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In ongoing studies in human breast carcinoma cells in tissue culture, we find that tumor necrosis factor α (TNF α) causes apoptosis which is preceded by accumulation of ceramide. This accumulation of ceramide involves activation of ICE-like proteases which are inhibitable by CrmA and by inhibitors of type 1 caspases (YVAD). On the other hand, the addition of ceramide to cells results in the induction of apoptosis which is inhibited by Bcl-2 and by inhibitors of the caspase-3-type proteases (DEVD). In further studies, we have determined that TNF α causes activation of neutral sphingomyelinase. We have isolated this enzyme from breast carcinoma cells and studied its regulation. These studies led to the identification of glutathione (GSH) as an inhibitor of this enzyme *in vitro*. Studies in cells show that TNF α causes a drop in glutathione levels which is coincident with activation of sphingomyelinase and the formation of ceramide. The addition of GSH or N-acetyl cysteine to TNF α treated cells results in replenishment of glutathione levels and prevention of ceramide formation. These studies are beginning to elucidate the mechanisms involved in activation of sphingomyelinase by TNF α in breast carcinoma cells.

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INTRODUCTION

Ceramide has recently emerged as an important endogenous candidate mediator of growth suppression; especially in response to cytotoxic agents and stress stimuli (1-3). Briefly, a number of extracellular agents have been found to induce ceramide formation. These include: Tumor Necrosis Factor alpha (TNFα), γ interferon, interleukin-1β, dexamethasone, Fas ligands, chemotherapeutic agents, and nerve growth factor (4). These agents cause the activation of membrane sphingomyelinases, which act on membrane sphingomyelin, causing the formation of ceramide. The addition of analogs of ceramide to cells causes either terminal cell differentiation, cell senescence, cell cycle arrest, or apoptosis. A role for the endogenous ceramide in mediating these biological effects in response to the above listed agents is further supported by the close association of formation of endogenous ceramide with these biological outcomes, the ability of exogenous ceramides to mimic these biologies, and the ability of agents that interfere with ceramide metabolism to modulate these responses (5,6).

The role of ceramide is further supported by recent insight into the mechanism of action of ceramide *in vitro* and in cells. Thus, *in vitro* ceramide has been shown to activate a serine/threonine protein phosphatase (7). This phosphatase is inhibited by okadaic acid, and okadaic acid appears to inhibit the effects of ceramide on apoptosis and growth suppression (8). Furthermore, an equivalent pathway has been demonstrated in yeast cells, whereby ceramide causes growth suppression of yeast (9). Genetic evidence was provided for the subunit composition of this phosphatase in yeast (10). Deletion of these subunits in yeast results in resistance to the activity of ceramide on growth suppression. Taken together, these studies suggest that ceramide in yeast works through activation of this phosphatase. Coupled with the inhibitor studies in mammalian cells, these studies would also suggest a role for this phosphatase in mediating the effect of ceramide on growth suppression.

In addition, ceramide has been shown to modulate a number of biochemical events in cells. For example, ceramide has been shown to activate the retinoblastoma gene father, down regulate the c-myc oncogene, inhibit phospholipase-D, modulate protein phosphorylation, activate protein kinases, and inhibit protein kinase Cα (8,11–14).

Major questions in this area of research center on: (1) What are the mechanisms involved in ceramide formation? (2) What are the mechanisms by which ceramide causes growth suppression? and (3) What is the precise role of ceramide in mediating growth suppression in response to TNFα, chemotherapeutic agents, and the other extracellular agents and stimuli that cause ceramide formation? The results presented in this document have aimed at addressing these issues. In particular, we have shown that TNF α induces activation of at least two distinct classes of proteases. The first class is involved in induction of ceramide levels and these are inhibited by CrmA and by YVAD. The second class is activated by ceramide and seems to act downstream to ceramide accumulation. Activation of these proteases is inhibited by Bcl-2 and by DEVD. In studies shown here, we demonstrate that there is a direct and mechanistic relationship between oxidative stress signaling and ceramide accumulation. We find that TNF α causes a drop in glutathione (GSH) levels and that this drop results directly in activation of neutral sphingomyelinase and accumulation of ceramide. These studies define for the first time a fundamental biochemical mechanism for coupling of the cytokine action to oxidative stress to ceramide formation and ceramide-mediated biology. These results should be of significance in defining mechanisms of tumor cell death.

BODY

Experimental Methods:

Tissue culture. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 0.2% sodium bicarbonate. G418 at 500 μg/ml was added to the CrmA cell line and its vector while hygromycin 150 μg/ml was added to the Bcl-2 cell line and its vector. Experiments were done in the absence of G418 or hygromycin. Cell viability was determined by the ability to exclude trypan blue.

Partial Purification of N-SMase -MCF7 cells grown to near confluence in 175 cm^2 flasks were harvested by trypsinization, washed with ice-cold phosphate buffered saline (PBS), pelleted by centrifugation, frozen in a methanol-dry ice bath and stored at -80°C until use. To obtain N-SMase, detergent solublized membrane proteins were prepared from homogenate from pooled cell pellets (2 x 10^9 cells) and resolved on a DEAE-Sepharose column (1 x 10 cm) connected to a Pharmacia FPLC system essentially as described (16), except that both buffers A and B contained Triton X-100 (0.005%, w/v). N-SMase was efficiently resolved from A-SMase in MCF7 cells (17) by the detergent extraction step and DEAE column, such that the final N-SMase preparation contained <1% of A-SMase activity under the assay conditions.

N-SMase Activity Assay--The activity of N-SMase was determined using a mixed micelle assay system as described (16). The reaction mixture contained enzyme preparation in 100 mM Tris-HCl, pH 7.4, 10 nmol [^{14}C]sphingomyelin (100,000 DPM), 0.1% Triton X-100 and 5 mM magnesium chloride in a final volume of 100 μl.

Measurement of Ceramide--Cells grown in 10 cm petri dishes were rinsed with ice-cold PBS and scraped into methanol. Cell lipids were extracted by the method of Bligh and Dyer (15). Ceramide content was determined using a modified diacylglycerol kinase assay as previously described (18).

SM level-- Cells were seeded at 2 X 10^5 cells/10 cm culture dish and grown for 48 hr. Then cells were switched to fresh complete medium containing [^{3}H]choline (1 μCi/ml). After 48 hrs, cells were switched again to fresh medium and chased for 2 hr before treatment with GSH and/or TNFa as described above. The level of SM was determined following a protocol essentially as described (19).

Measurement of GSH Level - Cells were seeded at 2 X 10^5 in 6 cm petri dishes in 4 ml of complete medium. Two days later, cells were treated with the desired agents as described above. Treated cells were detached by trypsinization, washed (3X) with ice-cold PBS and solublized in 150 μl of water. 5-sulphosalicylic acid was added to a final concentration of 2% and the supernatant was separated from the acid precipitated proteins by centrifugation. GSH content in the supernatant was determined by the Griffith (20) modification of the Tietze's enzymatic procedure (22) as described (16). Protein content was determined by the dye binding assay using bovine serum albumin as standard (23).

Western blot for PARP-Cells were scraped into medium, pelleted by centrifugation, and washed (1X) with ice cold PBS containing 1 mM PMSF. The cell pellet was resuspended in 50 μl of PBS-PMSF and solublized in 2X SDS sample buffer. Western blot for PARP was performed as described (18).

Cell viability-The viability of cells was determined by their ability to exclude trypan blue. The survival of cells was determined with the WST-1 cell proliferation reagent from Boehringer Mannheim. Cells were seeded at 10^3 cells/well/200 μl of complete medium in 96-well culture

plate. Two days later, cells were treated in quadruplicate with TNFa as described above. At the end of treatment, WST-1 reagent was added and after a 3 hr incubation period, the absorbance was measured at 450 nm with a multi-well plate reader as recommended by the manufacturer.

