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Enhanced glutathione biosynthetic capacity promotes resistance to As3+-induced apoptosis

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Abstract

Trivalent arsenite (As3+) is a known human carcinogen capable of inducing both cellular transformation and apoptotic cell death by mechanisms involving the production of reactive oxygen species. The tripeptide antioxidant glutathione (GSH) constitutes a vital cellular defense mechanism against oxidative stress. While intracellular levels of GSH are an important determinant of cellular susceptibility to undergo apoptotic cell death, it is not known whether cellular GSH biosynthetic capacity *per se* regulates As3+-induced apoptosis. The rate-limiting enzyme in GSH biosynthesis is glutamate cysteine ligase (GCL), a heterodimeric holoenzyme composed of a catalytic (GCLC) and a modifier (GCLM) subunit. To determine whether increased GSH biosynthetic capacity enhanced cellular resistance to As3+-induced apoptotic cell death, we utilized a mouse liver hepatoma (Hepa-1c1c7) cell line stably overexpressing both GCLC and GCLM. Overexpression of the GCL subunits increased GCL holoenzyme formation and activity and inhibited As3+-induced apoptosis. This cytoprotective effect was associated with a decrease in As3+-induced caspase activation, cleavage of caspase substrates and translocation of cytochrome c to the cytoplasm. In aggregate, these findings demonstrate that enhanced GSH biosynthetic capacity promotes resistance to As3+-induced apoptosis by preventing mitochondrial dysfunction and cytochrome c release and highlight the role of the GSH antioxidant defense system in dictating hepatocyte sensitivity to As3+-induced apoptotic cell death.

Keywords

arsenite; arsenic; glutathione; apoptosis; glutamate cysteine ligase; GCL

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The authors declare that there are no conflicts of interest

Introduction

Inorganic arsenic is a known human carcinogen associated with the development of cancers of the skin, bladder, lung, kidney and liver (Yoshida *et al*., 2004). Chronic exposure to low levels of trivalent arsenite (As3+) also promotes cell proliferation and malignant transformation in various cultured cell models *in vitro* (Zhao *et al*., 1997; Huang *et al*., 1999; Achanzar *et al*., 2002; Chien *et al*., 2004; Sens *et al*., 2004). In contrast, acute exposure to high concentrations of As3+ induces apoptotic cell death (Bode and Dong, 2002). Paradoxically, although As3+ is a known carcinogen, As3+ in the form of arsenic trioxide (ATO) is a highly effective chemotherapeutic in the treatment of acute promyelocytic leukemia (Bode and Dong, 2002; Miller *et al*., 2002). While the molecular mechanisms mediating As3+-induced transformation and apoptosis remain unclear, increased production of reactive oxygen species (ROS) has been implicated in both As3+-induced genotoxicity and cytotoxicity (Kitchin and Ahmad, 2003). Mammalian cells possess a number of antioxidant defense mechanisms to protect against oxidative stress. The tripeptide antioxidant glutathione (GSH) is a highly abundant and particularly effective means of protecting against oxidative injury (Griffith and Mulcahy, 1999). Interestingly, chronic exposure to sub-toxic doses of As3+ induces an adaptive response whereby cells become resistant to acute As3+-induced apoptosis (Romach *et al*., 2000; Brambila *et al*., 2002; Chien *et al*., 2004; Somji *et al*., 2006). This acquired tolerance to As3+ toxicity occurs in concert with malignant transformation, suggesting that this may provide a selective growth advantage during As3+-induced cellular transformation. The development of this resistant phenotype is associated with elevated intracellular GSH levels and increased expression of various detoxification and antioxidant enzymes, including enzymes involved in GSH biosynthesis, metabolism, and transport (Qu *et al*., 2001; Brambila *et al*., 2002; Chien *et al*., 2004; Coppin *et al*., 2008). While microarray studies have identified numerous gene products that could potentially mediate this apoptotic resistance (Chen *et al*., 2001a; Chen *et al*., 2001b; Hamadeh *et al*., 2002), inhibition of GSH biosynthesis alone is sufficient to sensitize As3+-transformed rat liver epithelial cells and human prostate cells to As3+-induced apoptosis (Liu *et al*., 2001a; Brambila *et al*., 2002). These findings provide compelling evidence that up-regulation of GSH homeostasis contributes to acquired tolerance to As3+ during chronic As3+ exposure.

GSH homeostasis is dependent on GSH biosynthesis, utilization and export. The inability of most cells to import GSH highlights the importance of *de novo* GSH biosynthesis in maintaining GSH homeostasis. GSH biosynthetic capacity is dependent on several factors, including substrate availability and glutamate cysteine ligase (GCL) activity (Griffith and Mulcahy, 1999). GCL mediates the rate-limiting step in GSH biosynthesis and is a heterodimeric holoenzyme composed of a catalytic (GCLC) and a regulatory (GCLM) subunit (Griffith and Mulcahy, 1999). Cellular GCL activity is governed mainly by the relative levels of the GCL subunits which are highly regulated by transcriptional control mechanisms (Wild and Mulcahy, 2000; Franklin *et al*., 2009; Lu, 2009). We and others have demonstrated that both acute and chronic exposure to sub-toxic concentrations of As3+ coordinately induce GCL subunit expression resulting in increased cellular GCL activity and GSH (Li *et al*., 2002; Schuliga *et al*., 2002; Pi *et al*., 2003; Coppin *et al*., 2008; Pi *et al*., 2008; Thompson *et al*., 2009). There is also strong evidence that alterations in GSH levels play an important role in dictating cellular sensitivity to As3+-induced apoptotic cell death (Bode and Dong, 2002; Miller *et al*., 2002). This is based on both comparative analyses and acute manipulation of cellular GSH levels. In this regard, increasing cellular GSH levels with the GSH precursor N-acetylcysteine (NAC) promotes cellular resistance to As3+-induced apoptosis, while depletion of cellular GSH levels with the GCLC inhibitor buthionine sulfoximine (BSO) dramatically potentiates As3+-induced apoptosis (Bode and Dong, 2002; Miller *et al*., 2002). Indeed, sufficiently elevating cellular GSH levels can abolish As3+-induced apoptosis, while depletion of cellular GSH is capable of enhancing As3+ toxicity in rat liver epithelial cells by an order

