Bcl-xL interrupts oxidative activation of neutral sphingomyelinase

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Abstract **Recent studies demonstrate a role for intracellular oxidation in the regulation of neutral sphingomyelinase (N-SMase). Glutathione (GSH) has been shown to regulate N-SMase in vitro and in cells. However, it has not been established whether the effects of GSH in cells are due to direct action on N-SMase. In this study, treatment of human mammary carcinoma MCF-7 cells with diamide, a thiol-depleting agent, caused a decrease in intracellular GSH and degradation of sphingomyelin (SM) to ceramide. The SM pool hydrolyzed in response to diamide belonged to the bacterial SMase-resistant pool of SM. Importantly, pretreatment of MCF-7 cells with GSH, *N*-acetylcysteine, an antioxidant, or GW69A, a specific N-SMase inhibitor, prevented diamide-induced degradation of SM to ceramide, suggesting that intracellular levels of GSH regulate the extent to which SM is degraded to ceramide and that this probably involves a GW69A-sensitive N-SMase. Unexpectedly, expression of Bcl-xL prevented tumor necrosis factor-α-induced SM hydrolysis and ceramide accumulation but not the decrease in intracellular GSH. Furthermore, Bcl-xL inhibited diamide-induced SM hydrolysis and ceramide accumulation but not the decrease in intracellular GSH. These results suggest that the site of action of Bcl-xL is downstream of GSH depletion and upstream of ceramide accumulation, and that GSH probably does not exert direct physiologic effects on N-SMase.**

Key words: Bcl-xL; Neutral sphingomyelinase; Glutathione; Ceramide

1. Introduction

Sphingomyelinase (SMase) hydrolyzes sphingomyelin (SM) to generate ceramide and phosphocholine, and several SMase isoforms have been described [1]. In particular, acid SMase (A-SMase) and neutral SMase (N-SMase) have been studied in detail, and both A-SMase and N-SMase are activated in response to cytokines and other agents [2–9].

Compared with A-SMase, the physiological function and the regulatory mechanism of N-SMase remain rather elusive. In spite of difficulties in its identification, there is evidence to support the role of various factors that can regulate N-SMase, especially glutathione (GSH) [10–12]. Recent studies have demonstrated a link between GSH and the breakdown of SM to ceramide based on the use of GSH precursors or GSH-depleting agents [5,13–24]. Furthermore, overexpression of GSH peroxidase-1 prevented activation of N-SMase, SM hydrolysis, and ceramide generation in response to doxorubicin [25]. These results suggest that the N-SMase/ceramide pathway and intracellular oxidation are closely linked and that intracellular GSH depletion might be upstream of N-SMase activation. Recently, GSH was shown to inhibit the activation of N-SMase in partially purified preparations. However, there is no evidence to support a direct physiologic effect of GSH on N-SMase.

Members of the Bcl-2 family of proteins, which play an important role in the regulation of apoptosis [26], have been shown to inhibit ceramide-induced apoptosis and ceramide accumulation [27–29]. Overexpression of Bcl-2 in MCF-7 cells did not prevent ceramide generation but inhibited the ability of exogenous ceramides to activate the distal caspases and to induce apoptosis, suggesting that Bcl-2 might act downstream of ceramide formation [5,29,30]. In contrast to Bcl-2, overexpression of Bcl-xL in MCF-7 cells has recently been found to interfere with ceramide accumulation and apoptosis induced by tumor necrosis factor-α (TNFα), suggesting that the action of Bcl-xL might be upstream of the formation of ceramides [29].

As described above, it is suggested that GSH plays a critical role in the N-SMase/ceramide pathway. Therefore, it became important to clarify the relationship between the intracellular levels of GSH, the Bcl-2 family of proteins and ceramide generation. Although there are some indications that Bcl-2 and Bcl-xL may function as antioxidants [31–35], it was shown that Bcl-2 had no effect on intracellular GSH depletion induced by TNFα in MCF-7 cells [5]. However, the role of Bcl-xL on this relationship has not yet been determined.

