TNFα-induced glutathione depletion lies downstream of $cPLA_2$ in L929 cells

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Abstract Both glutathione (GSH) depletion and arachidonic acid (AA) generation have been shown to regulate sphingomyelin (SM) hydrolysis and are known components in tumor necrosis factor α (TNFα)-induced cell death. In addition, both have hypothesized direct roles in activation of *N*-sphingomyelinase (SMase); however, it is not known whether these are independent pathways of *N*-SMase regulation or linked components of a single ordered pathway. This study was aimed at differentiating these possibilities using L929 cells. Depletion of GSH with L-buthionin-(*S,R*)-sulfoximine (BSO) induced 50% hydrolysis of SM at 12 h. In addition, TNF induced a depletion of GSH, and exogenous addition of GSH blocked TNF-induced SM hydrolysis as well as TNF-induced cell death. Together, these results establish GSH upstream of SM hydrolysis and ceramide generation in L929 cells. We next analyzed the L929 variant, C12, which lacks both cytosolic phospholipase A_2 ($cPLA_2$) mRNA and protein, in order to determine the relationship of $cPLA_2$ and GSH. TNF did not induce a significant drop in GSH levels in the C12 line. On the other hand, AA alone was capable of inducing a 60% depletion of GSH in C12 cells, suggesting that these cells remain responsive to AA distal to the site of $cPLA_2$. Furthermore, depleting GSH with BSO failed to effect AA release, but caused a drop in SM levels, showing that the defect in these cells was upstream of the GSH drop and SMase activation. When $cPLA_2$ was restored to the C12 line by expression of the cDNA, the resulting CPL4 cells regained sensitivity to TNF. Treatment of the CPL4 cells with TNF resulted in GSH levels dropping to levels near those of the wild-type L929 cells. These results demonstrate that GSH depletion following TNF treatment in L929 cells is dependent on intact $cPLA_2$ activity, and suggest a pathway in which activation of $cPLA_2$ is required for the oxidation and reduction of GSH levels followed by activation of SMases. © 2000 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Arachidonic acid; Apoptosis; Ceramide; Glutathione; Sphingomyelin

1. Introduction

The significance of apoptosis in development, wound healing, cancer pathogenesis, and cancer treatment has resulted in its recognition as one of the most regulated programs in the cell. Inducers of apoptosis include chemotherapeutic agents, radiation, Fas ligand, and tumor necrosis factor α (TNFα) [1]. Despite this multitude of inducers, there are common themes in apoptotic signaling including activation of caspases, generation of reactive oxygen species, and accumulation of ceramide. Regulation of ceramide production and metabolism in apoptosis is not well understood, partially due to the multiple metabolic pathways that converge on this lipid mediator [2,3]. Current work suggests that both activation of sphingomyelinases (SMases) as well as de novo ceramide synthesis may be involved [2–6]. Several direct and indirect mechanisms have been suggested for SMase activation including arachidonic acid (AA) generation and glutathione (GSH) depletion [7–10]. Activation of cytosolic phospholipase A_2 ($cPLA_2$) by TNF has been reported by a variety of laboratories, and has been suggested as a key factor in allowing TNF to induce death [11–13]. TNF has also been demonstrated to induce oxidant stress [14,15] and to cause a drop in GSH levels which precedes and regulates its cytotoxic effects [7,16,17].

We previously established that in TNFα-treated L929 cells, $cPLA_2$ and AA generation acted upstream of SMase activation [10]. These results were supported using a variant L929 cell line (C12) which lacks $cPLA_2$ and is resistant to TNF-induced death [18,19]. However, how AA then activates SMase was not understood. Both direct and indirect mechanisms were possible. Early work revealed that exogenous addition of AA stimulated sphingomyelin (SM) hydrolysis and ceramide generation. Methyl ester and an alcohol analog failed to have similar effects, suggesting specificity. However, as a variety of fatty acids were shown to have similar effects on neutral SMase. It was hypothesized that the AA effect did not require further AA metabolism to eicosanoids as such metabolism is not possible for the other fatty acids. In addition, AA activated SMase in crude homogenates, supporting the possibility of direct AA-mediated activation [9,20,21].

