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Glutathione Depletion Induces Spermatogonial Cell Autophagy

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ABSTRACT

The development and survival of male germ cells depend on the antioxidant capacity of the seminiferous tubule. Glutathione (GSH) plays an important role in the antioxidant defenses of the spermatogenic epithelium. Autophagy can act as a pro-survival response during oxidative stress or nutrient deficiency. In this work, we evaluated whether autophagy is involved in spermatogonia-type germ cell survival during severe GSH deficiency. We showed that the disruption of GSH metabolism with L-buthionine-(S,R)-sulfoximine (BSO) decreased reduced (GSH), oxidized (GSSG) glutathione content, and GSH/GSSG ratio in germ cells, without altering reactive oxygen species production and cell viability, evaluated by 2′,7′-dichlorodihydrofluorescein (DCF) fluorescence and exclusion of propidium iodide assays, respectively. Autophagy was assessed by processing the endogenous protein LC3I and observing its sub-cellular distribution. Immunoblot and immunofluorescence analysis showed a consistent increase in LC3II and accumulation of autophagic vesicles under GSH-depletion conditions. This condition did not show changes in the level of phosphorylation of AMP-activated protein kinase (AMPK) or the ATP content. A loss in S-glutathionylated protein pattern was also observed. However, inhibition of autophagy resulted in decreased ATP content and increased caspase-3/7 activity in GSH-depleted germ cells. These findings suggest that GSH deficiency triggers an AMPK-independent induction of autophagy in germ cells as an adaptive stress response. J. Cell. Biochem. 116: 2283–2292, 2015.

KEY WORDS: AUTOPHAGY; GERM CELLS; GLUTATHIONE

Spermatogenesis is a complex process in which spermatogonial stem cells become mature spermatozoa through a series of events involving mitosis, meiosis, and cell differentiation [Amann, 2008]. The high rate of cell division in this process implies correspondingly elevated rate of mitochondrial oxygen consumption by germ cells. The production of reactive oxygen species (ROS) is a physiological event that is required for the functional maturation and capacitation of spermatozoa [Griveau and Le Lannou, 1997; Aitken and Vernet, 1998; Agnihotri et al., 1999], through overproduction of ROS. This last event correlates with increased lipid peroxidation state and decreased sperm motility [Suleiman et al., 1996; Aitken et al., 2007; Agarwal et al., 2008], abnormal morphology [Aitken, 1994], and DNA damage in male germ cells [Aitken and Krausz, 2001]. The development and survival of these

Abbreviations: BSO, L-buthionine-(S,R)-sulfoximine; DCF, 2′,7′-dichlorodihydrofluorescein; DTT, dithiothreitol; GCL, glutamate-cysteine ligase; GSH, reduced glutathione; GSSG, oxidized glutathione; mtGSH, mitochondrial GSH; ROS, reactive oxygen species.

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cells depend on both their close contact with Sertoli cells and the antioxidant capacity of this epithelium [Dym and Fawcett, 1970; Dym and Fawcett, 1971; Boitani et al., 1983; Angulo et al., 2011; Abud and Hime, 2014].

Reduced glutathione (GSH) is the most abundant non-protein thiol in mammalian cells [Forman et al., 2010] and plays an essential role in many biological processes, including the synthesis of proteins and DNA, the transport of amino acids, post-translation regulation, and in protecting cells against oxidation [Meister, 1988; Sies, 1991]. Some of these biological processes are crucial for cell viability [Circu and Aw, 2012]. High concentrations of GSH have been reported in the male reproductive system [Grosshans and Calvin, 1985; Castellón, 1994]. After birth, GSH content progressively increases until the onset of spermatogenesis, playing a significant role in this process. High levels of GSH have been observed in pachytene spermatocytes, but low levels are found in round spermatids [Grosshans and Calvin, 1985; Bauché et al., 1994]. GSH has been shown to have a therapeutic role in male infertility [Kumar et al., 2000; Mora-Esteves and Shin, 2013].

Autophagy is a process that is essential for the maintenance of protein homeostasis through the lysosome machinery. This process begins with the sequestration and enclosure of part of the cytoplasm by double-membrane vacuoles, called autophagosomes. Autophagosomes fuse with lysosomes and the luminal contents are degraded by lysosomal enzymes for recycling [Ravikumar et al., 2010; Yang and Klionsky, 2010; Niedźwiedzka-Rystwej et al., 2013]. Autophagy has been described as a pro-survival response during metabolic or oxidative stress [Degenhardt et al., 2010; Yang and Klionsky, 2010]. The relationship between autophagy and GSH levels has been shown indirectly in a few studies [Chiang and Maric, 2011; Seo et al., 2011; Chandramani Shivalingappa et al., 2012; Desideri et al., 2012]. In three different carcinoma cells, nutrient starvation led to a significant decrease of intracellular GSH levels [Desideri et al., 2012]. This phenomenon was dependent on ABCC1-mediated GSH extrusion, along with glutamate–cysteine ligase (GCL) inhibition and, to a minor extent, the formation of GSH–protein-mixed disulfides that synergistically contributed to the modulation of autophagy [Desideri et al., 2012]. Furthermore, γ-interferon-inducible lysosomal thiol reductase (GILT)-deficient fibroblasts exhibit reduced GSH levels and a shift in the GSH/GSSG ratio toward the oxidized form [Chiang and Maric, 2011]. They also accumulate dysfunctional mitochondria and show increased autophagy [Chiang and Maric, 2011]. In a mouse macrophage cell line, GSH-depletion also induced autophagy when cells were treated with H_2O_2 [Seo et al., 2011]. In addition, methamphetamine-induced autophagy in the N27 dopaminergic cell line was associated with a GSH depletion. Pretreatment with *N*-acetyl cysteine (NAC) reduced the methamphetamine-induced GSH depletion and autophagy, while depletion of GSH with L-buthionine-(S,R)-sulfoximine (BSO) enhanced autophagy [Chandramani Shivalingappa et al., 2012]. However, GSH depletion can have damaging effects on hippocampal neurons by perturbing calcium homeostasis involving TRPM2 and TRPV1 channels, while NAC pretreatment may have neuroprotective activity against oxidative stress [Naziroglu et al., 2013; Ovey and Naziroglu, 2015].