To investigate the relationship between N-SMase, GSH and Bcl-xL, we examined the effect of Bcl-xL on diamide-, a thiol-depleting agent, and the TNFα-induced ceramide pathway using MCF-7 cells expressing Bcl-xL.

2. Materials and methods

2.1. Materials

RPMI 1640, fetal bovine serum (FBS) and trypsin-EDTA were from Life Technologies. Hygromycin was from Calbiochem. [*methyl*-

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Abbreviations: A-SMase, acid sphingomyelinase; N-SMase, neutral sphingomyelinase; SM, sphingomyelin; GSH, glutathione; TNFα, tumor necrosis factor-α; bSMase, bacterial sphingomyelinase; GSSG, oxidized GSH; NAC, *N*-acetylcysteine; FBS, fetal bovine serum; PBS, phosphate-buffered saline

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[3H]Choline chloride (1 mCi/ml, 85 mCi/mmol), [γ-32P]ATP (10 mCi/ml, 3000 Ci/mmol), and EN3HANCE spray were from NEN Life Science Products. Ceramide type III, bacterial SMase (bSMase) (from *Staphylococcus aureus*), GSH, oxidized GSH (GSSG), *N*-acetylcysteine (NAC), and diamide were from Sigma Chemical Company. Silica Gel 60 thin-layer chromatography (TLC) plates were from Whatman. Scintillation mixture Safety Solve was from Research Products International. All solvents were of AR grade and were from Mallinckrodt. Glaxo-Smith-Kline Research kindly provided us with GW69A.

2.2. Cell culture

The previously described cell line overexpressing Bcl-xL was derived from a TNFα-sensitive MCF-7 parental line [36]. Cells were in RPMI 1640 medium supplemented with 10% FBS. 150 μg/ml hygromycin was added to the Bcl-xL cell line and its vector. Experiments were done in the absence of hygromycin.

2.3. Metabolic labeling of cellular SM

Cells were seeded at 2×10^5 cells/100-mm Petri dish. After 2 days, the medium was changed and the cells were labeled with [*methyl*-3H]choline chloride (final specific activity: 0.5 μCi/ml) in RPMI 1640/10% FBS for 60 h. Cells were then washed once with phosphate-buffered saline (PBS) and chased with RPMI 1640/2% FBS for 2 h. After washing with PBS again, cells were treated with 0.5 mM diamide or 3 nM TNFα.

2.4. Drug treatment

Prior to 0.5 mM diamide or 3 nM TNFα, cells were incubated in RPMI 1640/2% FBS containing 100 mU/ml bSMase (for 25 min), 10 μM or 20 μM GW69A (for 45 min), 15 mM GSH (for 2 h) or 10 mM NAC (for 2 h). The final concentration of dimethyl sulfoxide (a solvent for GW69A) in the medium was 1.33% (v/v), which has no effect on cell viability. Each reagent was included in the medium throughout the subsequent incubations.

2.5. Measurements of [3H]choline-labeled SM

After removing the medium, cells were washed and scraped in PBS. Cells were pelleted, and total lipids were extracted according to the Bligh and Dyer method [37]. Total lipid extract was subjected to mild base hydrolysis [38]. [3H]SM was determined by TLC analysis in chloroform:methanol:12 mM $CaCl_2$ (90:52.5:12), followed by scraping and counting the radioactivity by liquid scintillation. SM levels were determined by measuring the amount (nmol) of inorganic phosphate present in the organic phase [39] and were normalized to total phosphate initially present.

2.6. Ceramide measurements

Cells were seeded at 2×10^6 cells/100-mm Petri dish in RPMI 1640/2% FBS, rested overnight, and then treated with 0.5 mM diamide or 3 nM TNFα. Cells were harvested in methanol, and lipids were extracted using the Bligh and Dyer method [37]. Aliquots of the organic phase (0.9 ml and 0.3 ml in duplicates) were dried down separately, and used, respectively, for ceramide and phosphate measurements [39]. The level of ceramide was determined by the diacylglycerol kinase assay as described [40].