In another line of investigation, we and others showed that GSH depletion and/or oxidation was a major mechanism for activation of neutral SMase in MCF-7 cells and other cell lines [7,22,23]. TNFα has been shown to result in GSH de-

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Abbreviations: AA, arachidonic acid; BSO, L-buthionin-(*S,R*)-sulfoximine; $cPLA_2$, cytosolic phospholipase A_2; GSH, glutathione; SM, sphingomyelin; SMase, sphingomyelinase; TNFα, tumor necrosis factor α

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pletion, *N*-SMase activation, and subsequent SM hydrolysis and ceramide accumulation. As exogenous ceramide does not result in GSH depletion and GSH does not protect from cell death induced by ceramide accumulation, GSH depletion is thought to lie upstream of ceramide [7]. Furthermore, GSH has been shown to inhibit *N*-SMase activity in vitro, suggesting a direct interaction between GSH and *N*-SMase [24]. On the other hand, some studies also suggest a direct effect of ceramide on the mitochondrial electron transport chain at complex III leading to the generation of reactive oxygen species and GSH depletion [25–27]. Thus, the relationship between GSH and ceramide may be cell-type specific, but requires further clarification.

To date, both $cPLA_2$ activation and GSH depletion have independently been shown to be required for activation of SMases. The goal of this study, therefore, was to determine whether these were independent mechanisms of *N*-SMase regulation or, alternately, if they were linked in a common pathway. We used murine fibroblast L929 cells, a classic model for TNF cytotoxic signaling, due to the exquisite sensitivity of these cells to TNFα. One advantage of this system over many other systems is that addition of cycloheximide or actinomycin D is not required, and therefore does not confuse the results. Moreover, the availability of the C12 variant (which lacks $cPLA_2$ mRNA and protein [18,19]) allows the ready determination of the role of $cPLA_2$. Using these tools, we show that depletion of cellular GSH lies downstream of AA generation, but upstream of SMase activation. These results argue for an indirect connection between $cPLA_2$ and SMase. More importantly, these results establish an important connection between $cPLA_2$ activation and GSH regulation, as they show these mediators as part of a single ordered pathway rather than independent regulators of SMase as previously suggested.

2. Materials and methods

2.1. Materials

Low glucose Cellgro Dulbecco's modified Eagle's medium, fetal calf serum (FCS), penicillin/streptomycin, and trypsin were purchased from Fischer Scientific (Atlanta, GA, USA). FCS was heat-inactivated at 55°C for 30 min before use. GSH and L-buthionin-(*S,R*)-sulfoximine (BSO; Sigma Chemical) were dissolved in medium, and pH corrected where necessary to 7.4. TNFα was kindly provided by Dr. Phil Pekala (East Carolina University). [^{3}H]Choline and [^{3}H]AA were obtained from NEN Technologies. C_6-ceramide was a kind gift of Dr. Alicia Bielawska, (MUSC, Charleston, SC, USA), and was dissolved in ethanol. All other reagents were obtained from Sigma.

2.2. Cell culture

L929 cells and C12 cells were maintained in 5% FCS with 60 mg/ml kanamycin sulfate at 37° in a 5% CO_2 incubator. For assays, cells were seeded at the indicated density and grown for 48–72 h. After washing with PBS, cells were re-fed with warm medium and rested for 1 h before treatment. Unless otherwise indicated, all treatments were performed in 5% FCS at 70–90% confluency. All experiments were done in duplicate with time-matched controls. Where treatments were done in ethanol, the total concentration of ethanol remained under 0.1%. Cells treated with GSH were maintained in freshly opened medium with 5% FCS and 25 mM HEPES.

2.3. SM measurement

Lipids were extracted utilizing the Bligh and Dyer method [28], and SM was measured by the method of Jayadev et al. [9], followed by normalization to phosphate.

2.4. GSH measurement

Intracellular GSH was measured by the Griffith protocol [29], with a few modifications as described [24].

2.5. AA release

Cells were plated at 2×10^4 in 12-well plates. After a 24-h incubation, the medium was replaced and 0.5 μCi/ml [^{3}H]AA added. Labeling was performed overnight. Prior to treatment, label was removed, and fresh warm medium added. After treatment, 500 μl of medium was removed and counted in a scintillation counter. All samples were in duplicate and contained time-matched controls.