RESULTS:

GSH Reversibly Inhibits Neutral SMase in vitro–Previously we found that GSH inhibited *in vitro* the N-SMase from Molt-4 leukemic cells (16). To study the role of GSH in TNFa signaling, we chose the human mammary carcinoma cell line MCF7 which is very sensitive to TNFa (17). We partially purified N-SMase from MCF7 cells following the procedure described for rat brain (16) and tested the effects of GSH on N-SMase *in vitro*. When, the enzyme was preincubated for 5 min at 37°C with 1-20 mM GSH followed by incubation with substrate for 30 min, a dose-dependent inhibition of N-SMase by GSH was observed with a greater than 95% inhibition observed with 3 mM of GSH (Fig. 1A). Preliminary experiments established that with the minimum preincubation time examined (1 min), GSH (3 mM) inhibited the enzyme activity by >80% (data not shown). The inhibition was specific for GSH since two other small thiol-containing molecules, dithiothreitol (DTT) and b-mercaptoethanol, at concentrations up to 20 mM, were ineffective (Fig 1A), and co-incubation of GSH with 5 or 20 mM DTT did not alter the inhibitory profile for GSH (Fig. 1B). When the N-SMase:GSH (4 mM) mixture was diluted by 3 or 5 fold, enzyme activity was recovered by 70% and 95% respectively, suggesting that the inhibition was reversible (Fig. 1C). GSH did not inhibit the acidic SMase (16) although both the N-SMase and acidic SMase have been suggested to be activated in cells treated with TNFa. These results demonstrate that physiologic levels of GSH (3-10 mM) totally inhibit N-SMase. The results also suggest that the sharp drop in cellular levels of GSH may relieve this inhibition and cause activation of N-SMase.

GSH Reversibly Inhibits Neutral SMase in cells–To investigate the effect of GSH on N-SMase in TNFa signaling, MCF7 cells were treated with TNFa (3 nM) for 2-24 hr and GSH levels were measured. As shown in Figure 2A, TNFa treatment resulted in an initial sharp drop in the level of GSH followed by a steady further decrease, with the first significant decrease observed at 8 hr post treatment. The most dramatic change in the level of GSH occurred between 8 and 10 hr post TNFa treatment where the GSH level plunged to nearly a third of that of control cells at the 10 hr time point. GSH levels then steadily decreased to 7% of control cells by 24 hr (Fig. 2A). At the 10 hr time point, cells treated with TNFa did not manifest any detectable sign of apoptosis. In particular, proteolysis of the "cell death substrate" poly(ADP-ribose)polymerase (PARP) was only detected at 12-24 hr (17). The TNFa-induced decrease in cellular GSH level was accompanied by an increase in the level of ceramide, with an initial significant rise observed at the 8-12 hr time points. Ceramide levels at 12 hr post TNFa treatment were twice control levels and reached more than 6 fold the control levels at 24 hr (Fig. 2B). When the effect of TNFa on cellular SM level was examined, significant SM hydrolysis was observed between 10 and 16 hr, and a 30% hydrolysis of SM was detected at 14 hr (Fig. 2C). These kinetics raise the possibility that GSH may be involved in the regulation of SM hydrolysis and ceramide generation in cells treated with TNFa.

Since GSH inhibited N-SMase *in vitro* at physiologically relevant concentrations and changes of GSH levels induced by TNFa proceeded ceramide accumulation and SM hydrolysis, we next investigated the effects of manipulating intracellular GSH levels on TNFa-induced SM hydrolysis and ceramide generation. First, replenishment of intracellular GSH by addition of 10 mM GSH to the culture medium prior to TNFa treatment (3 nM; 14 hr) completely prevented the TNFa-induced SM hydrolysis (Fig. 3A). Treatment of cells with GSH (10 mM) alone had no effect on SM levels. Second, GSH pretreatment significantly inhibited the accumulation of ceramide induced by TNFa (Fig. 3B). A nearly complete inhibition of ceramide increase was

observed at 12 hr post TNFa treatment (Fig. 3B). At 16 and 24 hr, ceramide accumulation induced by TNFa was inhibited by about 50%. Third, in addition to GSH, significant inhibition of TNFa-induced ceramide accumulation was also achieved by treatment of cells with GSH methylester and N-acetylcysteine (NAC) which is converted intracellularly to cysteine, a precursor of GSH biosynthesis (Fig. 3C). These results further support the notion that GSH levels regulate the activity of N-SMase in these cells.

Involvement of GSH in TNF-induced cell death- Next, the biological consequences of changes in GSH levels in response to TNFa were studied by examination of cell death and survival. Whereas TNFa induced significant cell death, as determined by the ability of cells to exclude trypan blue dye, partial rescue of cells was achieved by replenishment of intracellular GSH with GSH added to the cell culture medium. Preincubation of cells with 10 and 15 mM GSH for 2 hr prior to treatment with TNFa lowered TNFa-induced cell death from 58.5% to 35.5% and 26.5% respectively (Fig. 4A). The effect of TNFa on the viability of MCF7 cells was also determined using the WST-1 assay which measures the activity of the mitochondrial respiratory chain in viable cells. TNFa significantly reduced the survival of MCF7 cells, and a 50% reduction in survival was observed when cells were treated with 1 nM TNFa for 24 hr (Fig. 4B). Inclusion of 10 mM GSH enhanced the survival of TNFa-treated cells to 75-95% of control level (Fig. 4B).

Mechanistically, the effects of TNFa on PARP cleavage, a close marker of the apoptotic response, were evaluated. TNFa-induced cleavage of PARP was partially inhibited by GSH and NAC in a dose-dependent manner (Fig. 4C). Since PARP is a substrate for "execution" phase proteases such as CPP32/caspase-3, these results place GSH upstream of these proteases in the pathways leading to cell death.

The hypothesis that GSH levels regulate the activity of N-SMase in response to TNFa suggests that the product ceramide should function downstream of GSH and that it may not alter the cellular level of GSH. Bacterial SMases are known to induce a fast and significant elevation of intracellular ceramide by hydrolysis of membrane sphingomyelin. However, when MCF7 cells were treated with 300 mU/ml of bacterial SMase from Staphylococcus aureus or Bacillus cereus for up to 24 hr, no significant alteration in GSH levels was observed (Fig. 5A). Similarly, treatment of cells with 2.5-10 μM of the cell-permeable short chain C6- ceramide for up to 48 hr did not induce significant reduction in GSH level (Fig. 5B), although exogenous ceramide at these concentrations caused apoptosis in these cells (Fig, 5C). Conversely, pretreatment of cells with 10 mM GSH prior to ceramide treatment did not protect cells from death induced by ceramide (Fig. 5C), in sharp contrast to the ability of GSH to inhibit the hydrolysis of SM and ceramide accumulation induced by TNFa. These results place GSH upstream of N-SMase activation in the TNFa signaling pathway.

Relationship of GSH levels to caspase activation and to Bcl-2 - TNFa-induced accumulation of ceramide has been shown to be inhibited by the cow pox virus cytokine modifier protein (crmA) but not by bcl-2 (17), placing activation of SMases downstream of crmA-inhibitable proteases and upstream of bcl-2 inhibitable proteases. To investigate the relationship between crmA and GSH, MCF7 transfected with crmA or empty vector were treated with TNFa, and GSH levels were determined. No significant changes in GSH levels were observed in crmA-transfected cells treated with 3 nM TNFa over a time period ranging from 1 to 24 hr whereas a time dependent depletion of GSH was observed in the vector-transfected cells (Fig. 6A). The TNFa-induced depletion of GSH was also inhibited in a dose dependent manner by the substrate-based tetrapeptide inhibitor of ICE proteases, YVAD-chloromethylketone. Pretreatment of MCF7 cells with 50 μM YVAD prior to TNFa (3 nM) brought the GSH level from 20% of control to 80% of control (Fig. 6B). These results clearly demonstrate that the drop in GSH in response to TNFa is dependent upon activation of ICE-like proteases (such as caspases-8/FLICE).

Next, the effects of TNFa on the GSH levels in bcl-2-transfected MCF7 cells were examined following treatment with 1-10 nM TNFa for 14 hr. In both the bcl-2- and vector-transfected cells, TNFa, at a dose as low as 1 nM, caused a drop in GSH levels to 45% of that of untreated control cells (Fig. 7). Concentrations of TNFa greater than 1 nM and up to 20 nM further decreased GSH level to 20-30% of the control value. These results show that the drop in GSH levels is not downstream of the site of action of bcl-2.