2.7. Measurement of GSH and GSSG levels in cells

Cells were seeded at 2×10^6 cells/100-mm Petri dish in RPMI 1640/2% FBS, rested overnight, and then treated with 0.5 mM diamide or 3 nM TNFα. Treated cells were washed with ice-cold PBS, and harvested in 3.33% SSA buffer (3.33% 5-sulfosalicylic acid, 0.167 mM EDTA). The supernatant was separated from the acid-precipitated proteins by centrifugation. GSH and GSSG content in the supernatant were determined by the Griffith [41] modification of Tietze's enzymatic procedure [42]. GSH and GSSG content were normalized to total protein.

3. Results

3.1. Diamide induces the breakdown of SM to ceramide in MCF-7 cells

First, we investigated the effect of a thiol-depleting agent, diamide, on intracellular levels of GSH and breakdown of SM to ceramide in these cells. Diamide is known to reduce acutely the intracellular levels of GSH by its oxidation to GSSG [43–45]. Stimulation of MCF-7 cells with 0.5 mM diamide resulted in an immediate decrease in intracellular levels of GSH (Fig. 1a) and an increase of the intracellular levels of GSSG (Fig. 1b). The ratio of GSH/GSSG was therefore significantly reduced in diamide-treated cells (Fig. 3c). SM turnover of approximately 5% could be observed as early as 30 min following treatment of MCF-7 cells with 0.5 mM diamide, and maximal effects of up to about 20% SM hydrolysis were observed after 60 min of treatment (Fig. 2a). Thus, diamide induces acute hydrolysis of SM.

Next, we determined whether this SM pool hydrolyzed in response to diamide is on the outer leaflet of the plasma membrane and accessible to hydrolysis by exogenous SMase. To this end, cells were treated with bSMase in the presence or absence of diamide. bSMase induced significant hydrolysis of membrane SM (Fig. 2b). However, the effects of diamide were still observed in the presence of bSMase demonstrating that the pool of SM hydrolyzed in response to diamide is resistant to the action of bSMase and suggesting that it may not reside in the outer leaflet of the plasma membrane (Fig. 2b).

Furthermore, stimulation of cells with 0.5 mM diamide resulted in a time-dependent increase in the production of ceramide (Fig. 2c). Taken together, these experiments indicate that diamide can modulate the breakdown of SM to ceramide with significant induction of ceramide production. In addition, treatment of cells with NAC (10 mM) or extracellular application of GSH (15 mM) blocked diamide-mediated SM hydrolysis (Fig. 3a) and ceramide generation (Fig. 3b). These

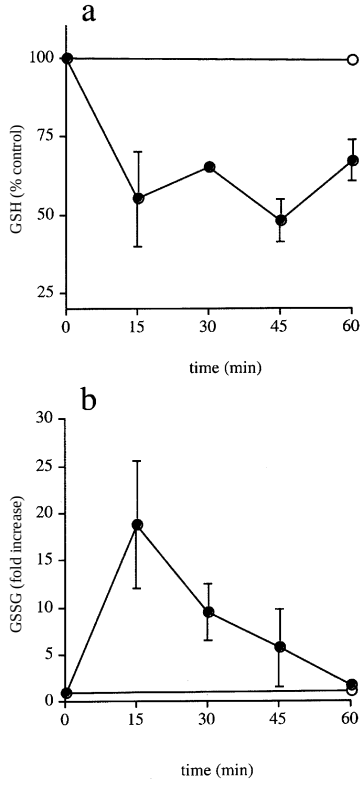

Fig. 1. Diamide-induced GSH depletion and GSSG increase in MCF-7 cells. MCF-7 cells were treated with (closed circles) or without (open circles) 0.5 mM diamide for the indicated times. GSH (a) and GSSG (b) levels were measured as described in Section 2. Results are mean ± S.D. of three independent experiments.