Intracellular GSH is a crucial factor in the normal development and survival of male germ cells. Under harsh environmental conditions, such as treatment with ethanol and heat stress, advanced-stage germ cells located in the adluminal compartment showed apoptosis in GSH-depleted testis tissue [Jana et al., 2010; Kanter et al., 2013]. Spermatogonia are located in the basal compartment of the seminiferous tubule and compared to other cells located in the adluminal compartment [Aitken and Clarkson, 1987; Aruldhas et al., 2005; Paul et al., 2009; Celino et al., 2011], spermatogonia are highly tolerant to ROS. In the present study, we investigated the role of autophagy in the survival of spermatogonia-type germ cells using the GC-1 cell line, under conditions of GSH depletion. Our results showed that disruption of GSH metabolism with BSO decreased GSH content in these cells, without altering ROS production and cell viability. These cells also showed AMPK-independent induced autophagy likely due to a downregulation of S-glutathionylated proteins, that are necessary for survival of GC-1 cells under these conditions.

MATERIALS AND METHODS

CELL CULTURE

GC-1 cells from American Type Culture Collection (ATCC, Catalog Number CRL-2053), similar to type B spermatogonia, were cultured in DMEM/F12 medium (Thermo Scientific HyClone, South Logan, UT) supplemented with 10% FBS (Thermo Scientific HyClone, South Logan, UT) [Angulo et al., 2008; Villarroel-Espíndola et al., 2013]. Cells were incubated at 37 °C with 5% CO_2.

GSH DEPLETION

Cells were incubated (2–24 h) in 1 mM L-buthionine-(S,R)-sulfoximine (Sigma–Aldrich, St. Louis, MO) [Guaiquil et al., 1997; Mardones et al., 2012; Maryon et al., 2013; Abdelhamid and El-Kadi, 2015]. For total GSH determination, 2×10^5 cells were washed twice with normal saline, lysed with 0.05 ml 0.4% Triton X-100, and the supernatant was processed using the recycling procedure and 5,5′-dithiobis-(2-nitrobenzoic acid) [Guaiquil et al., 1997]. Levels of both reduced and oxidized forms of GSH were determined using a GSH/GSSG-Glo™ assay according to the manufacturer's instructions (Promega, Eugene, OR). Briefly, this assay is a luminescence-based system for the quantification of total (GSH+GSSG), GSSG, and GSH/GSSG ratio in cultured cells. 1.5×10^4 cells were plated to each well of white frame and clear flat bottom 96-well tissue culture plates (Santa Cruz Biotechnology, Inc., Dallas, TX) 24 h before treatments. Changes in luminescence were measured with a Synergy 2 luminescence detection system (BioTek, Winooski, VT).

WESTERN BLOT ANALYSIS

Total protein extracts were obtained by homogenizing cells in NP-40 lysis buffer containing 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM sodium fluoride, 10 nM okadaic acid, and 1 mM sodium orthovanadate at 4 °C. Proteins were resolved by

SDS–PAGE, transferred onto PVDF membranes, and probed with the following primary antibodies: Mouse anti-tubulin (1:5000, Sigma-Aldrich, St. Louis, MO), Rabbit anti-GAPDH (1:5000, Sigma-Aldrich, St. Louis, MO), Mouse anti-GSH (1:2000, Abcam, Cambridge, United Kingdom), Rabbit anti-LC3 (1:1000, Cell Signaling Technology, Inc., Danvers, MA), Rabbit anti-Beclin-1 (1:1,000, Cell Signaling Technology, Inc., Danvers, MA), mouse anti-p62/SQSTM1 (1:2,000, Abnova, Taipel, Taiwan), mouse anti-phosphorylated p70s6k (Thr 389, 1:1,000, Cell Signaling Technology), Rabbit anti-p70s6k (1:1,000, Cell Signaling Technology), Rabbit anti-phosphorylated AMPk (Thr 172, 1:,1000, Cell Signaling Technology), and Rabbit anti-AMPK (1:1,000, Cell Signaling Technology). The blots were then incubated with a horseradish peroxidase-coupled secondary antibody (Thermo Scientific Pierce, South Logan, UT). ImageJ software (NIH, Bethesda, MD) was used for image densitometry.