of magnitude (Shimizu *et al*., 1998; Bode and Dong, 2002; Miller *et al*., 2002). While these findings clearly demonstrate that cellular sensitivity to As3+-induced apoptosis is inversely related to cellular GSH levels, they provide no information on the functional effects of altered GSH biosynthetic capacity *per se*.

GSH homeostasis is disrupted in most models of apoptotic cell death (Circu and Aw, 2008). This is mainly the result of the rapid depletion of cellular GSH levels due to extrusion of reduced GSH (Circu and Aw, 2008). However, altered GSH biosynthesis may also be a contributing factor as we have demonstrated that GCLC is a direct target for caspase-mediated cleavage during apoptosis (Siitonen *et al*., 1999; Pierce *et al*., 2000; Franklin *et al*., 2002; Franklin *et al*., 2003). While it is still unclear how altered GSH homeostasis affects apoptotic cell death, GSH is thought to function at the level of the mitochondrion to prevent the loss of mitochondrial membrane potential and release of pro-apoptotic factors into the cytoplasm (Circu and Aw, 2008). Consistent with this working model, we have found that increased GCL activity resulting from GCL overexpression inhibits TNF-induced apoptosis by maintaining mitochondrial integrity and preventing the release of cytochrome c (Botta *et al*., 2004). While TNF induces by cytochrome c release indirectly via Bid-mediated mitochondrial dysfunction, As3+ initiates cytochrome c release and activation of the apoptotic machinery by a direct effect on the mitochondrion (Larochette *et al*., 1999; Costantini *et al*., 2000). Based on the functional similarities of these cell death pathways, we believe that increased GSH biosynthetic capacity should also promote resistance to As3+-induced apoptosis.

In this study, we directly examined whether increased expression of the GCL subunits and enhanced GSH biosynthetic capacity promotes cellular resistance to apoptotic cell death utilizing an established mouse liver hepatoma (Hepa-1c1c7) cell line overexpressing GCLC and GCLM (Botta *et al*., 2004). Hepa-1c1c7 cells are an excellent model to selectively examine the effects of increased GCL subunit expression and GCL activity as the relative levels of the GCL subunits, and not substrate availability, are limiting for GSH biosynthesis (Shertzer *et al*., 1995). Furthermore, Hepa-1c1c7 cells overexpressing the GCL subunits exhibit increased GSH biosynthetic capacity, but only a modest increase in cellular GSH content (Botta *et al*., 2004). GCL overexpression was found to suppress As3+-induced translocation of cytochrome c to the cytoplasm and caspase activation, and inhibit As3+-induced apoptosis. These findings provide proof-of-principle that up-regulation of GSH biosynthesis could mediate apoptotic resistance, promote cell survival and provide a selective growth advantage during chronic As3 + exposure.

Materials and Methods

Reagents

$NaAsO_2$, 4′,6-diamidino-2-phenylindole (DAPI), and digitonin were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents were prepared in either H_2O, sterile phosphate-buffered saline (PBS) or DMSO. z-VAD-fmk was from Bachem Bioscience (Torrance, CA) and Ac-DEVDAMC, Ac-IETD-AMC, and Ac-LEHD-AMC were from Alexis Biochemicals (San Diego, CA).

Cell culture and treatments

Murine Hepa-1c1c7 cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM/F12 media (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured at 37C in a humidified atmosphere of 95% air/5% CO_2. Hepa-1c1c7 cell lines expressing murine *GCLC* and *GCLM* expression vectors (Hepa-CR17) or a pMC1-neo vector (Hepa-V3) (Stratagene, La Jolla, CA) were established as previously described (Botta *et al*., 2004). Cells were seeded at 1.0-1.5 ×

10^6 cells per 6-cm dish 18–24 h before treatment and all experiments were performed at 90-100% confluency.

Immunoblot analysis

Cells were harvested and lysed by a brief sonication on ice in TES/SB buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 250 mM sucrose, 20 mM boric acid, 1 mM L-serine) containing 1× Complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Lysates were clarified by centrifugation at 13,000 × g for 10 min at 4C and protein quantified by the Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of soluble protein (20 ug) were resolved on 10% SDSpolyacrylamide gels and transferred to PVDF membranes (Millipore, Bedford, MA). For analysis of GCL holoenzyme formation whole cell extracts were prepared in the absence of reducing equivalents and were resolved on native 10% PAGE gels in the absence of SDS. Samples for these studies were not boiled prior to gel loading and the gels were resolved in Tris/Glycine buffer lacking SDS at 4C prior to transfer to PVDF membranes. Membranes were blocked in Tris-buffered saline/0.1%Tween-20 (TBST) containing 5% non-fat milk prior to incubation with primary antibody in TBST containing 0.5% milk. Membranes were probed for GCLC, GCLM (Thompson *et al*., 1999; Franklin *et al*., 2002; Franklin *et al*., 2003; Thompson *et al*., 2009), βActin (Sigma), pro-caspase-3, cytochrome c (BD Biosciences, San Diego, CA), Bid (R&D Systems, Minneapolis, MN), GADD153 and ATF-3 (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-mouse-HRP and anti-rabbit HRP secondary antibodies (Amersham Biosciences) were used at 1:5,000 in TBST containing 0.5% milk. Antigen-antibody complexes were detected with Western Lightning Chemoluminescent Reagent (PerkinElmer, Boston, MA).