Finally the interrelation of GSH, bcl-2, crmA and TNFa-induced hydrolysis of SM was investigated. When SM levels were analyzed in crmA and bcl-2 transfected cells, bcl-2 had no effect on TNFa-induced SM hydrolysis, and this SM hydrolysis in bcl-2 cells was inhibited by pretreatment with GSH (Fig. 8). Cells with crmA, however, were completely resistant to TNFa-induced SM hydrolysis (Fig. 8). Thus, similar to the drop in GSH, activation of N-SMase is downstream of the site of action of crmA and most probably upstream of the site of action of bcl-2.

Relationship to Goals.

Task 1: To determine the mechanism by which TNF and chemotherapeutic agents cause activation of the sphingolipid pathway and cause accumulation of ceramide in breast cancer cells.

We have made substantial progress in defining the mechanisms of accumulation of ceramide, especially in response to TNF α in breast cancer MCF-7 cells. Specifically, we found previously that TNF α causes activation of neutral sphingomyelinase and consequent accumulation of ceramide. This is a delayed response that requires activation of ICE-like proteases which are inhibitable by CrmA and by YVAD. In the more recent studies described here, we have made a fundamental observation on a direct mechanism coupling TNF action to activation of neutral sphingomyelinase. From *in vitro* studies, we find that GSH results in inhibition of neutral sphingomyelinase. A drop in GSH levels would therefore result in activation of neutral sphingomyelinase in vitro and in cells. This was evaluated with TNF α which was shown in this study to cause a drop in cellular levels of GSH that are mechanistically related to activation of neutral sphingomyelinase.

Task 2: Define the role of ceramide in the regulation of breast cancer cells. In our studies we have shown that ceramide causes activation of proteases of the caspase-3 sub-family of death proteases. This activation is inhibited by bcl-2 and by tetrapeptide inhibitors such as DEVD. Taken together, these studies show that the ability of ceramide to induce apoptosis in MCF-7 cells occurs upstream of the site of action of bcl-2 and involves activation of cell death proteases. These conclusions are further supported by results from this study. The action of GSH on neutral SMase support a role for ceramide, formed through this pathway, in the regulation of proteases and cell death.

In ongoing and preliminary studies (not shown in this report), we find that an inhibitor of ceramidase which is an enzyme that clears the levels of ceramide, causes accumulation of ceramide levels in breast carcinoma cells. This inhibitor also results in death of these cells. This will be an area that will be developed significantly in next year's report.

Task 3 Determine the mechanism of action of ceramide and of agents that induce ceramide formation. These issues have been addressed in the first two years of study with definition of the effects of ceramide on apoptosis and on cell cycle arrest. The details of the cell cycle arrest and the involvement of Rb in this pathway will be further developed in next year's report. Ongoing studies are also directed at examining the role of phosphatases as direct targets for ceramide and in mediating at least some of the effects of ceramide on PKC, caspases and cell death.

CONCLUSIONS

Studies in the first year of the proposal demonstrated that operation of the sphingomyelinase-ceramide pathway in MCF-7 cells which is activated by temoxiphen and TNF α. Since the effects of TNF α were early and more robust, we concentrated primarily on this agent to activate the apoptotic pathways in MCF-7 cells. We also documented the ability of TNF α and ceramide to cause apoptosis in this cell line, and studies were initiated evaluating the effects of ceramide and TNF α on cell cycle arrest in this cell line.

During the second year of the proposal, we made major connections involving TNF α, ceramide, and major known regulators of apoptotic responses. In particular, we showed that TNF α causes the activation of at least two distinct classes of proteases. The first class of proteases which are inhibited by CrmA and by YVAD, and therefore belong to the ICE-like proteases, is activated in response to TNF α and is required for activation of neutral sphingomyelinase and accumulation of ceramide. The second class of proteases which is inhibited by BEVD is probably comprised of caspase-3-type cell death proteases. These proteases are activated by ceramide and by TNF α. The action of TNF α and of ceramide on these proteases is inhibited by DEVD and is inhibited by Bcl-2. Taken together, these studies suggested a sequence of events whereby TNF α causes activation of proximal proteases that result in accumulation of ceramide which then acts through a Bcl-2 inhibitable mechanism to activate downstream cell death proteases.

In this third year of research, we have taken the studies to a more biochemical level and applied those results to a cellular biochemical level. Specifically, we found that GSH plays a critical role in coupling the action of TNF α with activation of the sphingomyelinase-ceramide pathway. These results show that TNF α causes a drop in the cellular levels of glutathione. Because our in vitro studies show that glutathione inhibits neutral sphingomyelinase and physiologically relevant concentrations of GSH, these studies suggested to us that this drop in cellular levels of GSH may relieve inhibition of neutral sphingomyelinase and cause activation of this enzyme and subsequent accumulation of ceramide. This was further substantiated by examining the effects of replenishing glutathione following TNF action. This results in inhibition of activation of sphingomyelinase and a drop in ceramide levels.

Taken together, these studies are providing important mechanistic insight both at an *in vitro* biochemical level and at a cellular level into how the apoptotic pathway operates in breast cancer cells, especially in response to TNF α as a primary activator of cell death in these cells. Our studies show for the first time a direct correlation and mechanistic interaction between oxidative stress (which results in a drop in GSH levels) and the ceramide pathway of apoptosis regulation.

Future studies will aim at further understanding of this apoptotic pathway. In addition, those studies will aim at developing our lead studies with the inhibitor of ceramidase as a possible tool to induce ceramide levels and therefore to activate the apoptotic pathway in breast carcinoma cells.

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APPENDIX

FIGURE LEGENDS

Fig. 1. GSH inhibits N-SMase from MCF7 cells. A, GSH, but not dithiothreitol (DTT) or b-mercaptoethanol(b-ME), inhibits N-SMase. N-SMase, partially purified from membranes of MCF7 cells, was preincubated for 5 min at 37°C in 20 mM Tris-HCl, pH 7.5 with the indicated concentrations of GSH, dithiothreitol (DTT) or b-mercaptoethanol (b-ME) followed by the addition of [^{14}C]-sphingomyelin (100,000 DPM, 5 nmol) in 100 mM Tris-HCl, pH 7.5 containing 0.1% Triton X-100 and 5 mM $MgCl_2$ (total volume: 100 µl). The reaction proceeded for 30 min at 37°C and was stopped by the addition of 1.5 ml chloroform:methanol (2/1, v/v) and 0.2 ml of water. After phase separation, a portion of the upper phase containing the product [^{14}C]choline phosphate was counted for radioactivity by liquid scintillation. B, co-incubation with DTT does not affect inhibition of N-SMase by GSH. N-SMase was preincubated for 5 min at 37°C with the indicated concentrations of GSH and 0, 5 or 20 mM DTT. C, reversibility of inhibition of N-SMase by GSH. N-SMase was preincubated with 4 mM GSH for 5 min, diluted by three (1:3) or five (1:5) fold with 20 mM Tris-HCl, pH 7.5, and then assayed for activity. Results were corrected based on equal amounts of enzyme preparations used for each condition. Results shown are averages of duplicate determination and are representative of three separate experiments.

Fig. 2. Effect of TNFa on cellular content of GSH, ceramide and SM. A, GSH levels. MCF7 cells were treated with 3 nM TNFa for the indicated time intervals. Total cellular GSH was measured with an enzymatic kinetic assay as described under "Experimental Procedures", normalized against total cellular protein, and expressed as a percentage of the time-matched controls. Results are expressed as percent of time-matched controls and are means ± S. D. of duplicate determinations of three separate experiments. B, ceramide levels. Lipids were extracted from cells left untreated or treated with 3 nM TNFa for the indicated time intervals and ceramide content was determined using the bacterial diacylglycerol kinase assay as described under "Experimental Procedures". C, SM levels. MCF7 cells (50% confluent) were pre-labeled with [^{3}H]choline chloride (0.5 µCi/ml, 80 Ci/mmol, American Radiolabeled Chemicals) for 48 hr. Cells were washed PBS, rested in fresh complete medium for 2 hr and then treated with 3 nM TNFa for the indicated time intervals. Total cellular lipids were harvested, SM content was determined as described under "Experimental Procedures", and normalized against total cellular protein. Results for B and C are expressed as percent of time-matched controls and are means ± S. D. of duplicate determinations of four separate experiments.