IMMUNOFLUORESCENCE STUDIES

Cells seeded onto poly-L-lysine-coated coverslips were fixed for 30 min in PBS containing 3.7% (w/v) paraformaldehyde. After fixation, cells were permeabilized for 20 min with PBS containing 0.3% (v/v) Triton X-100. Blocking and incubation with primary and secondary antibodies were carried out in PBS containing 3% (w/v) BSA and 0.15% (v/v) Triton X-100. Coverslips were washed in PBS and 0.3% (v/v) Triton X-100, and mounted onto glass slides using DAKO fluorescence mounting medium. Fluorescence images were obtained with an Olympus FV1000 confocal microscope (Universidad Austral de Chile). We used primary antibodies to LC3 and then Alexa Fluor® 488-coupled secondary antibody (Invitrogen, Carlsbad, CA). Nuclei were stained with DAPI (Invitrogen).

CELL VIABILITY

Intracellular ATP content was determined using a CellTiter-Glo® Luminescent Cell Viability Assay (Promega), in accordance with the manufacturer's instructions. The integrity of the plasma membrane was assessed by determining the ability of cells to exclude propidium iodide (PI, Sigma–Aldrich, 1 μg/ml). The level of PI incorporation was quantified in a Becton Dickinson FAC-Scan flow cytometer; 10.000 events were recorded for each sample. Caspase-3/7 activity was determined using a caspase-Glo® 3/7 assay (Promega) according to the manufacturer's instructions. Briefly, this assay provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. This substrate is cleaved to release aminoluciferin, a substrate of luciferase. For ATP and Caspase 3/7 assays 1.5×10^4 cells were plated to each well of white frame and clear flat bottom 96-well tissue culture plates (Santa Cruz Biotechnology) 24 h before treatments. Changes in luminescence were measured with a Synergy 2 luminescence detection system (BioTek).

ROS PRODUCTION

1.5×10^4 cells were plated to each well of black frame and clear flat bottom 96-well tissue culture plates (Santa Cruz Biotechnology). After treatment with or without BSO, cells were loaded with 10 μM 2′,7′-dichlorodihydrofluorescein (DCF; Life Technologies, Norway) fluorescent dye in PBS pH 7.4 for 30 min at 37 °C. The fluorescent dye was replaced by prewarmed incubation buffer (IB). Changes in fluorescence were measured with a Synergy 2 fluorescence detection system (BioTek).

EXPRESSION OF RESULTS AND STATISTICAL ANALYSIS

Values are represented as mean ± SD of three independent experiments or as examples of representative experiments performed on at least three separate occasions. Data were analyzed by ANOVA and comparisons between groups were performed using a protected Kruskal–Wallis. A value of $P < 0.05$ was set as the limit of statistical significance.

RESULTS

GSH DEPLETION DOES NOT AFFECT ROS PRODUCTION AND CELL VIABILITY IN GC-1 GERM CELLS TREATED WITH BSO

GC-1, a spermatogonia cell line, was treated with BSO, a potent inhibitor of GCL, a rate-limiting enzyme essential for GSH biosyn-

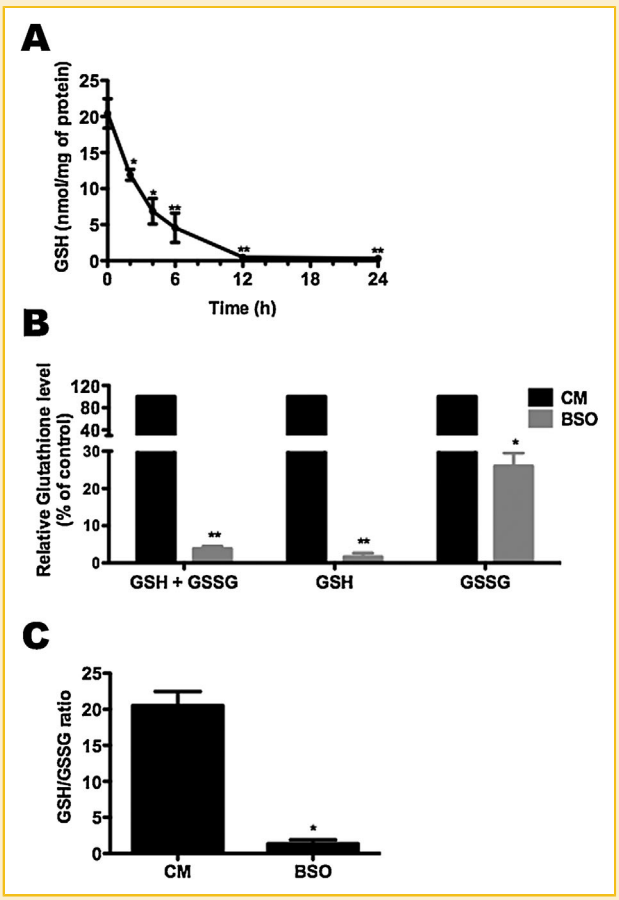

Fig. 1. BSO induces GSH depletion in GC-1 germ cells. Cells were treated with BSO (1 mM) for 24 h. Complete medium (CM) was used as a negative control. (A) GSH content was measured at 2, 4, 6, 12, and 24 h. (B) Total glutathione (GSH+GSSG), reduced glutathione (GSH), and oxidized glutathione (GSSG) were measured at 24 h using the luciferin–luciferase assay. (C) GSH/GSSG ratio. Data were mean ± SD of three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ versus CM or 0 h (untreated cells).