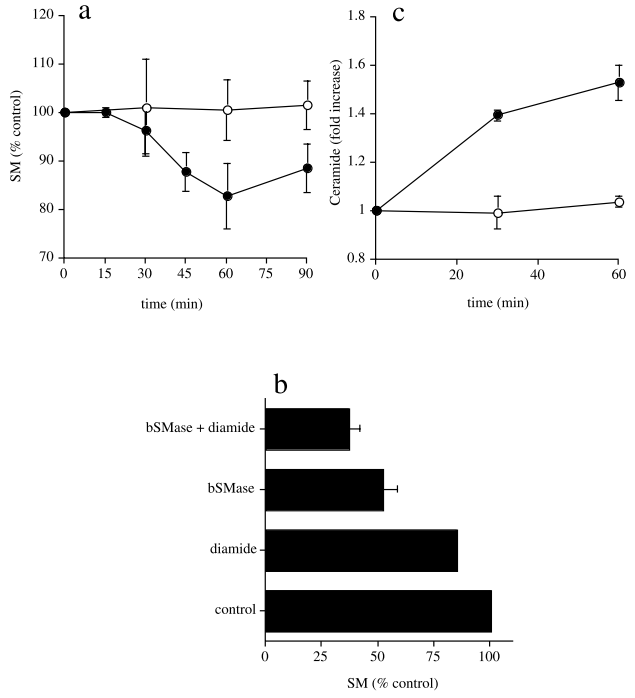

Fig. 2. Diamide-induced SM hydrolysis and ceramide generation in MCF-7 cells. a: Diamide-induced SM hydrolysis in MCF-7 cells. Cells were then treated with (closed circles) or without (open circles) 0.5 mM diamide for the indicated time. SM content was analyzed as described in Section 2. b: Treatment of MCF-7 cells with diamide (for 1 h) and bSMase. Cells were treated with vehicle, 0.5 mM diamide, 100 mU/ml bSMase, or diamide plus bSMase. Prior to 0.5 mM diamide, cells were incubated in RPMI 1640/2% FBS containing 100 mU/ml bSMase for 25 min. bSMase was included in the medium throughout the subsequent incubations. c: Lipids were extracted from cells untreated (open circles) or treated (closed circles) with 0.5 mM diamide for the indicated time, and ceramide content was determined as described in Section 2. Results are mean ± S.D. of three independent experiments.

results demonstrate that intracellular levels of GSH are important regulators of breakdown of SM to ceramide.

In order to investigate if these effects are due to activation of N-SMase, we utilized GW69A, a recently developed specific inhibitor of N-SMase [46]. GW69A blocked diamide-mediated SM hydrolysis (Fig. 3a) and ceramide generation (Fig. 3b) but not diamide-induced reduction of GSH/GSSG ratio (Fig. 3c), suggesting that the intracellular levels of GSH regulate the extent to which SM is degraded to ceramide and that this probably involves a GW69A-sensitive N-SMase.

3.2. *Bcl-xL blocks TNFα-induced SM hydrolysis and ceramide generation at a site downstream of GSH depletion in MCF-7 cells*

Next, we investigated the effects of Bcl-xL on TNFα-induced SM hydrolysis, ceramide accumulation and changes of GSH levels. First, significant SM hydrolysis was observed between 10 and 15 h after treatment with 3 nM TNFα, and a 30% hydrolysis of SM was detected at 15 h (Fig. 4a). However, Bcl-xL completely inhibited SM hydrolysis. Second, stimulation of MCF-7 cells with TNFα resulted in a time-dependent increase in the production of ceramide (Fig. 4b). Bcl-xL overexpression completely inhibited ceramide accumulation in response to TNFα (Fig. 4c). Third, a time-dependent depletion of GSH was observed in both wild type and Bcl-xL cells treated with 3 nM TNFα. These changes were indistinguishable from the effects of TNFα in wild type cells (Fig. 4d), suggesting that Bcl-xL does not affect the decrease in GSH levels in response to TNFα. These results suggested that Bcl-xL probably acts between the decrease in GSH levels and generation of ceramide.