2.6. Viability determination

Viability was determined using the cresyl violet assay. Briefly, cells were seeded in 12-well plates at 2×10^4/well. After 48 h incubation, the media were replaced and cells were treated. Upon completion of treatment, media were removed and cells washed twice with ice-cold PBS. Cells were fixed and stained with 0.5% cresyl violet, 4% formaldehyde, and 30% methanol. Excess stain was removed, and the fixed cells were washed twice for 5 and 10 min with PBS. Dye was eluted with 33% acetic acid, and an aliquot removed for spectrophotometric analysis at OD_{600}.

3. Results and discussion

3.1. Effects of BSO on GSH levels and SMase activity

GSH depletion has been reported to lie upstream of SM hydrolysis in MCF-7 cells [7]. However, GSH may also be

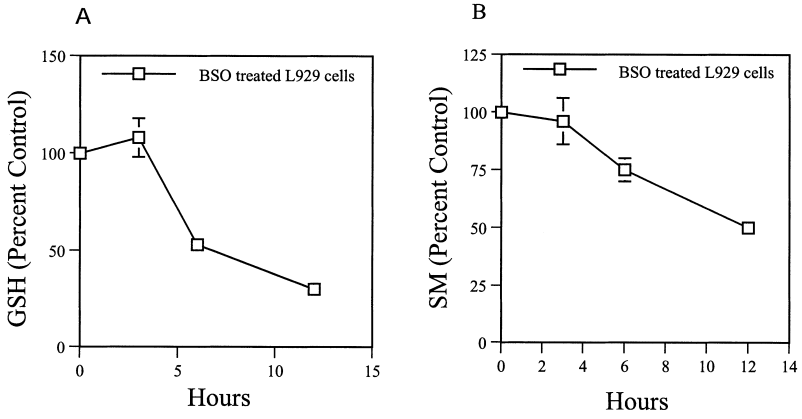

Fig. 1. Effect of BSO on GSH and SM levels in L929 cells. A,B: Cells were seeded in duplicate, treated with 100 μM BSO, and harvested concomitantly at the indicated times. Time-matched vehicle controls were also harvested at each point. Results are from three separate experiments and are expressed as mean ± S.D. If not visible, error bars are smaller than the diameter of the points. A: Cells treated with 100 μM BSO and assayed for GSH at the indicated times with results expressed as percent control. B: SM hydrolysis was measured in cells treated with 100 μM BSO at the indicated times with results expressed as percent control.