Fig. 3. GSH inhibits SM hydrolysis and ceramide accumulation induced by TNFa. A, GSH inhibits TNFa-induced SM hydrolysis. MCF7 cells were pre-labeled with [3H]choline chloride for 48 hr, washed with PBS, and rested for 2 hr in fresh complete medium. Cells were then pretreated for 2 hr with GSH (10 mM final concentration) before treatment with 3 nM TNFa for 14 hr. SM content was analyzed as described under "Experimental Procedures". Results are expressed as percent of time-matched controls and are means ± S. D. of duplicate determinations of four separate experiments. *$p<0.005$ compared to TNFa-treated cells. B, GSH inhibits TNFa-induced ceramide accumulation. MCF7 cells were pretreated with 10 mM GSH for 2 hr followed by 3 nM TNFa for the indicated time intervals. Cellular lipids were extracted and ceramide levels were determined. Results are expressed as percent of time-matched control. *$p<0.005$ compared to TNFa-treated cells (open circle). C, TNFa-induced ceramide accumulation is inhibited by GSH methylester (GSH-ME) and N-acetylcysteine (NAC). MCF7 cells were pretreated with 10 mM GSH-ME or NAC for 2 hr followed by 3 nM TNFa for 16, or 24 hr. For B and C, ceramide content was determined as described under "Experimental Procedures" and results are expressed as percent of time-matched controls and are means ± S. D. of duplicate determinations of three separate experiments.

Fig. 4. Elevation of intracellular GSH levels prevents apoptosis induced by TNFa. A, protection of TNFa-induced death by GSH. MCF7 cells were pretreated for 2 hr with 5-15 mM GSH followed by treatment with 3 nM TNFa for 24 hr. Cell death was measured by trypan blue exclusion. Results are means ± SD of triplicate determinations from three separate experiments. B, GSH inhibits TNFa-induced decrease in cell survival. MCF7 cells cultured in 96-well plate were pretreated for 2 hr with 10 mM GSH followed by treatment with 1-5 nM TNFa for 24 hr. Afterwards, cells were incubated with the WST-1 reagent for 3 hr, and cell survival was analyzed following the manufacturer's instructions. Results are expressed as percent of control and are means ± SD of quadruplicate determinations from three separate experiments. C, GSH and NAC inhibit TNFa-induced PARP proteolysis. MCF7 cells were pretreated for 2 hr with 10-20 mM GSH followed by treatment with 3 nM TNFa for 18 hr. Cells were scraped into and lysed in SDS sample buffer. The proteins were analyzed with a 6% SDS-PAGE gel and PARP cleavage was analyzed by Western blot as described under "Experimental Procedures". Blot shown is representative of three separate experiments.

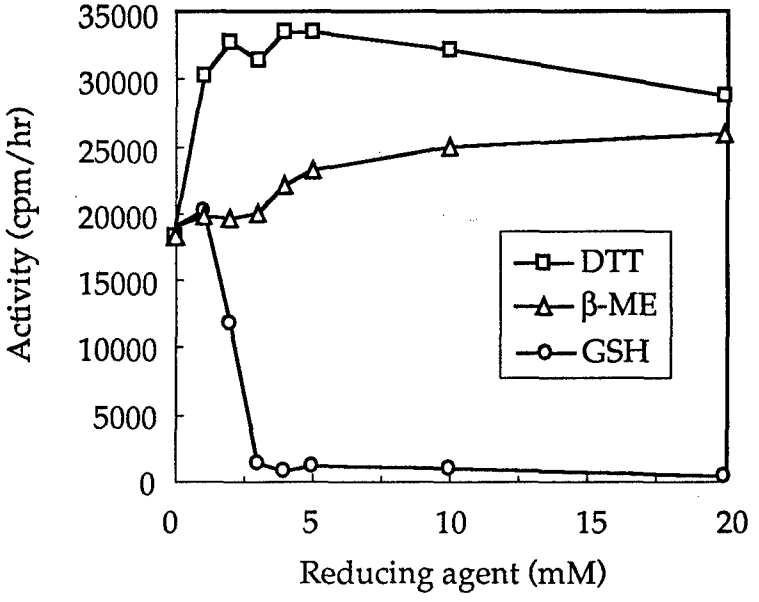
Fig. 5. Ceramide does not deplete cellular GSH and GSH does not protect from death induced by ceramide. A, bacterial SMase does not affect GSH level. MCF7 cells were treated with 300 mU/ml of bacterial SMase from Staphylococcus aureus (S. aureus) or Bacillus cereus (B. cereus) for the indicated time intervals. B, synthetic ceramide does not affect GSH level. MCF7 cells were treated with 2.5-10 μM C6 ceramide for up to 48 hr in RPMI 1640 containing 2% FBS. GSH levels were measured as described under "Experimental Procedures". The amount of ethanol used in B as vehicle for ceramide delivery (0.05% final concentration) did not induce any significant changes in GSH levels when compared to untreated cells. Results in A and B are expressed as percent of control and are mean ± S. D. of duplicate determination of three separate experiments. C, GSH does not protect cells from synthetic ceramide-induced death. MCF7 cells were pretreated for 2 hr with 10 mM GSH followed by treatment with 1-10 μM C6 ceramide for 24 or 48 hr in RPMI 1640 containing 2% FBS. Cell death was measured by trypan blue exclusion. Results are means ± S. D. of duplicate determination from three experiments.

Fig. 6. CrmA blocks GSH depletion induced by TNFa. A MCF7 cells overexpessing crmA are resistant to TNFa-induced depletion of GSH. CrmA-, or vector-transfected MCF7 cells were treated with 3 nM TNFa for 2-24 hr. B, YVAD inhibits the TNFa-induced depletion of GSH. MCF7 cells were pretreated for 2 hr with 12.5-50 μM YVAD followed by treatment with 3 nM TNFa for 12 hr. GSH levels were determined as described under "Experimental Procedures", expressed as percent of time-matched controls, and are means ± S. D. of duplicate determinations of three experiments.

Fig. 7. Bcl-2 has no effect on GSH depletion induced by TNFa. B, Time course for the effect of TNFa on GSH level in crmA-transfected MCF7 cells. bcl-2-, crmA-, or corresponding vector-transfected MCF7 cells were treated with 1-20 nM TNFa for 12 hr, and GSH levels were measured. Results are expressed as percent of control and are means ± S. D. of duplicate determinations of three experiments.

Fig. 8. CrmA, but not bcl-2, inhibits the TNFa-induced SM hydrolysis. MCF7 cells transfected with bcl-2, crmA or its vector, were pre-labeled with [3H]choline chloride for 48 hr, washed with PBS, and rested for 2 hr in complete medium. Cells were then treated with 10 mM GSH, followed by 3 nM TNFa for 14 hr. Cell lipids were harvested and SM content was determined as described under "Experimental Procedures". Results are expressed as percent of control, and are means ± S. D. of duplicate determinations of three experiments..