3.3. *Bcl-xL blocks diamide-induced SM hydrolysis and ceramide generation at a site downstream of GSH depletion in MCF-7 cells*

The above results suggested that Bcl-xL might act between the decrease in GSH levels and the breakdown of SM to ceramide. To further investigate this possibility, we examined the effects of Bcl-xL on diamide-induced SM hydrolysis, ceramide accumulation and changes of GSH levels. First, in the Bcl-xL-

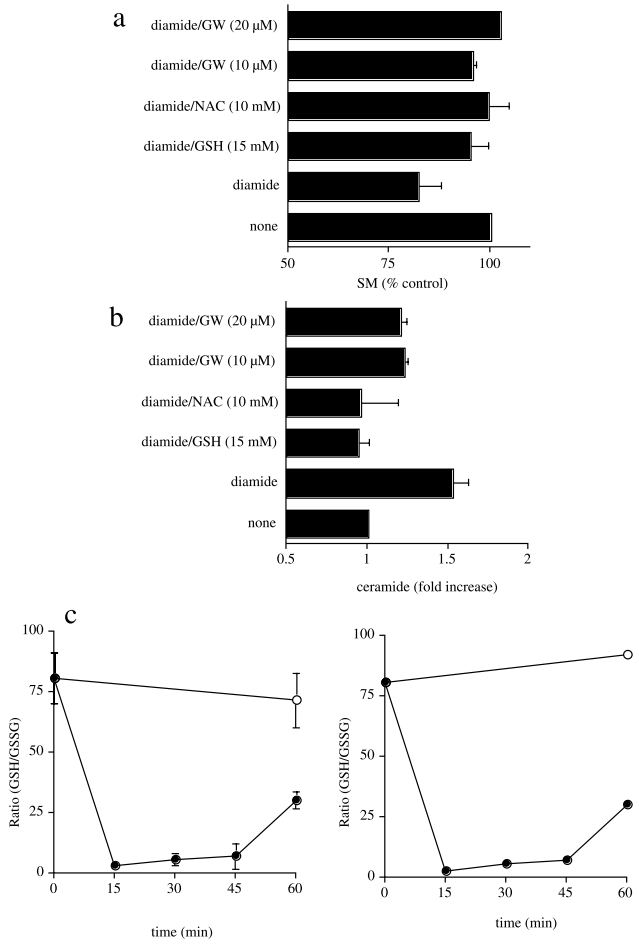

Fig. 3. The effects of GSH, NAC and GW69A on diamide-induced SM hydrolysis and ceramide generation in MCF-7 cells. a: GSH, NAC and GW69A inhibit diamide-induced SM hydrolysis. Cells were treated with or without 0.5 mM diamide for 1 h in the presence or absence of GW69A, GSH, or NAC. SM content was analyzed as described in Section 2. b: GSH, NAC and GW69A inhibit diamide-induced ceramide elevation. Cells were treated with or without 0.5 mM diamide for 1 h, and ceramide content was determined as described in Section 2. c: The effects of GW69A on diamide-induced reduction of the GSH/GSSG ratio. MCF-7 cells were treated with (closed circles) or without (open circles) 0.5 mM diamide for the indicated time. GSH and GSSG levels were measured as described in Section 2. Prior to 0.5 mM diamide, cells were incubated in RPMI 1640/2% FBS with (right panel) or without (left panel) 20 μM GW69A (for 45 min). Results are mean ± S.D. of three independent experiments.

transfected cells, a depletion of intracellular GSH and an increase of intracellular GSSG were observed following treatment with 0.5 mM diamide (Fig. 5a–c). These changes were indistinguishable from the effects of diamide in wild type cells, suggesting that Bcl-xL does not affect the decrease in GSH levels in response to diamide. Next, the effects of Bcl-xL on diamide-induced SM hydrolysis and ceramide generation were examined. Bcl-xL completely inhibited SM hydrolysis (Fig. 5d) and partially inhibited ceramide generation in response to 0.5 mM diamide (Fig. 5e). These results suggest that the site of action of Bcl-xL is downstream of GSH depletion and upstream of ceramide accumulation, and that Bcl-xL does not have a major antioxidant function.