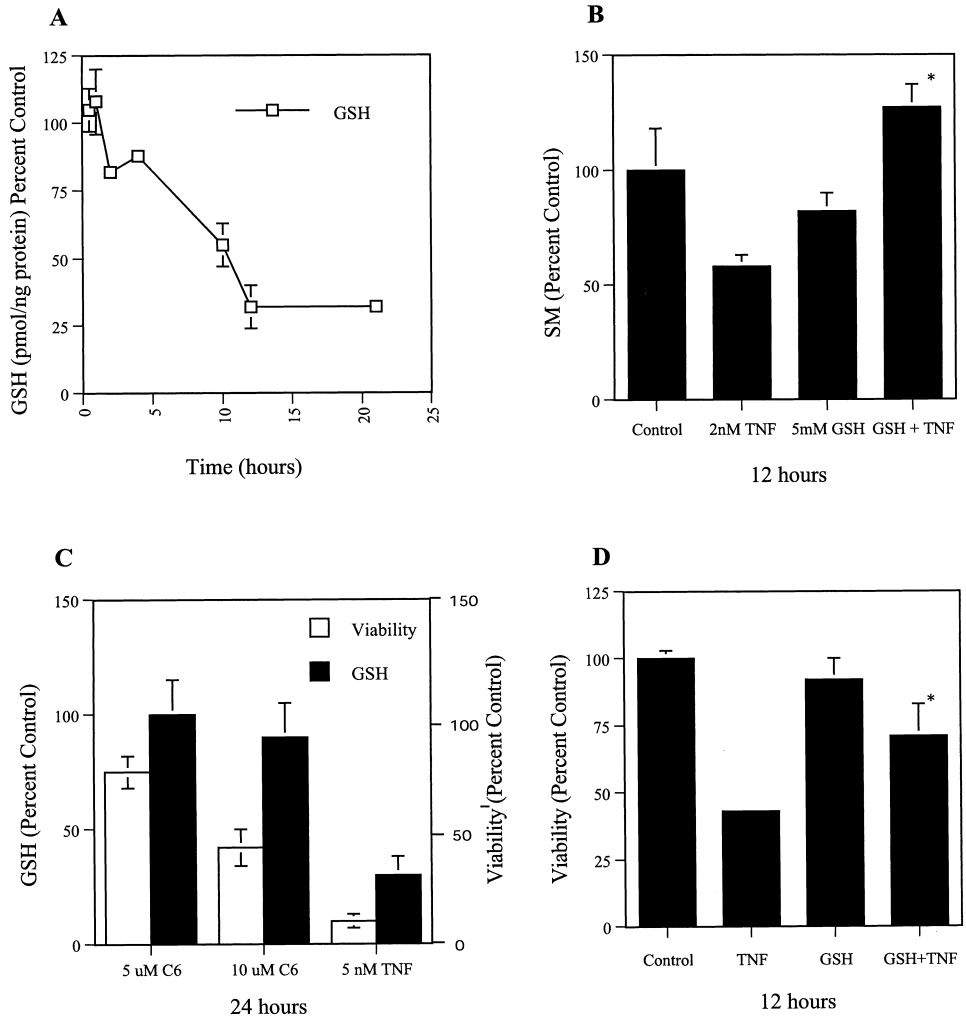

Fig. 2. Effects of GSH repletion on TNF-induced SM hydrolysis, ceramide accumulation and viability. A: Cells treated with 5 nM TNF were harvested at the indicated times and assayed for GSH. Results are from three individual experiments, each done in duplicate, and are expressed as mean percent time-matched control values ± S.D. If not visible, error bars are smaller than the diameter of the points. B: Cells were steady-state labeled with 0.5 μCi/ml [^{3}H]choline for 48 h, then pre-incubated with 5 mM GSH for 2 h prior to treatment with 5 nM TNF for 12 h. SM levels were measured as described in Section 2. Results are the average of three separate experiments ± S.D. A *t*-test confirmed GSH significantly protected from TNF-induced hydrolysis with $^*P < 0.05$. C: L929 cells were treated with 5 nM TNF and 5 or 10 μM C_6-ceramide (C6) for 24 h, and harvested for GSH measurements and viability as determined using the cresyl violet assay. Results are graphed on a double *y*-axis with both GSH and viability values expressed as percent vehicle control. Results are from three individual experiments, and are expressed as mean ± S.D. D: Cells were pre-incubated with 5 mM GSH for 2 h prior to treatment with 5 mM TNF for 12 h. Viability was determined using the cresyl violet assay, with results expressed as the average of three experiments ± S.D. A *t*-test confirmed GSH significantly protected from TNF-induced hydrolysis with $^*P < 0.05$.

depleted subsequent to ROS production downstream of ceramide effects on the mitochondrial electron transport chain [25–27]. Since $cPLA_2$ has been shown to regulate SMase in L929 cells, it became necessary to determine if GSH depletion is also involved in regulating SMase in L929 cells. To examine GSH regulation of SMase, GSH depletion was induced with BSO, which blocks GSH production by inhibiting γ-glutamyl cysteine synthase. In L929 cells at 6 h, 100 μM BSO reduced GSH levels by 50%, and by 12 h 80% of total cellular GSH was depleted (Fig. 1A). Concomitantly, BSO also induced SM hydrolysis. Approximately 20% hydrolysis of SM was observed by 6 h, and by 12 h 50% of SM was lost (Fig. 1B). These results show that GSH depletion is sufficient to induce SM hydrolysis in L929 cells as previously reported in MCF-7 cells.

3.2. *Effects of GSH repletion on TNF-induced SM hydrolysis, ceramide accumulation and viability*

To examine if GSH depletion plays a role in response to TNFα in L929 cells, cells were stimulated with 5 nM TNF and assayed for GSH over a 21-h time-course (Fig. 2A). Treatment of L929 cells with 5 nM TNF induced a sustained loss of GSH beginning 2 h after treatment with a 20% drop and continuing until 12 h, leveling at 30% of control values. These results demonstrate a significant and sustained depletion of GSH beginning at 2 h in L929 cells.