To detect translocation of cytochrome c from the mitochondria to cytosol, cells were fractionated by a rapid digitonin lysis procedure (Single *et al*., 1998). Cells were incubated on ice for 5 min in digitonin lysis buffer (DLB; 75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose) containing 20 ug digitonin per 1×10^6 cells. Lysates were centrifuged at 13,000 × g for 5 min at 4C. Supernatants were recovered and the pellets lysed by sonication in DLB and centrifuged as described above. Soluble protein (10 ug) from the fractions was resolved on 15% SDS-polyacrylamide gels and analyzed for cytochrome c by immunoblotting as described above.

GSH and GCL activity assays

Total GSH content (GSH + GSSG) was determined by a modification of the Tietze assay (Baker *et al*., 1990). Cell extracts were prepared by sonication in TES/SB as described above (Thompson *et al*., 1999; Franklin *et al*., 2002; Franklin *et al*., 2003) and GSH levels were determined against a standard curve of GSSG and levels calculated per μg of soluble protein in the original cell extract. This value was utilized to determine the relative change in intracellular GSH levels compared to untreated samples. GCL activity was measured by a fluorescence-based NDA assay as described previously (White *et al*., 2003).

Apoptosis and caspase assays

Apoptosis was quantified by assessing apoptotic nuclear morphology of DAPI-stained cells (Pierce *et al*., 2000). Cells were harvested, fixed in 70% ethanol and either analyzed immediately or stored at −20C. After a brief centrifugation, cells were resuspended in a solution containing 0.5% Nonidet P-40 and 10 ug/ml DAPI and analyzed by fluorescence microscopy. Caspase activities were measured using fluorogenic substrates as previously described (Franklin *et al*., 1998; Franklin *et al*., 2002; Franklin *et al*., 2003). Soluble protein extract (5–25 μg) was added to an equal volume of 2× caspase cleavage buffer (40 mM PIPES, pH 7.2, 200 mM NaCl, 20% sucrose, 0.2% CHAPS, 20 mM DTT) containing 40 μM fluorogenic substrate (Ac-DEVD-AMC for caspase-3, AcIETD-AMC for caspase-8, or Ac-LEHD-AMC

for caspase-9). Reactions were carried out at 37C, and fluorescence was monitored on a SpectraMax Gemini EM (Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Substrate autofluorescence was subtracted from each point, and specific activity was calculated against a standard curve of AMC (Sigma, St. Louis, MO).

Statistical analysis

Data are presented as averages +/− SEM of at least three experiments. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA). Results were compared by one-way or two-way ANOVA with Tukey's post test and mean differences were considered significant when $p < 0.05$.

Results

As3+-induced apoptosis in parental Hepa-1c1c7 cells

As3+ exhibits clear dose-dependent effects in most cultured cell systems, with low levels promoting cell proliferation and transformation and higher concentrations inducing apoptosis (Bode and Dong, 2002). To determine whether Hepa-1c1c7 cells were sensitive to As3+-induced apoptosis, cells were treated with varying concentrations of As3+ for 16 h and apoptosis was quantified by assessing apoptotic nuclear morphology of DAPI-stained cells (Pierce *et al*., 2000). In contrast to previous studies (Elbekai and El-Kadi, 2004), we found that As3+ was highly cytotoxic in the Hepa-1c1c7 cell line. As3+-induced apoptotic cell death was dose-dependent with near maximal apoptosis occurring at 20 uM As3+ (Fig. 1A, left panel), while evidence of necrotic cell death was observed at higher concentrations (data not shown). For this reason, 20 uM As3+ was employed for all future studies. As3+-induced apoptosis in Hepa-1c1c7 was also time-dependent, with significant apoptotic cell death (~20%) occurring within 12 h of treatment and ~80% of cells appearing apoptotic within 24 h (Fig. 1A, right panel). As3+-induced apoptosis was accompanied by a coordinate increase in the activities of caspases-3/8/9 as measured utilizing fluorogenic caspase substrates (Fig. 1B). The time- and dose-dependent activation of these caspases correlated well with the relative level of apoptotic cell death. Importantly, As3+-induced apoptosis was found to be caspase-dependent as pretreatment with the pan-caspase inhibitor z-VAD-fmk nearly abolished As3+-induced apoptotic cell death (Fig. 1C). These findings demonstrate that Hepa-1c1c7 cells are sensitive to As3+-induced apoptotic cell death, which occurs in a caspase-dependent manner.