4. Discussion

Depletion of endogenous GSH by diamide induces the breakdown of SM to ceramide, which is blocked by NAC, GSH and GW69A, suggesting that depletion of GSH is closely related to regulation of N-SMase activation. Importantly, the results show that Bcl-xL inhibits diamide-induced breakdown of SM to ceramide and TNFα-induced ceramide accumulation, but does not modulate diamide- and TNFα-induced GSH depletion, suggesting that the site of action of Bcl-xL is downstream of GSH depletion and upstream of ceramide accumulation.

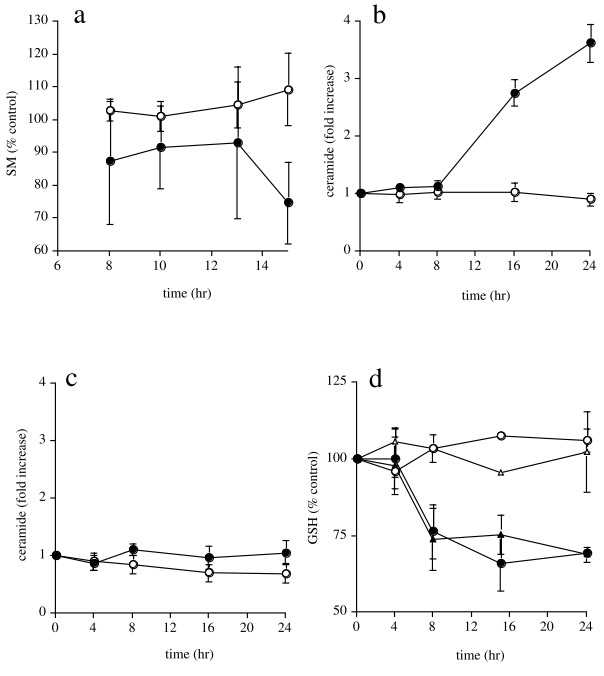

Fig. 4. The effects of Bcl-xL on TNFα-induced SM hydrolysis, ceramide elevation, and GSH depletion. a: Bcl-xL inhibits TNFα-induced SM hydrolysis. Bcl-xL- (open circles) or vector- (closed circles) transfected MCF-7 cells were then treated 3 nM TNFα for the indicated time. SM content was analyzed as described in Section 2. b,c: Bcl-xL inhibits TNFα-induced ceramide elevation. Bcl-xL- (c) or vector- (b) transfected MCF-7 cells were treated with (closed circles) or without (open circles) 3 nM TNFα for the indicated time, and ceramide content was determined as described in Section 2. d: Bcl-xL has no effect on TNFα-induced GSH. Shown is the time course for the effect of TNFα on GSH level in Bcl-xL- (triangles) or vector- (circles) transfected MCF-7 cells. Bcl-xL- or vector-transfected MCF-7 cells were treated with (closed) or without (open) 3 nM TNFα for the indicated time, and GSH levels were measured as described in Section 2. Results are mean ± S.D. of three independent experiments.