A


35000
30000
25000
20000
15000
10000
5000
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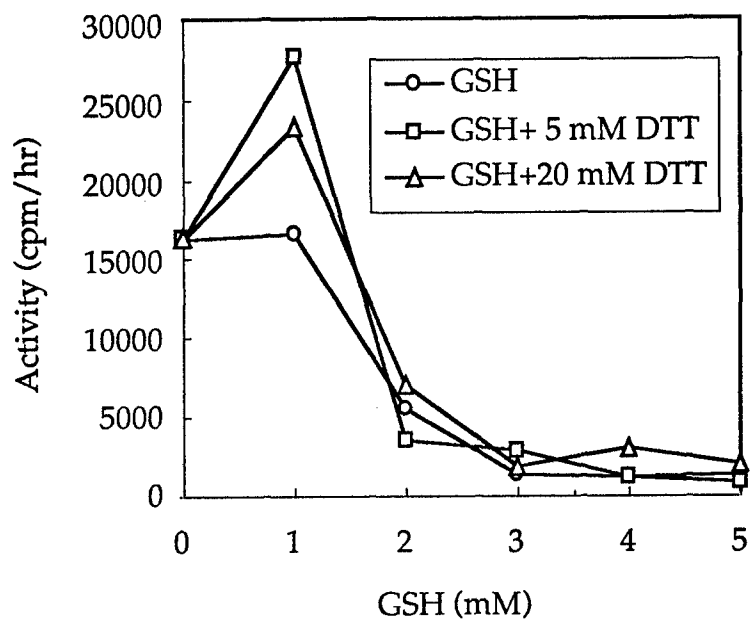
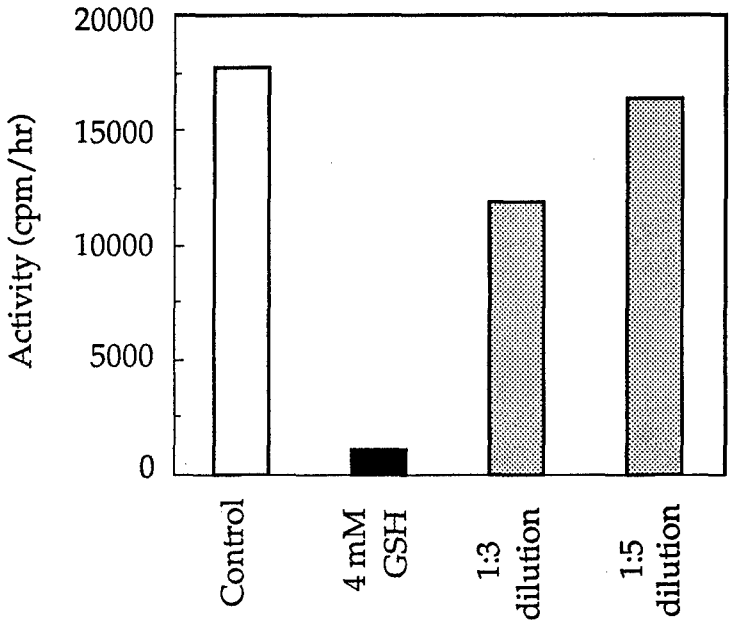
Activity (cpm/hr)
0
5
10
15
20
Reducing agent (mM)
DTT
β-ME
GSH

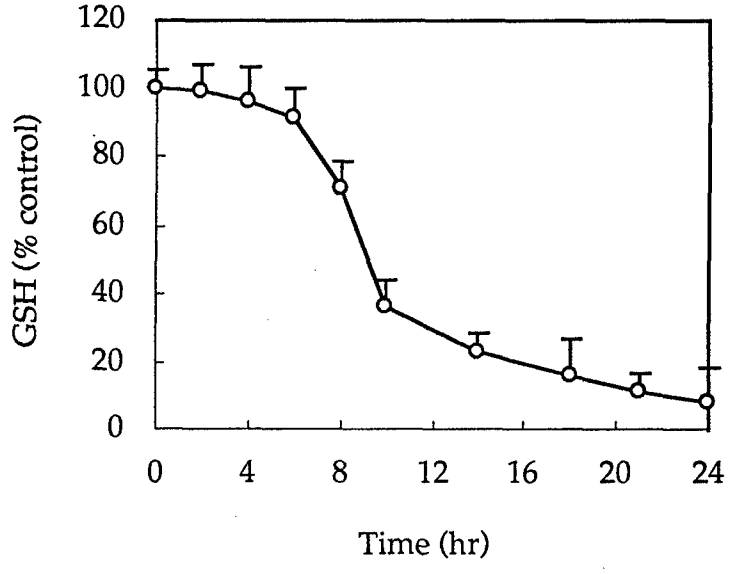
B


30000
25000
20000
15000
10000
5000
0
Activity (cpm/hr)
0
1
2
3
4
5
GSH (mM)
GSH
GSH+ 5 mM DTT
GSH+20 mM DTT

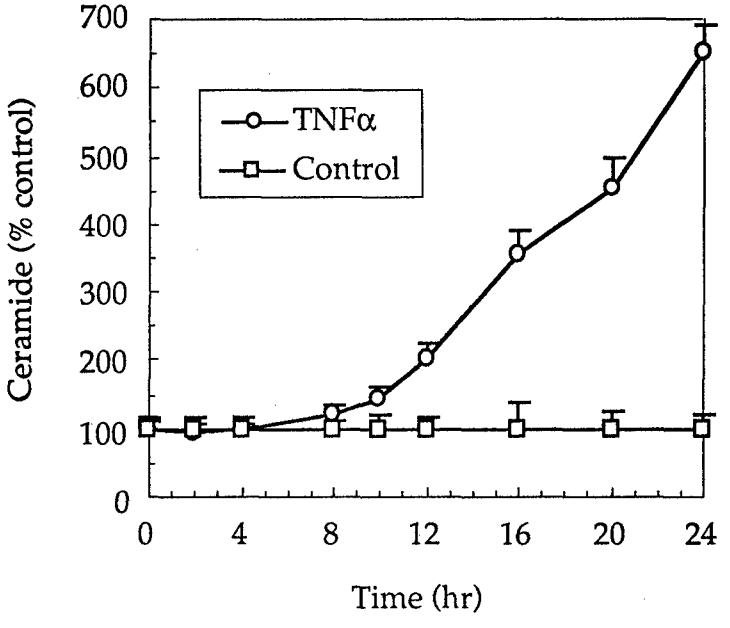
C


20000
15000
10000
5000
0
Activity (cpm/hr)
Control
4 mM GSH
1:3 dilution
1:5 dilution