Hepa-1c1c7 cells overexpressing murine *GCLC* and *GCLM* exhibit enhanced GSH biosynthetic capacity

While As3+-induced apoptosis is highly sensitive to intracellular GSH levels (Bode and Dong, 2002), the goal of this study was to examine whether enhanced GSH biosynthetic capacity *per se* promotes resistance to As3+-induced apoptosis. To directly examine this hypothesis, we employed a clonally derived Hepa-1c1c7 cell line overexpressing both murine *GCLC* and *GCLM* (Hepa-CR17) (Botta *et al*., 2004). The Hepa-1c1c7 cell line was chosen for these studies as GCL subunit expression, and not cysteine availability, is limiting for GSH biosynthesis in these cells (Shertzer *et al*., 1995). While GCL subunit expression is under the control of a heavy metal-inducible metallothionein promoter in the Hepa-CR17 cell line, basal GCL subunit expression and GCL activity are constitutively elevated in CR17 cells and all studies were performed in the absence of heavy metals (Botta *et al*., 2004). As previously reported (Botta *et al*., 2004), Hepa-CR17 cells expressed 2-3 fold more GCLC and GCLM protein than the vector control cell line (Hepa-V3) (Fig. 2A). Overexpression of the GCL subunits also led to increased GCL holoenzyme formation as detected by resolving whole cell extracts by native gel electrophoresis and immunoblotting for GCLC (Fig. 2B). Importantly, increased GCL subunit expression and GCL holoenzyme formation was associated with a significant ~2.5-

fold increase in cellular GCL activity (Fig. 2C), but only a modest increase in GSH content (Fig. 2D).

Enhanced GSH biosynthetic capacity promotes resistance to As3+-induced apoptosis

To determine whether GCL overexpression altered Hepa cell sensitivity to As3+-induced apoptosis, V3 and CR17 cells were treated with 20 uM As3+ for various time periods and analyzed for apoptotic cell death (Pierce *et al.*, 2000). Similar to our findings in the parental Hepa-1c1c7 cell line (Fig. 1), treatment of Hepa-V3 cells with 20 uM As3+ resulted in a time-dependent induction of apoptosis as judged by cytoplasmic shrinkage, membrane blebbing, and the appearance of condensed nuclei on cell staining with DAPI, with ~75% of the cells displaying apoptotic nuclear morphology at 24 h (Fig. 3, open bars). In contrast, Hepa-CR17 cells were highly resistant to As3+-induced apoptosis, with only ~15% of cells exhibiting apoptotic nuclear morphology at 24 h (Fig. 3, closed bars).

GCL overexpression attenuates As3+-induced cytochrome c release and caspase-9 activation

As3+-induced apoptosis occurs via activation of the intrinsic cell death pathway resulting in mitochondrial dysfunction, release of cytochrome c and other apoptotic factors, and activation of caspase-9 (Larochette *et al.*, 1999; Costantini *et al.*, 2000). In an attempt to elucidate the molecular mechanism(s) mediating the protective effects of GCL overexpression, we initially examined whether increased GSH biosynthesis suppressed As3+-induced apoptosis at the level of the mitochondria by assessing As3+-induced translocation of cytochrome c in the Hepa-V3 and CR17 cell lines. Cells were treated with As3+ and cytosolic and mitochondrial fractions isolated and analyzed for cytochrome c content by immunoblotting. As shown in Figure 4A, As3+ treatment caused the time-dependent translocation of cytochrome c from the mitochondrial fraction to the cytosolic fraction in Hepa-V3 cells. In contrast, As3+-induced cytochrome c translocation in the Hepa-CR17 cell line was attenuated and delayed. As cytochrome c leads to caspase-9 activation via apoptosome formation (Riedl and Salvesen, 2007), we determined whether suppression of cytochrome c release was associated with a concomitant decrease in As3+-induced activation of caspase-9. As3+ caused the time-dependent activation of LEHDase activity in the Hepa-V3 cell line, which was significantly reduced in Hepa-CR17 cells (Fig. 4B). These findings support a model in which enhanced GSH biosynthetic capacity suppresses As3+-induced apoptosis by preventing cytochrome c release from the mitochondria and caspase-9 activation.

GCL overexpression attenuates As3+-induced activation of caspases-3/8

Caspase-9 leads to the activation of effector caspases, such as caspase-3, that mediate execution of the apoptotic program (Riedl and Salvesen, 2007). Caspase-3 also serves in an amplification loop via feedback activation of caspase-8, which can then enhance mitochondrial dysfunction and apoptotic cell death via cleavage and activation of Bid (Slee *et al.*, 1999). To further elucidate the molecular mechanism(s) mediating the protective effects of GCL overexpression, we examined whether increased GSH biosynthesis suppressed activation of these caspase pathways. Similar to parental Hepa-1c1c7 cells, As3+ induced the time-dependent activation of both caspase-3 and -8 activities in the Hepa-V3 cell line (Fig. 5A and 5B, open bars). In contrast, As3+-induced activation of these caspases was significantly reduced in the CR17 cell line (Fig. 5A and 5B, closed bars). The decrease in As3+-induced DEVDase activity in the CR17 cell line correlated with reduced processing and cleavage of the 32 kDa pro-caspase-3 to its 17 kDa active fragment (Fig. 5C, upper panel). Similarly, the cleavage and loss of Bid, an established endogenous caspase-8 target protein (Li *et al.*, 1998), was dramatically reduced in the Hepa-CR17 cell line (Fig. 5C, bottom panel). Importantly, cytochrome c release, caspase activation, and the cleavage of cellular caspase targets correlated both temporally and in

magnitude with the onset of As3+-induced apoptotic morphology and cell death in the V3 and CR17 cell lines. In aggregate, these findings indicate that enhanced GSH biosynthetic capacity suppresses As3+-induced mitochondrial dysfunction, cytochrome c release, and activation of the caspase cascade.