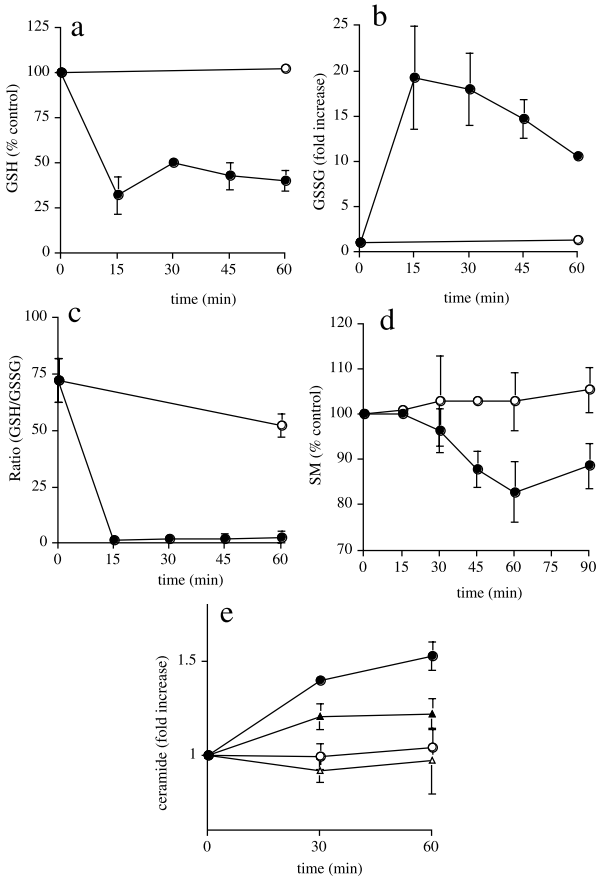

Fig. 5. The effects of Bcl-xL on diamide-induced SM hydrolysis, ceramide elevation, and GSH depletion. Bcl-xL-transfected MCF-7 cells were treated with (closed circles) or without (open circles) 0.5 mM diamide for the indicated time. GSH (a) and GSSG (b) levels were measured as described in Section 2. c: The ratio GSH/GSSG. d: Bcl-xL inhibits diamide-induced SM hydrolysis. Bcl-xL- (open circles) or vector- (closed circles) transfected MCF-7 cells were treated with 0.5 mM diamide for the indicated time. SM content was analyzed as described in Section 2. e: Lipids were extracted from Bcl-xL- (triangles) or vector- (circles) transfected MCF-7 cells untreated (open) or treated (closed) with 0.5 mM diamide for the indicated time, and ceramide content was determined as described in Section 2. Results are mean ± S.D. of three independent experiments.

These studies have several interesting and important implications. First, the current results, showing that diamide induced the breakdown of SM to ceramide, demonstrate that intracellular levels of GSH and/or reactive oxygen species regulate the degradation of SM to ceramide. Recent studies have shown that depletion of intracellular levels of GSH is closely related to activation of N-SMase and ceramide formation [5,10–25]. Our results also support these observations. Second, our results, showing that Bcl-xL and GW69A inhibit the breakdown of SM to ceramide, but do not modulate the depletion of GSH in response to diamide, support an indirect mechanism of action for GSH inhibition of N-SMase. These findings raise the question of what are the mechanisms coupling oxidation/depletion of GSH to the activation of N-SMase. This may involve undetermined molecule(s) whose own oxidation may regulate N-SMase. Third, our results show that Bcl-xL inhibited SM hydrolysis and ceramide accu-

mulation in response to diamide and TNFα, and that Bcl-xL did not block diamide- and TNFα-induced GSH depletion, suggesting that Bcl-xL acts downstream of the decrease in GSH levels and that Bcl-xL does not function as a major antioxidant. The mechanism of action of Bcl-xL remains unknown, and further investigations are clearly needed to answer this question. Fourth, these results demonstrate a close relationship between ceramide accumulation, GSH depletion, and the action of Bcl-xL (Fig. 3). Given the presumed mitochondrial site of action of Bcl-2 and its related proteins [47], they raise an important question as to the subcellular localization of ceramide generation [48,49]. Recent studies have suggested the presence of ceramide metabolism in mitochondria. Taken together, it is suggested that ceramide might be generated in mitochondria, and that N-SMase might be localized in mitochondria or in close proximity to mitochondria (mitochondria-associated membranes).

In conclusion, we have demonstrated that diamide and TNFα induce the breakdown of SM to ceramide and that Bcl-xL inhibits this pathway at a site downstream of depletion of GSH and upstream of ceramide accumulation. The mechanism and relevance of the inhibitory action of Bcl-xL on N-SMase activation should be the subject of future studies.

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