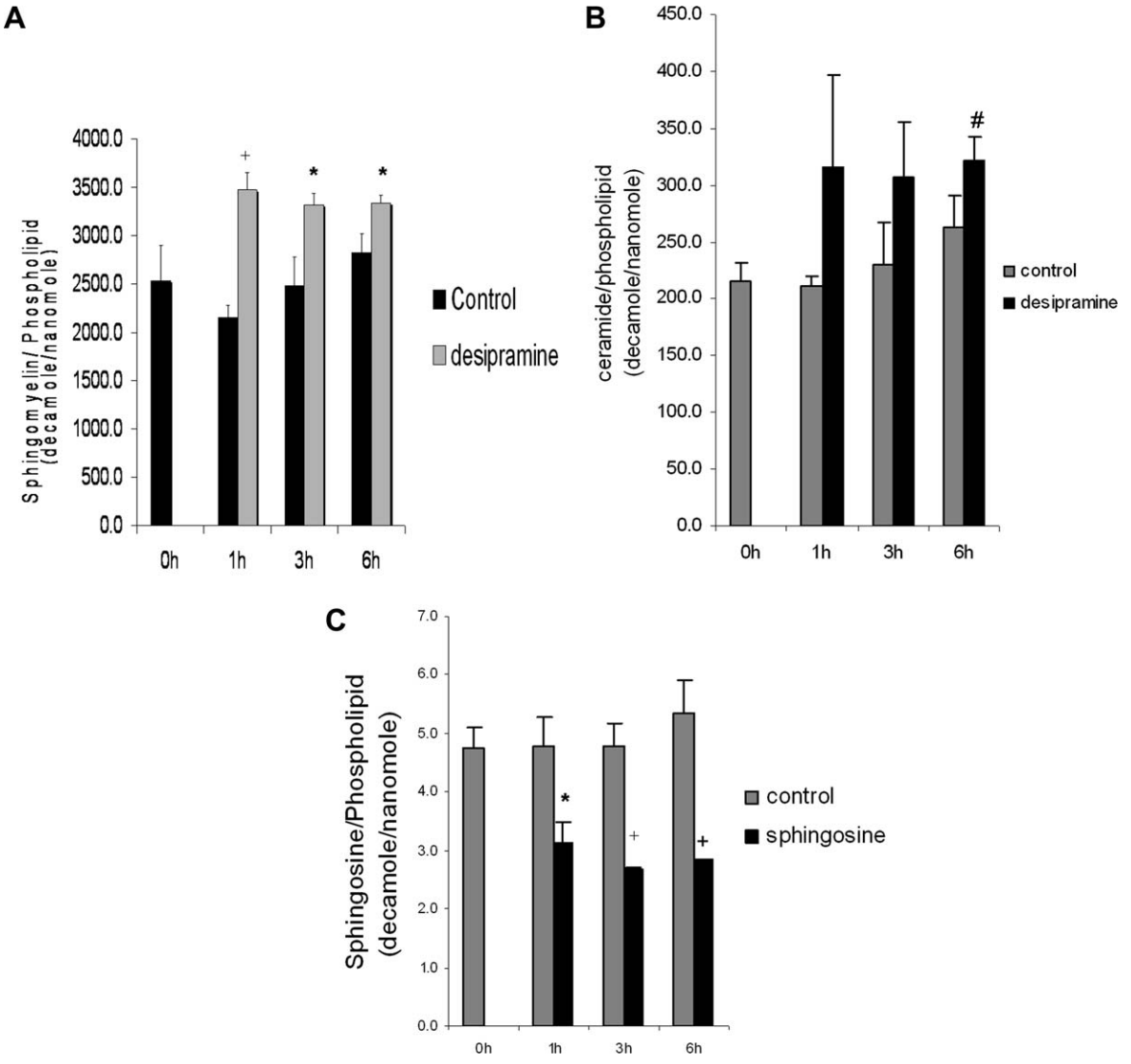

Fig. 3. The effect of desipramine on sphingolipid levels. Changes in sphingomyelin (A), ceramide (B), and sphingosine (C) levels in DU145 cancer cells following treatment with 10 μM desipramine for the indicated times were determined by mass spectrometry as described in Section 2. Results are expressed as mean + S.D. for at least two different experiments. Statistical significance between desipramine treatment and matched control is determined using statistical student's *t*-test. # represents $p < 0.05$, *represents $p < 0.005$, and + represents $p < 0.0005$.

since acid ceramidase has been shown to be over-expressed in cancer and to play an important role in mediating resistance to chemotherapeutics [18,19,22].

Next, we investigated the mechanism of downregulation of acid ceramidase by desipramine. We demonstrate that downregulation is not due to decreased transcription. Next, the effect of desipramine on acid ceramidase could not be reproduced by other agents that neutralize lysosomal pH (NH_4Cl, bafilomycin A_1), suggesting that lysosomal pH dissipation on its own cannot account for desipramine effect on acid ceramidase. Finally, the specific cathepsin B/L inhibitor CA074ME [14,15] inhibited acid ceramidase degradation following incubation with desipramine suggesting that cathepsins B and/or L are involved in the degradation of acid ceramidase following incubation with desipramine.

Since desipramine downregulates two key enzymes with opposite effects on ceramide metabolism, it became important to determine the net effect of desipramine on basal levels of ceramide in cells. We found that in the prostate cancer cell line DU145, desipramine led to accumulation of sphingomyelin and ceramide. Importantly, desipramine induced a significant drop in the levels of sphingosine in DU145 cancer cells, suggesting that desipramine can be used as an inhibitor of sphingosine generation by acid ceramidase. Thus, desipramine affects the level of more than one functionally active sphingolipid molecule, namely, sphingomyelin, sphingosine, and ceramide with the latter two being both implicated in apoptosis pathways [4]. Therefore, based on the above, we recommend that care be exercised in interpreting results with desipramine due to the multiple effects of this molecule on sphingolipid modulating enzymes and their sphingolipid products.

Although the literature on acid ceramidase is limited compared to acid sphingomyelinase, accumulating evidence suggests that acid ceramidase is over-expressed in cancer and that its over-expression mediates resistance to apoptosis induction [18,19]. Therefore, interest on developing and testing

drugs that inhibit acid ceramidase is mounting with the specific goal to develop drugs that sensitize cancer cells to chemotherapeutic agents or radiation by inhibiting acid ceramidase allowing ceramide levels to accumulate [20,21]. This suggests that this class of amphiphilic drugs which downregulate acid ceramidase might be combined with radiation or chemotherapy to enhance ceramide upregulation and produce a more potent therapeutic event. Since desipramine is an approved drug for use in depression therapy such a clinical trial would be possible to perform.

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