To determine if GSH depletion is necessary for SM hydrolysis, cells were pre-incubated with 5 mM GSH for 2 h prior to treatment with TNFα. GSH repletion completely blocked SM hydrolysis. At 12 h, TNF alone induced a 40% drop in SM while GSH repletion totally prevented SM hydrolysis

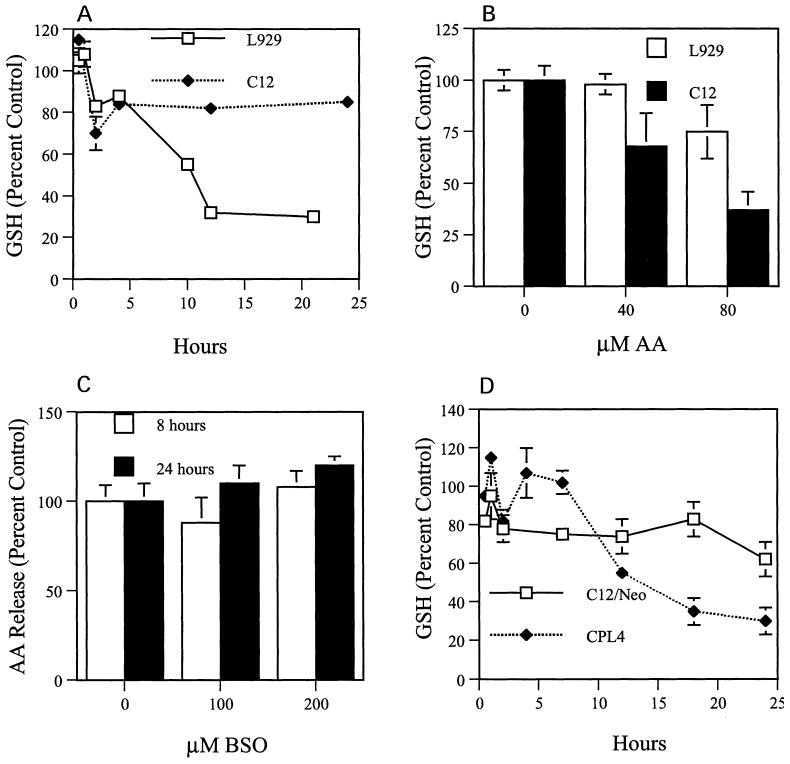

Fig. 3. Effects of $cPLA_2$ and AA on GSH levels. A: C12 and L929 cells treated with 5 nM TNF were harvested at the indicated times and assayed for GSH. Results are from four separate experiments, and are expressed as mean percent control ± S.D. B: Cells were treated with 40 and 80 μM of AA for 18 h and cells harvested for GSH measurements. Results are from three separate experiments performed in duplicate, and are expressed as mean ± S.D. Asterisks indicate results significantly different from control as determined by *t*-test ($P < 0.05$). C: Cells labeled with [^{3}H]AA were treated with 100 μM and 200 μM BSO for 8 and 24 h, and AA release determined. D: C12/neo and CPL4 cells were seeded simultaneously and treated with 5 nM TNF over a 24-h time-course. At each time-point, GSH levels were measured. Time-matched controls were used, and each point done in duplicate. Results are from three separate experiments, and are expressed as mean percent control ± S.D.

(Fig. 2B). These results indicate that GSH depletion is necessary for TNF-induced SM hydrolysis in L929 cells.

To examine the effects of ceramide on GSH levels and viability, cells were treated with 5 and 10 μM C_6-ceramide for 24 h. TNF killed almost 90% of cells by 24 h, and produced a 70% drop in GSH, whereas both 5 and 10 μM ceramide killed 60% of cells with no drop in GSH levels (Fig. 2C). The observation that GSH depletion is necessary for SM hydrolysis and ceramide generation coupled with the inability of ceramide to deplete GSH suggest that GSH depletion lies upstream of SM hydrolysis and ceramide generation in L929 cells.

To determine if GSH repletion protected cells from TNF-induced cell death, cells were pre-incubated with 5 mM GSH for 2 h prior to treatment with 5 nM TNF for 12 h. GSH partially protected cells from cytotoxicity as determined by the cresyl violet assay, such that with GSH repletion, viability was restored to 80% of control compared with 40% of control in cells treated with TNF alone (Fig. 2D). Taken together, these results (Fig. 2C,D) show that GSH depletion is necessary for TNF-induced cytotoxicity, whereas ceramide generation is sufficient for the decrease in viability observed in response to TNF treatment, again suggesting that GSH depletion lies upstream of ceramide generation in L929 cells.