Caspase inhibition does not prevent As3+-induced cytochrome c release

The results in Figure 1 demonstrate that As3+-induced apoptosis in Hepa-1c1c7 cells is dependent on caspase activation. However, while As3+-induced cytochrome c release and caspase activation are both suppressed in Hepa-CR17 cells, it is not clear which of these events mediates the protective effects of GCL overexpression. To determine whether enhanced GSH biosynthesis protects against As3+-induced apoptosis by preventing cytochrome c release or suppressing caspase activity, we examined the effects of the pan-caspase inhibitor z-VAD-fmk on As3+-induced cytochrome c release. Pretreatment with zVAD-fmk abolished cellular caspase activity as judged by the inhibition pro-caspase-3 cleavage and processing and cleavage of GCLC, a known caspase-3 target protein (Franklin *et al.*, 2002) (Fig. 6, upper panels). However, in contrast to GCL overexpression, caspase inhibition had no effect on As3+-induced cytochrome c release (bottom panels). These differential effects suggest that enhanced GSH biosynthetic capacity attenuates As3+-induced apoptosis by preventing cytochrome c release at a site upstream of caspase activation.

GCL overexpression does not abolish As3+-induced protein expression

As3+ metabolism involves GSH conjugation reactions and it is possible that GCL overexpression reduces As3+-induced apoptosis via transporter-mediated efflux of As3+ as a GSH conjugate (Leslie *et al.*, 2004). To test this hypothesis, we examined whether other As3+-induced cellular responses were also attenuated in the Hepa-CR17 cell line. As3+ induces the expression of ATF-3 and GADD153 in a variety of cell types (Guyton *et al.*, 1996; Fawcett *et al.*, 1999). Immunoblot analysis of ATF-3 and GADD153 expression indicates that As3+ treatment causes a similar potent and prolonged induction of ATF-3 and GADD153 protein expression in both the Hepa-V3 and Hepa-CR17 cell lines (Fig. 7). Thus, while GCL overexpression inhibits As3+-induced apoptosis, it does not abolish all As3+-induced cellular responses.

Discussion

Inorganic As3+ is a known human carcinogen and the development of relevant cultured cell models has permitted examination of the molecular events associated with As3+-induced transformation. Chronic low level exposure to As3+ leads to malignant transformation *in vitro* which is associated with the development of As3+ adaptation, whereby cells become resistant to acute As3+ toxicity (Liu *et al.*, 2001a; Brambila *et al.*, 2002; Leslie *et al.*, 2004; Coppin *et al.*, 2008). Microarray studies indicate that this adaptation involves the up-regulation of numerous detoxification and antioxidant enzymes, including proteins involved in GSH biosynthesis, metabolism, and efflux (Chen *et al.*, 2001a; Liu *et al.*, 2001a; Lu *et al.*, 2001; Hamadeh *et al.*, 2002; Liu *et al.*, 2004). In this study, we directly examined whether increased cellular GSH biosynthetic capacity alone could inhibit As3+-induced apoptosis and account for this resistant phenotype. For these studies we employed Hepa-1c1c7 cells stably transfected with both GCL subunits, which resulted in a 2-3-fold increase in GCLC and GCLM protein expression and an ~2.5 fold increase in GCL activity compared to vector-transfected cells (Fig. 2, (Botta *et al.*, 2004)). These relative increases correlate well with those observed in As3+-transformed cells in culture (Coppin *et al.*, 2008). However, in contrast to As3+-transformed cells which often contain large increases in GSH levels (Qu *et al.*, 2001; Brambila *et al.*, 2002; Chien *et al.*, 2004; Coppin *et al.*, 2008), GCL overexpression only led to a modest increase in GSH levels. Importantly, GCL overexpression nearly abolished As3+-induced

apoptosis in Hepa-1c1c7 cells. While cellular GSH levels can dictate cellular sensitivity to As3+-induced apoptosis, it has been postulated that increased GCL activity and cellular GSH biosynthetic capacity rather than elevated GSH levels mediates the development of apoptotic resistance in transformed cells (Yang *et al.*, 2002). Thus, reestablishing cellular GSH homeostasis after acute As3+ exposure via rapid resynthesis of depleted GSH stores may play a more significant role in mediating the cytoprotective effects of GCL overexpression than elevation of static GSH levels *per se*. The ability of GCL overexpression to prevent As3+-induced apoptosis while having only a modest effect on cellular GSH levels provides support for this hypothesis.

The ability of enhanced GSH biosynthetic capacity to suppress As3+-induced apoptosis could be derivative of either the antioxidant or metabolic/detoxification properties of GSH. GSH could directly scavenge As3+-induced ROS or act indirectly as a co-factor in glutathione peroxidase-mediated reduction of hydrogen or lipid peroxides (Griffith and Mulcahy, 1999). Alternatively, suppression of As3+-induced toxicity could be mediated via GST-mediated GSH conjugation and detoxification of As3+ (Griffith and Mulcahy, 1999). Indeed, transporter-mediated efflux of As3+ as a GSH conjugate is thought to contribute to As3+ adaptation (Liu *et al.*, 2001a; Leslie *et al.*, 2004; Coppin *et al.*, 2008). However, As3+-induced ATF-3 and GADD153 expression were not suppressed in Hepa-CR17 cells, suggesting that the anti-apoptotic effects of GCL overexpression were not due to GSH-mediated metabolism/detoxification of As3+, which would inhibit all As3+-mediated cellular responses. Thus, while GCL overexpression inhibits As3+-induced apoptosis, it does not abolish all As3+-induced signaling events that may be required for malignant transformation (Hamadeh *et al.*, 2002; Trouba *et al.*, 2002). However, it is not known whether acquired tolerance to As3+ occurs during, or in response to As3+-induced transformation. This is an important distinction when attempting to ascertain whether this resistant phenotype provides a selective growth advantage and contributes to As3+-induced malignant transformation.