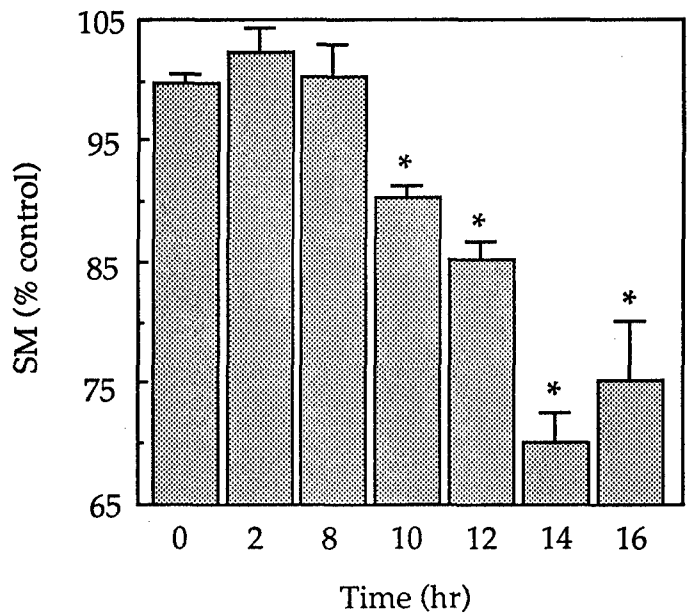
A

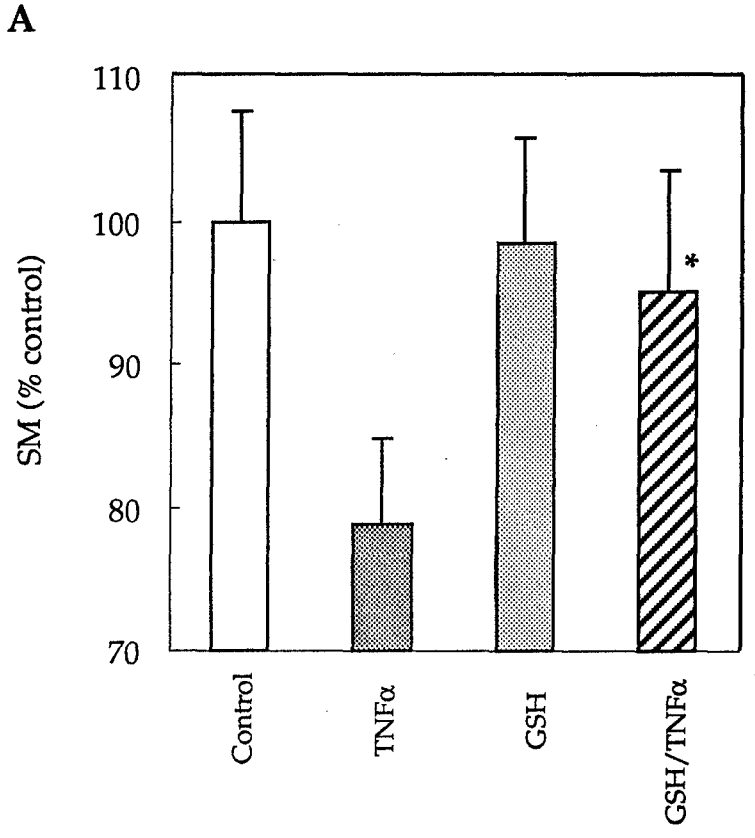


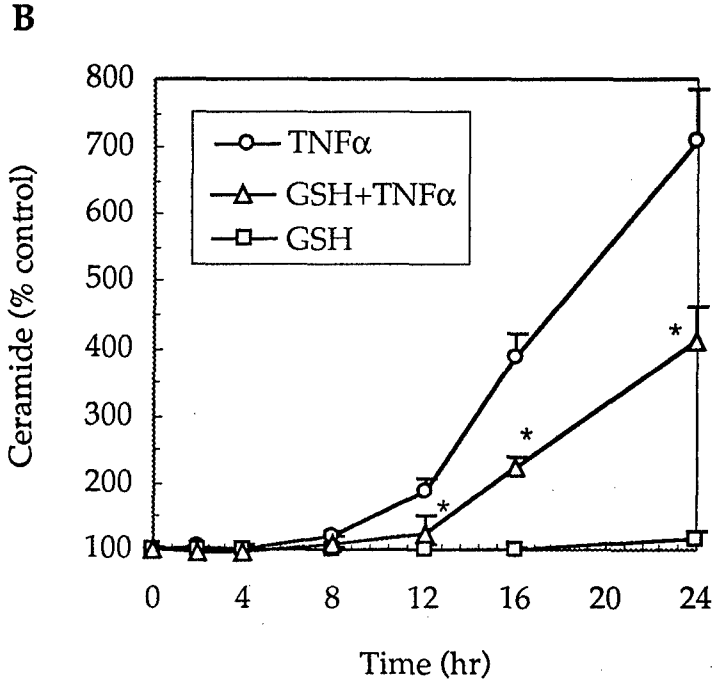
B

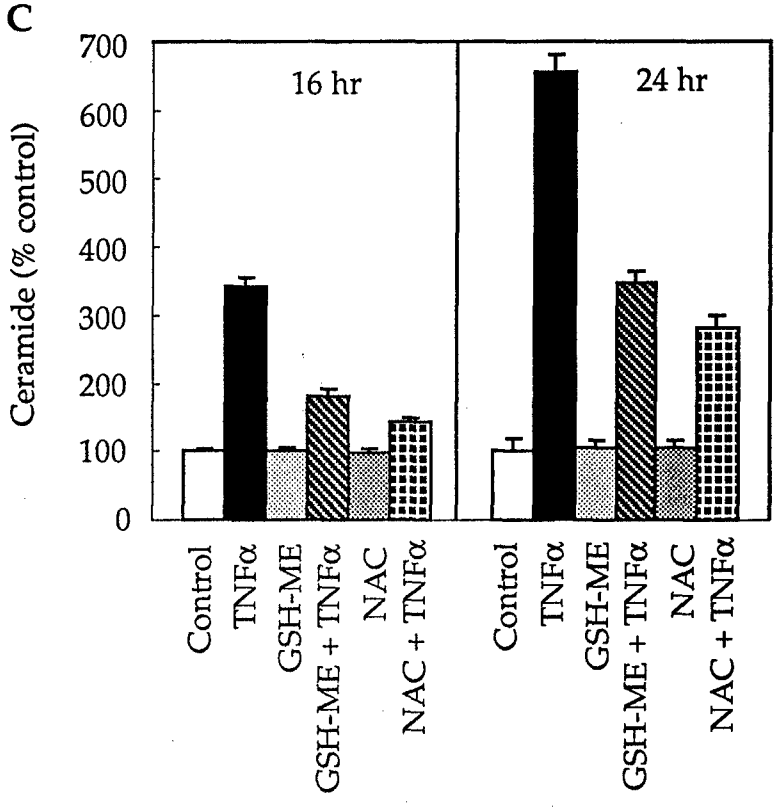

700
600
500
400
300
200
100
0
Ceramide (% control)
TNFα
Control
0
4
8
12
16
20
24
Time (hr)

C

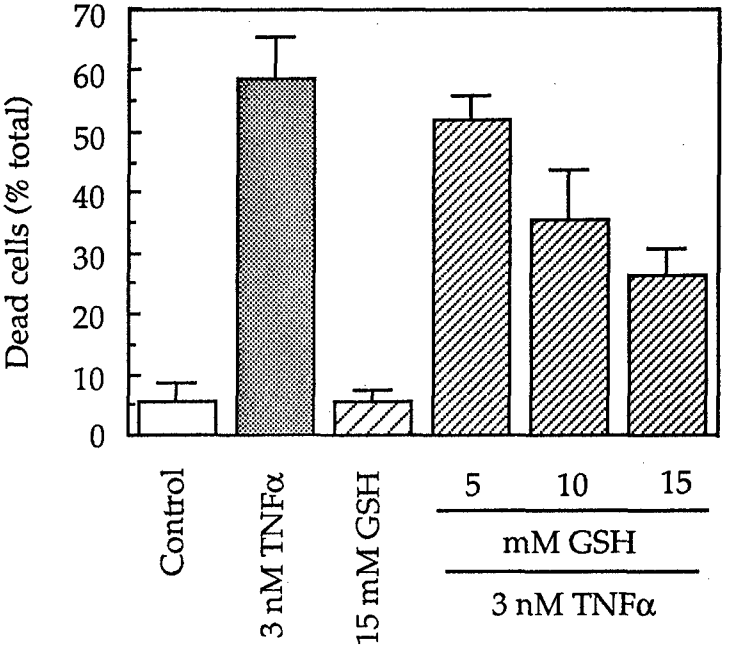



A
110
100
90
80
70
SM (% control)
*
Control
TNFα
GSH
GSH/TNFα


B
800
700
600
500
400
300
200
100
Ceramide (% control)
TNFα
GSH+TNFα
GSH
*
*
*
0
4
8
12
16
20
24
Time (hr)


C
Ceramide (% control)
700
600
500
400
300
200
100
0
16 hr
24 hr
Control
TNFα
GSH-ME
GSH-ME + TNFα
NAC
NAC + TNFα
Control
TNFα
GSH-ME
GSH-ME + TNFα
NAC
NAC + TNFα

A


Dead cells (% total)
70
60
50
40
30
20
10
0
Control
3 nM TNFα
15 mM GSH
5
10
15
mM GSH
3 nM TNFα