thesis [Griffith and Biol, 1979]. Over 90% of GCL is present in the cytoplasm, hence BSO effectively depletes cytoplasmic GSH [Griffith and Meister, 1985]. The intracellular GSH content in control GC-1 cells was 20 nmol/mg of protein; when GC-1 cells were treated with 1 mM BSO for 24 h, intracellular GSH rapidly decreased in a time-dependent manner and reached a value near 0 after treatment with 1 mM BSO for 12 h, and was undetectable 24 h after treatment (Fig. 1A). We used a more sensitive luminescence method to evaluate the content of reduced (GSH) and oxidized (GSSG) glutathione in the presence of BSO and observed a decrease in both GSH and GSSG (Fig. 1B). The GSH/GSSG ratio also decreased when these cells were treated with BSO (Fig. 1C). BSO treatment increases ROS production and affects cell viability [Chen et al., 2010; Findeisen et al., 2011; Kim et al., 2013]. To evaluate this in our model, we loaded GC-1 germ cells with 10 μM DCF for 30 min after BSO treatment and then measured the change in fluorescence over a 15 min period (Fig. 2A). Total fluorescence was measured at 30 min after loading cells with this probe (Fig. 2B). No change was observed in ROS production when GC-1 cells were depleted of GSH. Cell viability was determined by measuring ATP content (Fig. 2C) and by propidium iodide dye exclusion using a flow cytometer (Fig. 2D) in cultures of GSH-depleted GC-1 cells. No changes were seen, in either case, during GSH depletion.

GSH DEPLETION INDUCES AUTOPHAGY VIA mTOR SIGNALING IN GC-1 SPERMATOGONIAL CELLS

Modification of the soluble protein LC3 (or Atg 8) by phosphatidylethanolamine (PE) is essential for the formation of mammalian autophagosomes [Kabeya et al., 2000; Tanida et al., 2004]. The lipidated form of LC3 (called LC3II) is widely used as a specific marker for monitoring autophagy. Addition of PE accelerates the electrophoretic mobility of LC3 compared with LC3I and changes its subcellular distribution within cells [Mizushima et al., 2010; Klionsky et al., 2012]. p62/SQSTM interacts with both LC3 and ubiquitinated proteins and is selectively incorporated into autophagosomes to be degraded by autophagy machinery by delivering protein aggregates. Beclin-1 (or Atg 6) is a key regulator of autophagy by regulating PtdIns3KC3-dependent generation of phosphatidylinositol 3-phosphate (PtdIns(3)P) and the subsequent recruitment of additional Atg proteins involved in autophagosome formation [Kang et al., 2011]. In order to evaluate whether GSH depletion induces autophagy in GC-1 cells, we treated cultured GC-1 cells with 1 mM BSO for 24 h. Western blots were performed to monitor changes in the protein levels of LC3, p62, and Beclin-1 (Fig. 3A and B). No changes were observed in the protein levels of LC3I, LC3II, p62, and Beclin-1 in GC-1 cells during GSH depletion. An autophagy flux assay was applied to determine the total amount of