As3+ initiates activation of the apoptotic machinery via direct effects on mitochondrial membrane proteins and subsequent release of pro-apoptotic factors from the mitochondria (Larochette *et al.*, 1999; Costantini *et al.*, 2000). While both enhanced GSH biosynthetic capacity and caspase inhibition prevented As3+-induced apoptosis, only GCL overexpression inhibited cytochrome c release. This is consistent with previous studies demonstrating that caspase activation is not necessary for cytochrome c release during chemical-induced apoptosis (Sun *et al.*, 1999). These findings also suggest enhanced GSH biosynthesis inhibits As3+-induced apoptosis at the level of cytochrome c release and not via inhibition of downstream caspase activation. Furthermore, while As3+ does not cause a significant depletion of mitochondrial GSH (Bustamante *et al.*, 2005), As3+-induced mitochondrial dysfunction is thought to be in equilibrium with mitochondrial GSH levels (Costantini *et al.*, 1996). GSH biosynthesis occurs in the cytoplasm and mitochondrial GSH levels are maintained by high affinity transport of cytosolic GSH across the mitochondrial inner membrane (Lash, 2006). While mitochondrial pools of GSH are distinct from cytosolic pools and are preserved even when cytosolic GSH levels are significantly depleted (Soderdahl *et al.*, 2003), mitochondrial GSH levels are nonetheless sensitive to changes in cytosolic GSH biosynthesis (Chen *et al.*, 2007). Recent studies indicate that Bcl-2 also regulates an essential mitochondrial GSH pool that plays a critical role in maintaining mitochondrial redox homeostasis (Zimmermann *et al.*, 2007). Importantly, mitochondria have been identified as the source of As3+-induced ROS production (Pourahmad *et al.*, 2003) and GSH plays a critical co-factor role in glutathione peroxidase-mediated reduction of hydrogen peroxide and lipid peroxides within the mitochondria (Orrenius *et al.*, 2007). GSH also serves as a co-factor for mitochondrial localized glutaredoxin 2 that reduces protein disulfide and mixed disulfides (Orrenius *et al.*, 2007). Thus, GSH may serve to maintain mitochondrial redox homeostasis and counteract the deleterious effects of As3+-induced mitochondrial-generated ROS, preventing mitochondrial dysfunction

and release of pro-apoptotic factors such as cytochrome c. Interestingly, mitochondrial damage and subsequent ROS production may also play a causal role in As3+-induced genotoxicity and carcinogenesis (Liu *et al.*, 2001b; Liu *et al.*, 2005).

In summary, these findings indicate that enhanced GSH biosynthesis resulting from increased expression of the GCL subunits can inhibit As3+-induced apoptosis. This cytoprotective effect may play an important role in the development of apoptotic resistance during As3+-induced malignant transformation which is associated with increased GCL subunit expression and GSH homeostasis and can be reversed by inhibition of GCL activity (Liu *et al.*, 2001a; Brambila *et al.*, 2002). However, it is important to note that As3+ adaptation is associated with the up-regulation of a number of proteins involved in GSH metabolism that could also contribute to this resistant phenotype, including various GSTs and ABCC transporters (Chen *et al.*, 2001a; Liu *et al.*, 2001a; Lu *et al.*, 2001; Hamadeh *et al.*, 2002; Liu *et al.*, 2004). In fact, acquired tolerance to As3+-induced toxicity can be reversed by inhibition of GCL, GST or ABCC1 transporter activity (Liu *et al.*, 2001a; Brambila *et al.*, 2002). Thus, while enhanced GSH biosynthesis may play an important role in apoptotic resistance in As3+-transformed cells, additional proteins involved in GSH metabolism and efflux likely contribute to this resistant phenotype.

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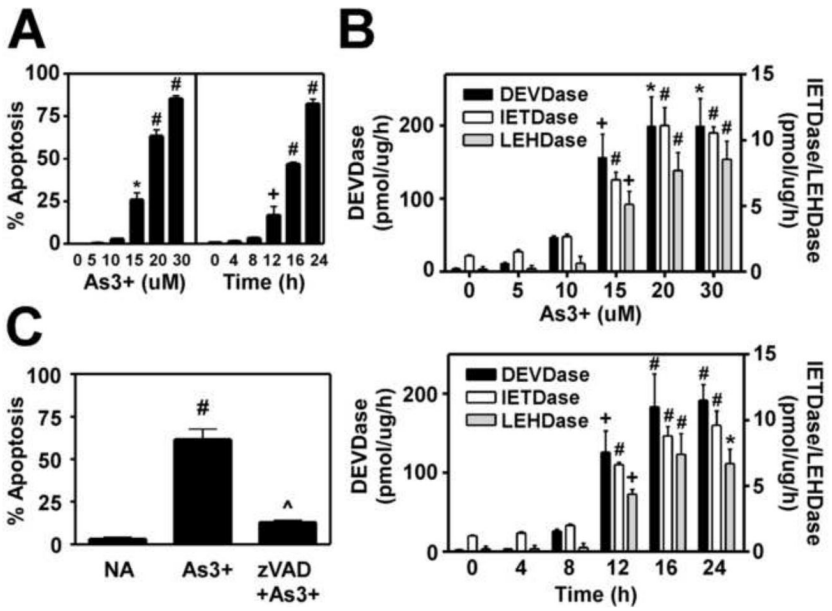