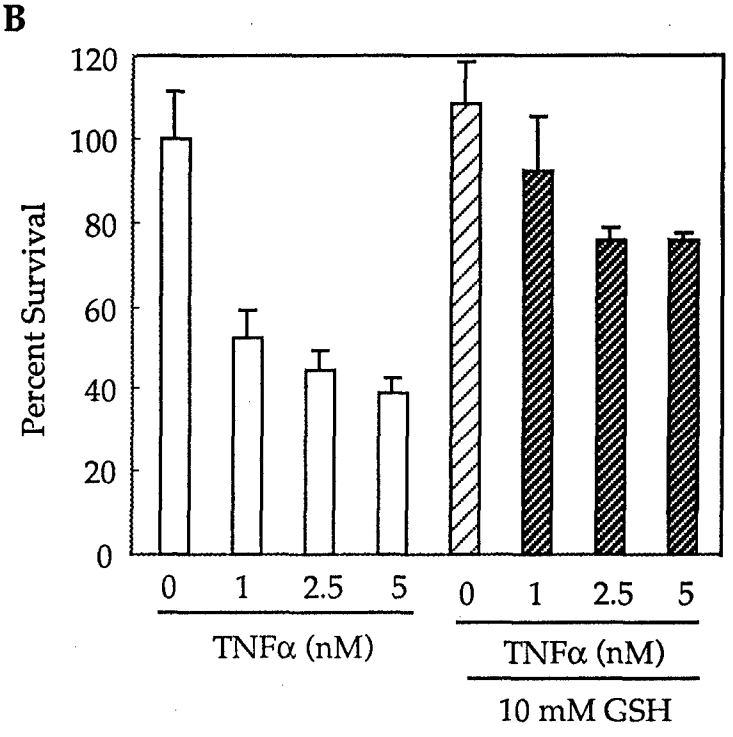
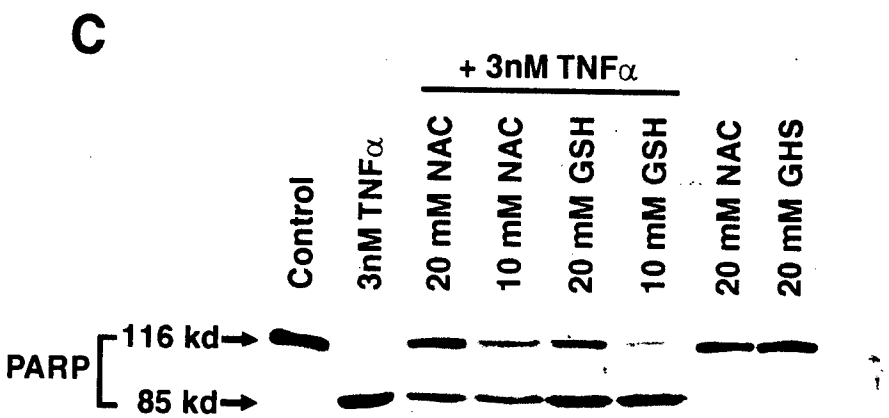
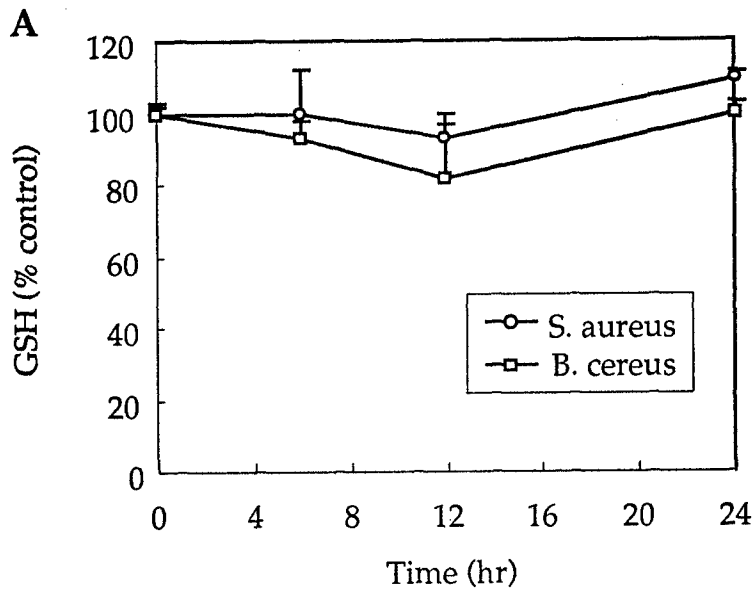
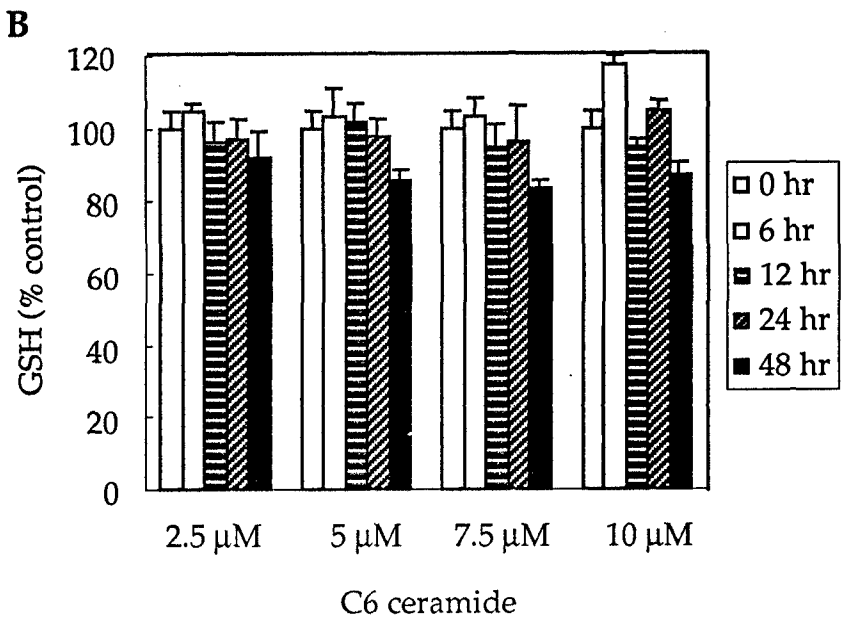

B
Percent Survival
120
100
80
60
40
20
0
0 1 2.5 5
TNFα (nM)
0 1 2.5 5
TNFα (nM)
10 mM GSH

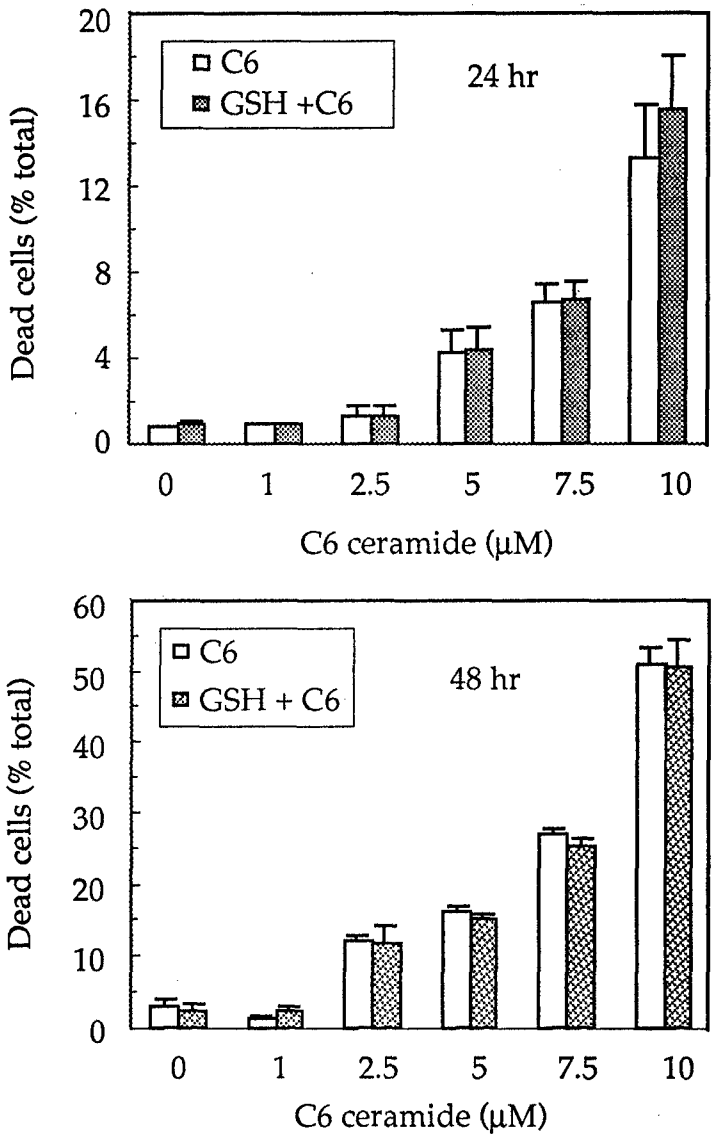
Figure 4C


C
+ 3nM TNFα
Control
3nM TNFα
20 mM NAC
10 mM NAC
20 mM GSH
10 mM GSH
20 mM NAC
20 mM GHS
PARP
116 kd
85 kd