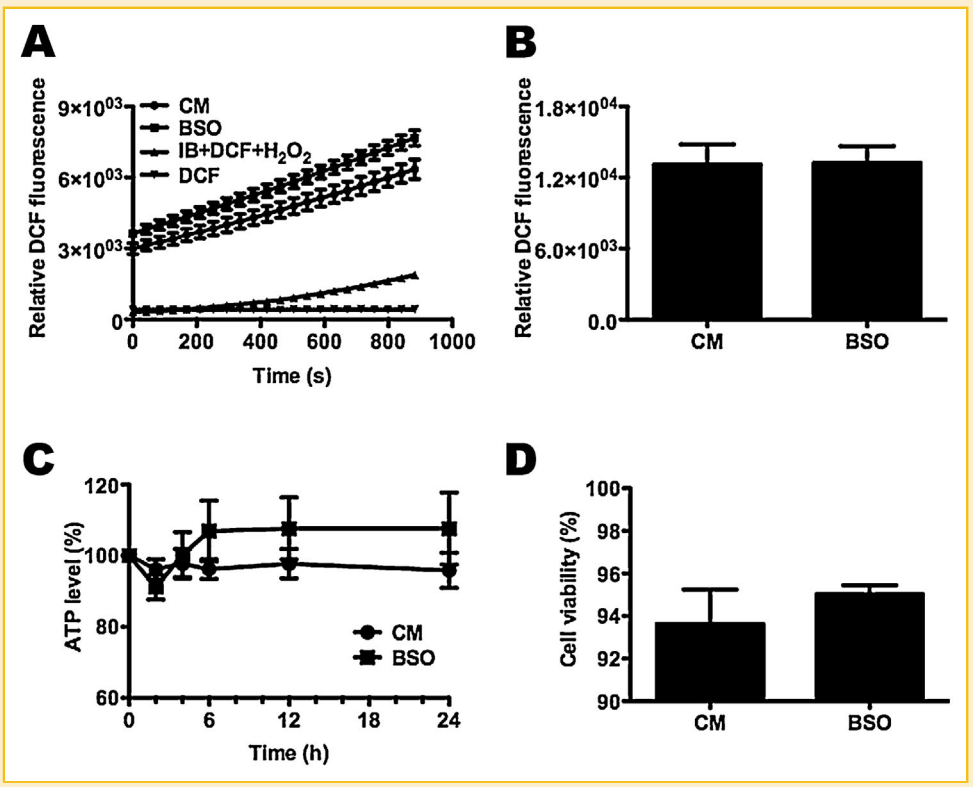

Fig. 2. GSH depletion does not affect ROS production and cell viability. Cells were treated with BSO (1 mM) for 24 h. Complete medium (CM) was used as a negative control. (A) Cells were loaded with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCF) following BSO treatment. Intracellular ROS release was quantified via changes in DCF fluorescence over a 15-min period. (B) DCF fluorescence was quantified at 30 min after loading the probe. (C) Intracellular ATP levels were determined by luminescence using the luciferin–luciferase assay at each time interval. (D) Cell viability was measured by the ability of cells to exclude propidium iodide (PI), determined using flow cytometry. Data were mean ± SD of three independent experiments. IB, incubation buffer.

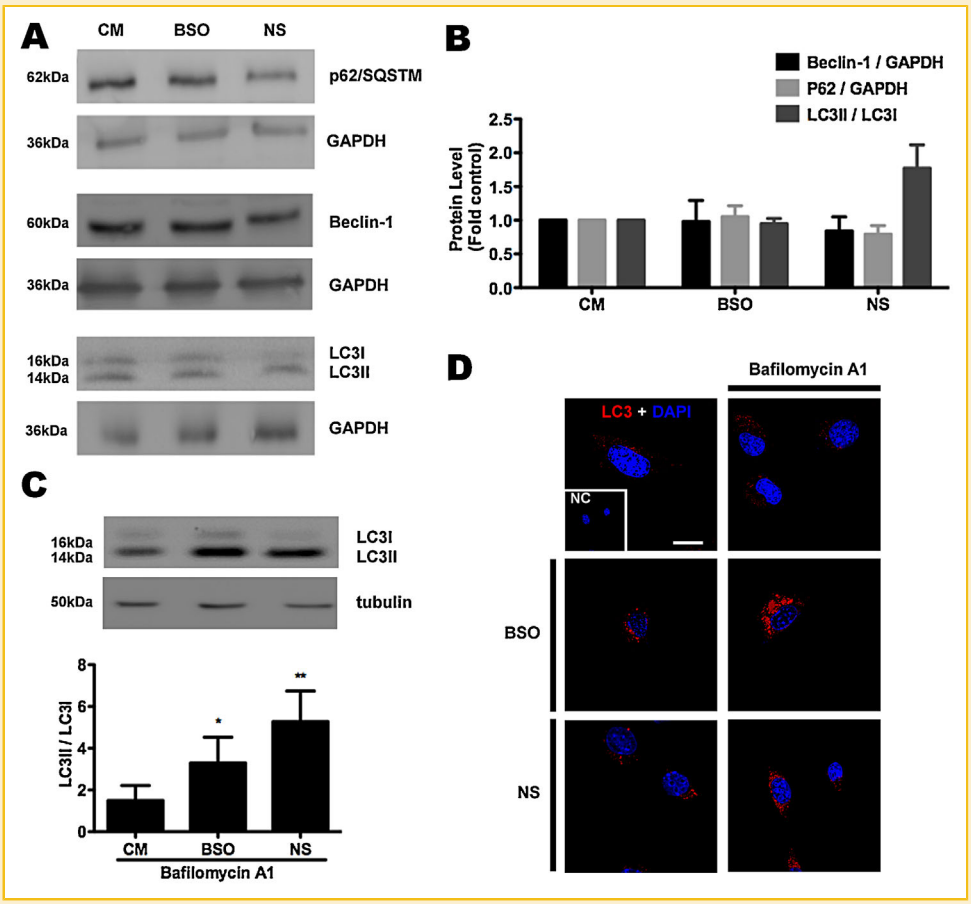

Fig. 3. GSH depletion induces autophagy in GC-1 germ cells. Cells were treated with BSO (1 mM) for 24 h. Nutrient starvation (NS) was used as a positive control and complete medium (CM) was used as a negative control. (A, B) Cell lysates were subjected to Western blot against p62, Beclin-1, LC3, and GAPDH. The figure corresponds to a representative blot (A) and quantitative analysis of p62/GAPDH, Beclin-1/GAPDH, and LC3II/LC3I, the ratio of LC3II over LC3I was estimated by comparing LC3II/GAPDH values over LC3I/GAPDH. (B). Data are mean ± SD of three independent experiments. (C) Autophagic flux was measured in the presence of bafilomycin A1 (50 nM: top) and quantification of LC3 processing is shown (bottom). Data are mean ± SD of three independent experiments. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus bafilomycin A1. (D) LC3 localization in the absence or presence of bafilomycin A1 was determined by immunofluorescence using a specific antibody. Negative control without first antibody (NC). Nuclei were stained with DAPI. Scale bar: 20 μm.