Table 1
Effect of repleting GSH on TNF-induced AA release

Treatment	Percent control
Control	100.0 ± 4.3
5 mM GSH	94.2 ± 1.6
5 nM TNF	153.3 ± 2.0
5 nM TNF+5 mM GSH	154.4 ± 0.5

L929 cells labeled with [^{3}H]AA were treated with 5 mM GSH for 2 h before addition of 5 nM TNF. AA release was determined after 12 h of TNF treatment. Results are representative of three separate experiments performed in duplicate, and are expressed as mean percent control ± S.D.

3.3. Role of $cPLA_2$ in TNF-induced depletion of GSH

Having established that GSH depletion was necessary for TNF-induced SM hydrolysis, we next sought to investigate whether or not there was a connection between AA production and GSH depletion as both have been independently shown to activate SMase [8,9,21,30]. For these studies, we used the TNF-resistant L929 variant (C12) detective in $cPLA_2$ [18,19]. Using this cell line, we previously demonstrated that

Table 2
Effect of BSO on GSH levels and SM hydrolysis in C12 cells

	Control	100 μM BSO	200 μM BSO
GSH	100 ± 6.4	41.0 ± 12.3	20.0 ± 14.7
SM	100 ± 1.3	79.4 ± 1.5	71.0 ± 0.3

C12 cells were treated with BSO for 12 h and assayed for GSH and SM as described. For SM measurements, cells were pre-labeled with [^{3}H]choline for 72 h. Results are representative of three separate experiments performed in duplicate, and are expressed as mean percent control ± S.D.

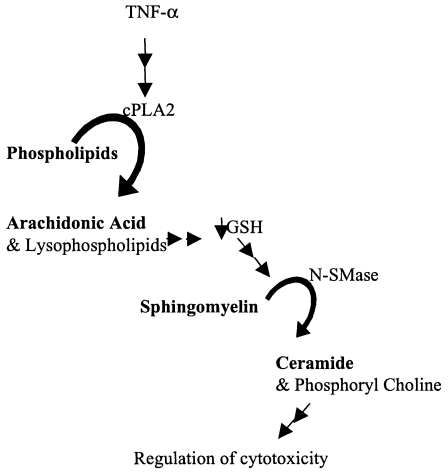

Fig. 4. Proposed scheme of TNF-induced cell death in L929 cells. According to this scheme, TNF activates $cPLA_2$ which then generates AA and lysophospholipids. This in turn is required for the depletion of GSH, possibly as a result of oxidation of GSH. Depletion of GSH is then required for the activation of SMase, SM hydrolysis, and ceramide generation. Ceramide in turn regulates many downstream cell growth and death responses.

$cPLA_2$ was necessary for TNF-induced SMase activation in L929 cells [10]. Although TNF induced a small sustained 20% drop in GSH, the GSH levels did not fall to the lower values seen in L929 cells (Fig. 3A). Therefore, $cPLA_2$ activity is necessary for TNF-induced GSH depletion in L929 cells.

To determine if GSH depletion was necessary for $cPLA_2$-mediated AA production, we measured TNF-induced AA production with and without GSH repletion (5 mM) 2 h prior to TNF treatment for 12 h. A 1.5-fold increase in AA production in response to TNF was observed that was unaffected by GSH levels (Table 1). Therefore, while $cPLA_2$ activity is necessary for GSH depletion, GSH depletion is not necessary for AA production.

To determine if the product of $cPLA_2$ activity, AA, is not only necessary but also sufficient to induce GSH depletion, we next examined GSH levels in cells treated with 40 and 80 μM AA for 18 h, concentrations that have been found previously to be required for cytotoxicity [31]. Treatment with 80 μM AA reduced GSH levels by 30% in L929 and by 60% in C12 cells (Fig. 3B). Based on these results, AA is sufficient for GSH depletion in L929 cells. In addition, we observed C12 cells to be more sensitive to AA than L929 cells.