Figure 1. Time- and dose-dependent As3+-induced apoptosis in Hepa-1c1c7 cells

(A and B) Hepa-1c1c7 cells were treated for 16 h with the indicated concentrations of As3+ or with 20 uM As3+ for the time periods indicated. (A) As3+-induced apoptosis was quantified by assessing apoptotic nuclear morphology of DAPI-stained cells (Pierce *et al.*, 2000). (B) Caspase activities were determined as described in the Methods section utilizing the fluorogenic substrates Ac-DEVD-AMC (caspase-3), AcIETD-AMC (caspase-8), and Ac-LEHD-AMC (caspase-9). (C) Cells were pretreated with z-VAD-fmk (50 uM) for 1 h prior to treatment with As3+ (20 uM) for 16 h and apoptosis quantified as described above. Data presented are averages +/- SEM of at least three experiments. + $p < 0.05$, * $p < 0.01$, # $p < 0.001$, indicates a significant difference compared to untreated control, and ^ $p < 0.001$ indicates a significant difference from As3+-only treated cells.

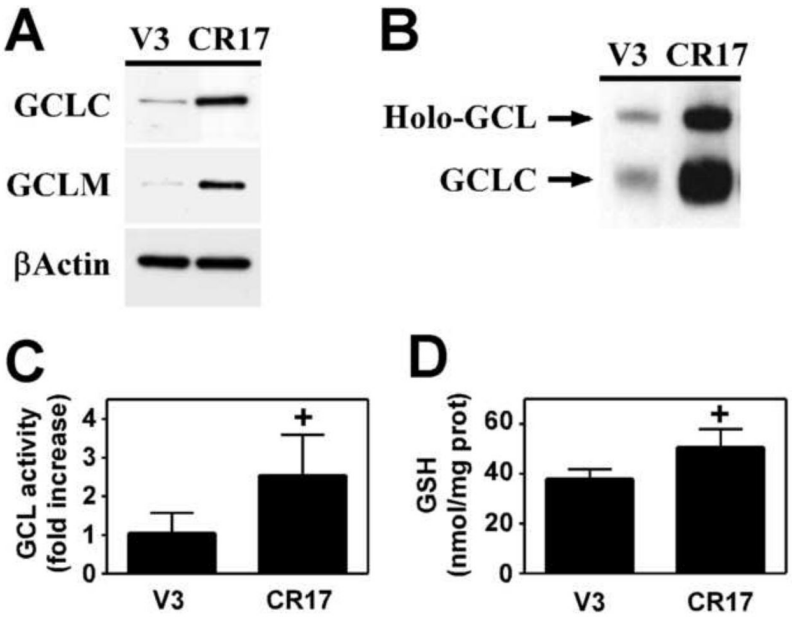

Figure 2. Hepa-1c1c7 cells overexpressing GCLC and GCLM exhibit enhanced GCL activity

Hepa-1c1c7 cells were transfected with pMC1-neo vector alone (V3) or together with *GCLC* and *GCLM* expression vectors (CR17) and stable cell lines established as previously described (Botta *et al.*, 2004). (A) GCLC, GCLM, and βActin protein expression were analyzed by immunoblotting. (B) GCL holoenzyme formation was assessed by native gel electrophoresis and immunoblotting for GCLC. (C) GCL activity was measured by a fluorescence-based NDA assay as previously described (White *et al.*, 2003). (D) Total GSH levels (GSH + GSSG) were measured by a modified Tietze assay (Baker *et al.*, 1990). Data presented are averages +/− SEM of at least three experiments. + $p < 0.05$, indicates a significant difference compared to the Hepa-V3 vector control cell line.