LC3II accumulated. We used bafilomycin A1, a lysosomal proton pump inhibitor, to disrupt the autophagosome–lysosome fusion step (Fig. 3C). We observed a rise in the LC3II/LC3I ratio in glutathione-depleted cells, a measurement used to evaluate induction of autophagy, similar when the cells were cultured in a nutrient-starved medium. To confirm these results, we performed an immunofluorescence study against LC3 in the presence or absence of bafilomycin A1 (Fig. 3D). Under these conditions, GSH depletion was accompanied by the accumulation of punctate, high-intensity anti-LC3 immunoreactive material. In order to investigate the involvement of the AMPK-mTOR signaling pathway during GSH depletion, we evaluated the phosphorylation of AMPK (Thr 172) and the mTOR substrate p70s6k at different times in the presence of BSO. Decreased levels of phospho-p70s6k (Thr389) were observed after 2 h of BSO treatment (Fig. 4A), suggesting an inactivation of the mTOR complex. Regarding the AMPK pathway (Fig. 4B), no significant changes were observed in its phosphorylation status. These results suggest that GSH deficiency triggers an AMPK-independent autophagy in germ cells.

LOSS OF S-GLUTATHIONYLATED PROTEIN PATTERNS IN GSH-DEPLETED GC-1 GERM CELLS

S-glutathionylation is involved in cell signaling by modulating protein function and by protecting proteins from oxidation [Xiong et al., 2011; Pastore and Piemonte, 2012]. In order to reveal any changes in S-glutathionylated protein patterns by GSH depletion, we investigated these post-translational modifications by Western blotting using an anti-GSH antibody (Fig. 5). Protein pattern were completely lost when cells were treated with BSO for 24 h compared with control cells and during nutrient-starved conditions. This suggests that GSH depletion triggers autophagy in GC-1 cells perhaps by downregulating S-glutathionylated proteins.

AUTOPHAGY IS NECESSARY FOR GC-1 GERM CELL LINE SURVIVAL UNDER CONDITIONS OF GSH DEPLETION

Autophagy is a pro-survival response associated with a number physiological events [Degenhardt et al., 2010; Yang and Klionsky, 2010]. To evaluate whether autophagy is the mechanism by which

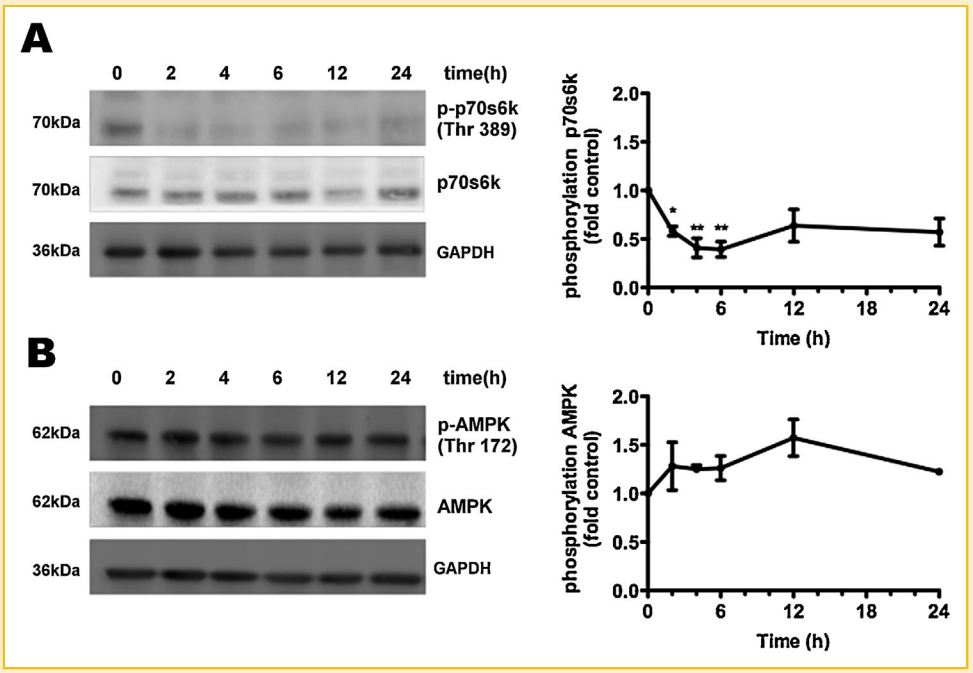

Fig. 4. GSH depletion-induced autophagy is AMPK independent in GC-1 germ cells. Cells were exposed to BSO (1 mM). Cell lysates were subjected to Western blot against, (A) p70s6k, (B) AMPK, and (A, B) GAPDH, at each time interval, where p indicates the phosphorylated forms. The figure corresponds to representative blots (left) and quantitative analysis of relative protein content (right). Data are mean ± SD of three independent experiments. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus 0 h (untreated cells).