To determine if GSH depletion is sufficient for $cPLA_2$ activity and AA release, cells labeled with [^{3}H]AA were treated with 100 and 200 μM BSO, and AA release was determined. There were no significant changes in AA release with GSH depletion (Fig. 3C). As a control, we measured BSO-induced GSH depletion and SM hydrolysis in C12 cells to confirm that these parameters were normal (Table 2). Taken together, these results demonstrate that AA production is upstream of GSH depletion.

To further substantiate this conclusion, we hypothesized that restoration of $cPLA_2$ activity to C12 cells should restore TNF-induced GSH depletion. C12/neo (vector only control cells) and CPL4 (C12 cells transfected with $cPLA_2$) were seeded simultaneously and treated with 5 nM TNF. Cells were harvested and GSH levels measured over a time-course of 24 h. GSH levels decreased to levels comparable to those found in wild-type L929 cells while the vector control levels remained at 80% of control (Fig. 3D). In addition, as previously reported, CPL4 cells partially regained sensitivity to TNF-induced cytotoxicity [10]. TNF induced 60% death in CPL4 cells versus 15% in C12 and 85% in L929 (data not shown). Taken together, these studies show that $cPLA_2$ is necessary for TNF-induced GSH depletion and subsequent cytotoxicity in L929 cells. Therefore, $cPLA_2$ activity lies upstream of GSH depletion and SM hydrolysis.

3.4. Conclusions

The first conclusion to be derived from these results is that GSH depletion lies upstream of SM hydrolysis in L929 cells. This is supported by the facts that (i) GSH depletion occurred prior to SM hydrolysis; (ii) pharmacologically depleting GSH with BSO induced SM hydrolysis, and therefore, GSH depletion is sufficient for SM hydrolysis; (iii) exogenous GSH treatment prevented TNF-induced cytotoxicity and SM hydrolysis, and therefore, GSH depletion is necessary for SM hydrolysis; (iv) C_6-ceramide treatment does not affect GSH levels, and therefore, GSH depletion is not downstream of ceramide formation. These results extend previous studies on the relationship of GSH and SMase [7,22,23], and they begin to define a general mechanism tying oxidative stress/GSH metabolism to activation of SMases.

These studies also provide strong evidence that GSH depletion in L929 cells is downstream of $cPLA_2$ and dependent on $cPLA_2$ activity. Thus, C12 cells, which lack $cPLA_2$ activity, failed to drop GSH upon TNF stimulation. In addition, exogenous AA was sufficient to induce GSH depletion, whereas manipulating GSH levels pharmacologically failed to affect AA levels. Most convincingly, restoring $cPLA_2$ activity to C12 cells also restored GSH depletion in response to TNF. Together, these results are most consistent with GSH depletion lying downstream of $cPLA_2$, with activation of $cPLA_2$ being required for the depletion in the levels of GSH seen in L929 cells after TNF treatment.

These results suggest the following scenario (see Fig. 4). TNF induces $cPLA_2$ activation, resulting in AA release. $cPLA_2$ activity in turn results in GSH depletion, which, finally, initiates SM hydrolysis. The ceramide induced by this system then goes on to play a role in regulating cytotoxicity [7,10,23,32].

The connection of $cPLA_2$ to GSH leads to further questions on possible mechanisms for this connection. The complexity of AA signaling and metabolism offers several possibilities. AA itself may play a direct role in regulating GSH depletion, through regulation of metabolism or uptake/efflux of GSH. Other research has suggested metabolism to arachidonoyl CoA may be involved in AAs effect on SMase activity [21]. Alternatively, the eicosanoids produced from AA metabolism may play a role. Several groups have reported a role for lipoxygenase enzymes in TNF-induced cytotoxity [13,33–35], and a recent report suggests lipoxygenase activity may be important in apoptosis induced by thiol depletion [36]. Another option for connecting $cPLA_2$ and GSH depletion stems from the nature of AA metabolism. Many enzymes responsible for metabolizing AA produce hydroperoxides and other oxidative species as by-products [37]. It is possible that these oxidative species go on to induce the depletion of GSH, either before or after oxidation to GSSG [38]. Transporters for both oxidized and reduced GSH are known, and in Fas-induced

GSH depletion, GSH is pumped out of the cell [39], although the nature of the molecular species transported was not determined. Thus, it is possible that persistent activation of $cPLA_2$ and downstream enzymes may result in an oxidative load in the cell that results in depletion of GSH. Ongoing studies in our laboratory are investigating these possibilities.

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