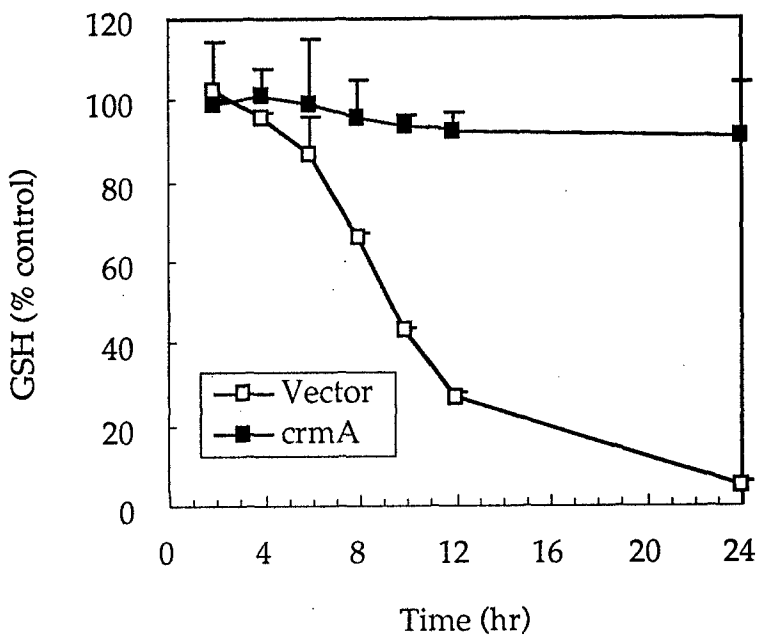

A
120
100
80
60
40
20
0
GSH (% control)
0
4
8
12
16
20
24
Time (hr)
S. aureus
B. cereus

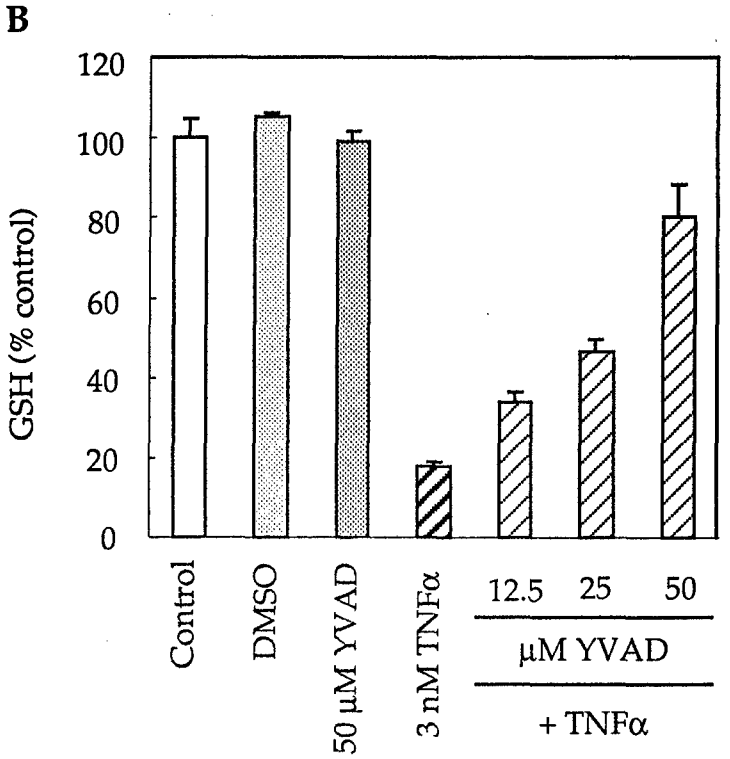

B
120
100
80
60
40
20
0
GSH (% control)
0 hr
6 hr
12 hr
24 hr
48 hr
2.5 μM
5 μM
7.5 μM
10 μM
C6 ceramide

C

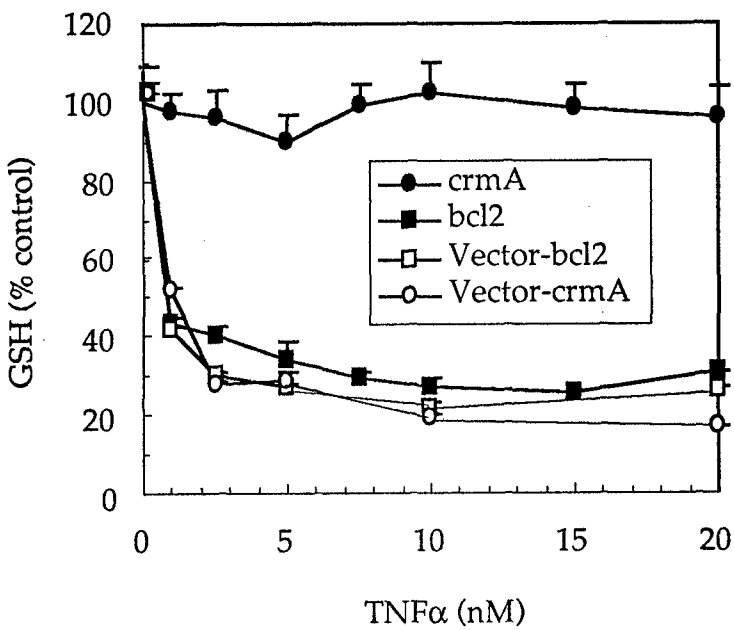

20
16
12
8
4
0
Dead cells (% total)
C6
GSH +C6
24 hr
0
1
2.5
5
7.5
10
C6 ceramide (μM)
60
50
40
30
20
10
0
Dead cells (% total)
C6
GSH + C6
48 hr
0
1
2.5
5
7.5
10
C6 ceramide (μM)

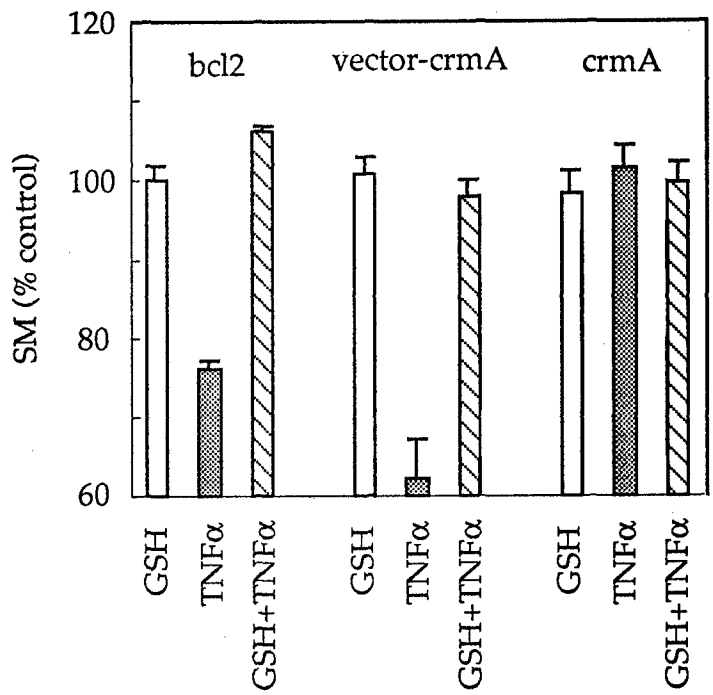
A


120
100
80
60
40
20
0
GSH (% control)
Vector
crmA
0
4
8
12
16
20
24
Time (hr)


B
GSH (% control)
120
100
80
60
40
20
0
Control
DMSO
50 μM YVAD
3 nM TNFα
12.5
25
50
μM YVAD
+ TNFα

Liu et al Figure 7




120
100
80
60
SM (% control)
bcl2
vector-crmA
crmA
GSH
TNFα
GSH+TNFα
GSH
TNFα
GSH+TNFα
GSH
TNFα
GSH+TNFα

Recd 2/25/2000

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 17 Feb 00

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SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical report written for Grant DAMD17-94-J-4266 and DAMD17-94-J-4301. Request the limited distribution statement for Accession Document Numbers ADB222576, ADB221779, and ADB235519, be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Virginia Miller at DSN 343-7327 or by email at virginia.miller@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management