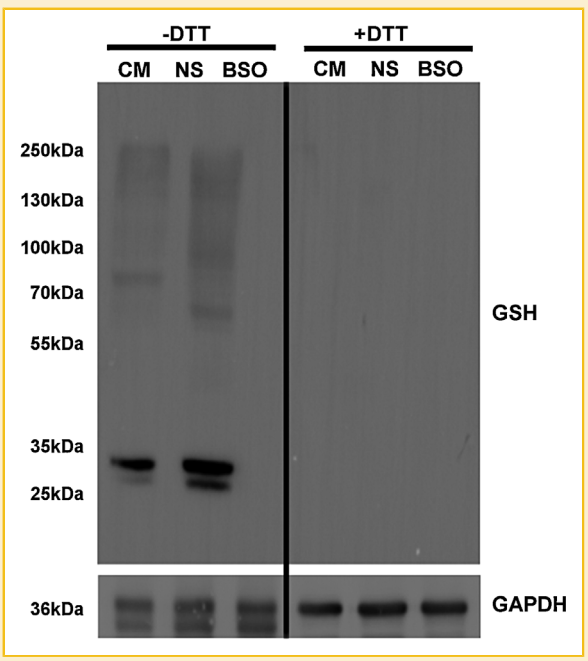

Fig. 5. S-glutathionylated protein pattern loss after GSH depletion in GC-1 germ cells. Cells were treated with BSO (1 mM) for 24 h. Nutrient starvation (NS) was used as a positive control of autophagy and complete medium (CM) was used as a negative control. Each treatment was resolved by SDS–PAGE in the absence (−DTT) or presence (+DTT) of 100 mM DTT (Dithiothreitol). Proteins (100 μg) were loaded on 8% SDS–PAGE and were then subjected to Western blot against GSH and GAPDH. The figure corresponds to a representative blot from three independent experiments.

spermatogonial germ cells survive during GSH deficiency, we measured the ATP content in GC-1 cells treated with bafilomycin A1 in the presence or absence of BSO for 24 h (Fig. 6A). We observed a difference in the ATP content between treatment times of 2 h and 12 h. To avoid the effect of bafilomycin A1 at 24 h, we also preincubated germ cells with or without BSO for 12 h and thereafter they were exposed to bafilomycin A1 for 12 h. ATP levels (Fig. 6B) and caspase-3/7 activity (Fig. 6C) were measured in order to evaluate cell viability during these treatments. No change was observed in ATP levels, although an increased caspase-3/7 activity was seen in cells treated with BSO and bafilomycin A1. These data suggest that an acute reduction in intracellular GSH concentrations may promote apoptosis in male germ cells (GC-1 cell line), although autophagy acts as a pro-survival response during GSH deficiency.

DISCUSSION

In this study, we evaluated the effect of GSH depletion on the survival of spermatogonial germ cells. Spermatogonia are located in the basal compartment of the seminiferous tubule and are highly tolerant to ROS compared to other germ cells [Paul et al., 2009]. Our results suggest that GSH deficiency stimulates autophagy in spermatogonial germ cells by an AMPK-independent manner, likely due to a downregulation of S-glutathionylated proteins. Moreover, induction of autophagy seems to act as a pro-survival response. Under conditions of GSH deficiency, no changes in ROS production and ATP levels were observed. These results are consistent with the high levels of Cu/Zn- and Zn-superoxide dismutase reported in

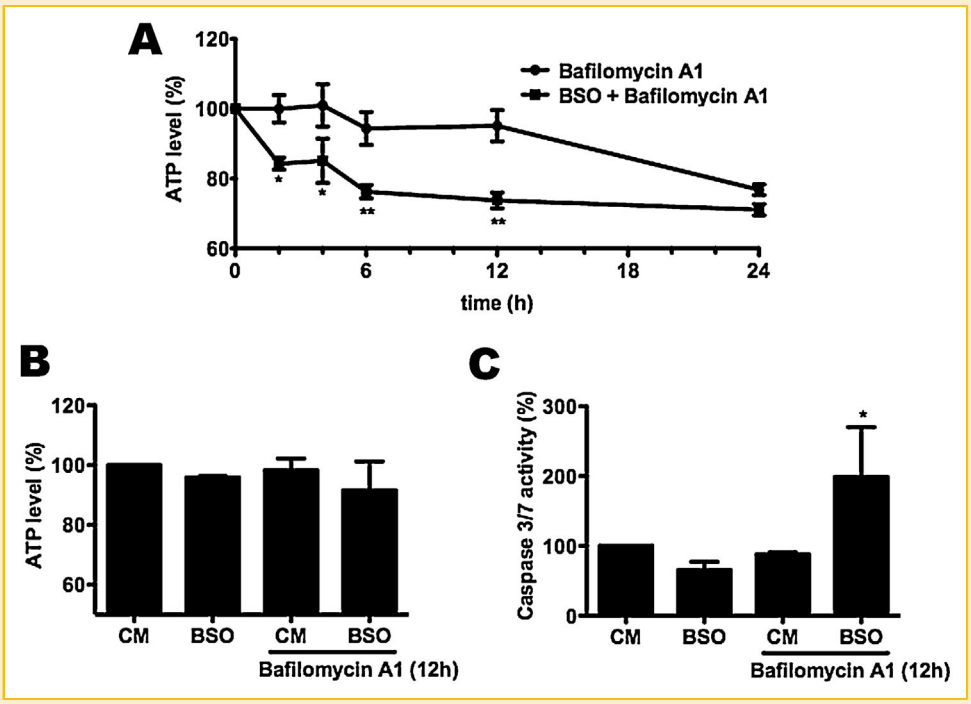

Fig. 6. Autophagy is necessary for GC-1 germ cell survival under conditions of GSH depletion. (A) Cells were treated with bafilomycin A1 (50 nM) and BSO (1 mM) or bafilomycin alone (50 nM) for a period of 24 h. Intracellular ATP levels were determined by luminescence using the luciferin–luciferase assay at each time interval. (B, C) Preincubation of cells with or without BSO (1 mM) for 12 h and subsequent exposure to bafilomycin A1 (50 nM) for 12 h. Intracellular ATP levels (B) and caspase-3/7 activity (C) were determined by luminescence using the luciferin–luciferase assay. Data were mean ± SD of three independent experiments. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus bafilomycin A1.

spermatogonia cells [Celino et al., 2011]. The high activity of these antioxidant enzymes probably confers resistance during GSH depletion. Nevertheless, Chi et al. [2007] showed that in glutathione-depleted NSC34 motor neuron-like cells incubated with ethacrynic acid (EA, chemical-mediated glutathione depletion) for up to 12 h or BSO for up to 48 h, ROS production, assayed with DCF probe, was higher with EA than BSO. ROS production in BSO-treated cells was similar to the control. [Chi et al., 2007]. Autophagy has been described as a pro-survival response during oxidative stress [Lee et al., 2012] and associated with GSH metabolism in several studies [Deffieu et al., 2009; Seo et al., 2011; Desideri et al., 2012]. Nevertheless, this present study directly correlates GSH level with autophagy induction in mammalian cells. We found an increased LC3II/LC3I ratio in autophagic flux assays and a change in the localization of endogenous LC3 protein in GC-1 cells during GSH deficiency (Fig. 3). No changes were observed in Beclin-1 level during glutathione depletion and nutrient starvation. However, we have supporting data that demonstrate Beclin-1 increase during nutrient starvation at 6 h in HeLa cells (data not shown). Several studies have demonstrated a Beclin-1-independent autophagy [Scarlatti et al., 2008; Tian et al., 2010; Grishchuk et al., 2011]. Regarding BSO treatment, it is unclear whether this event is dependent or independent of Beclin-1. No changes were observed in p62 levels over the 24 h period of BSO treatment. However, decreased p62 was observed when cells were incubated for 48 h in BSO (data not shown). p62 participates in the clearance of dysfunctional mitochondria during autophagy induction [Manley et al., 2013]. Cellular GSH is compartmentalized into distinct pools within the cytosol, mitochondria, endoplasmic reticulum, and nucleus [Meister, 1991]. Mitochondrial GSH (mtGSH) is an independent redox pool with a matrix GSH turnover rate of 30 h [Jocelyn and Kamminga, 1974; Garcia-Ruiz and Fernandez-Checa, 2006]. The method of GSH depletion used in this study does not completely deplete mitochondrial and nuclear GSH. Other agents, including EA, have been used to effectively deplete cellular, mitochondrial, and nuclear GSH [Keelan et al., 2001; Rizzardini et al., 2003]. We also saw no involvement from the AMPK pathway, and ATP levels remained unaltered in this process, suggesting that the mechanism of GSH depletion-induced autophagy is independent of energy metabolism. S-glutathionylation is the formation of a disulfide bond between GSH and cysteine residues of proteins and was initially described as a protein oxidation process. However, other studies showed that S-glutathionylation regulates protein function at post-translational levels [Ziegler, 1985; Gallogly and Mieyal, 2007; Dalle-Donne et al., 2009; Hill and Bhatnagar, 2012]. The downregulation of S-glutathionylation observed in our study may have a number of biological implications, including the regulation of autophagy in spermatogonial germ cells. The crosstalk between autophagy and apoptosis is very complex and still controversial. Different types of interplay (partnership or antagonist) between the autophagy and apoptosis have been indicated [Eisenberg-Lerner et al., 2009]. Autophagy antagonizes apoptotic cell death by promoting cell survival, through the removal

of damaged mitochondria (source of ROS) by catabolizing cellular macromolecules to provide energy fuels for the starved cell or by limiting ER stress through the degradation of protein aggregates. These processes block apoptotic response. Our results suggest that autophagy acts as a pro-survival response during GSH deficiency. We also observed an increase in caspase-3/7 activity in GSH-depleted GC-1 germs cells treated with bafilomycin A1, which blocks the fusion step of autophagy. The cytochrome P-450 2E1(CYP2E) plays an important role in the toxicity of ethanol, drugs, and carcinogens and is activated under various pathophysiological conditions, such as diabetes and obesity [Tanaka et al., 2000]. Wu and Caderbaum, [2013] showed that autophagy acts as a protective mechanism for the toxicity produced by several agents that activate CYP2E. They also observed a decrease in viability of HepG2 E47 cells when these cells were treated with BSO and 3-methyladenine [Wu and Cederbaum, 2013], a specific inhibitor of PI3K class III protein, which is used to block the initiation step of autophagy. These data agree with our results in GC-1 cells and suggest that autophagy may act as pro-survival response during glutathione deficiency.

This is the first study that evaluates the relationship between GSH depletion and autophagy induction in spermatogonial germ cells. Our findings indicate that low GSH levels act as a signal to activate the autophagy machinery. The exact molecular mechanism remains to be explored in future studies, but the present data suggest that a downregulation of the S-glutathionylation modification may activate this process in germ cells. Induction of autophagy in spermatogonial cells may also represent another mechanism that provides tolerance during stressful conditions, such as GSH deficiency.

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