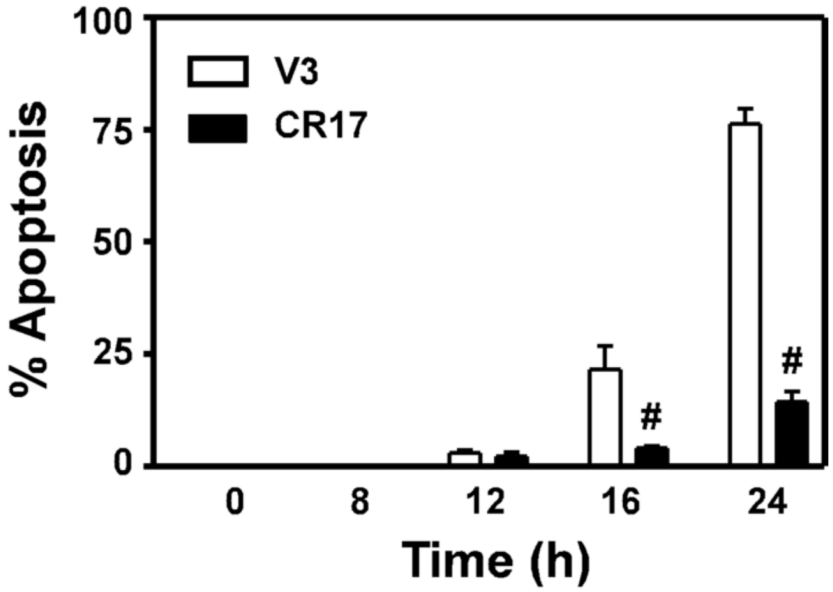

Figure 3. Enhanced GSH biosynthetic capacity inhibits As3+-induced apoptosis

Hepa-V3 and Hepa-CR17 cells were treated with 20 uM As3+ for the time periods indicated. Cells were stained with DAPI and apoptosis was quantified by assessing apoptotic nuclear morphology by fluorescence microscopy. Data presented are averages +/− SEM of at least three experiments. # $p < 0.001$, indicates a significant difference compared to As3+-induced apoptosis in Hepa-V3 cells at that time point.

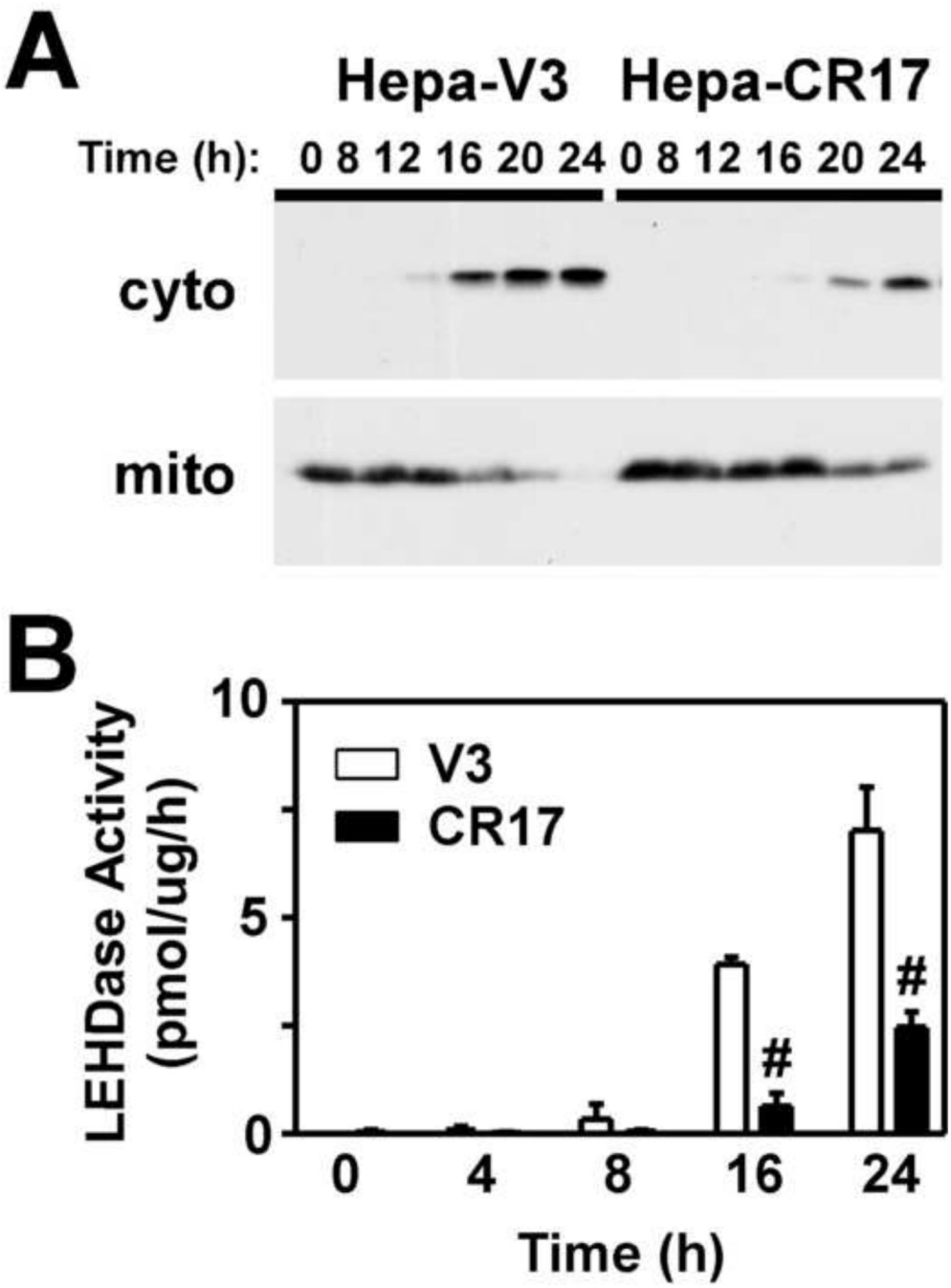

Figure 4. Overexpression of GCL inhibits As3+-induced cytochrome c release and caspase-9 activation

Hepa-V3 and Hepa-CR17 cells were treated with 20 uM As3+ for the time periods indicated. (A) Cytosolic and mitochondrial fractions were isolated by a digitonin lysis procedure (Single *et al*., 1998) and cytochrome c expression in the fractions was assessed by immunoblotting. (B) Caspase-9-like activity was measured utilizing the fluorogenic substrate Ac-LEHD-AMC. The data presented in B are averages +/− SEM of at least three experiments. # $p < 0.001$, indicates a significant difference compared to As3+-induced caspase activity in Hepa-V3 cells at that time point.

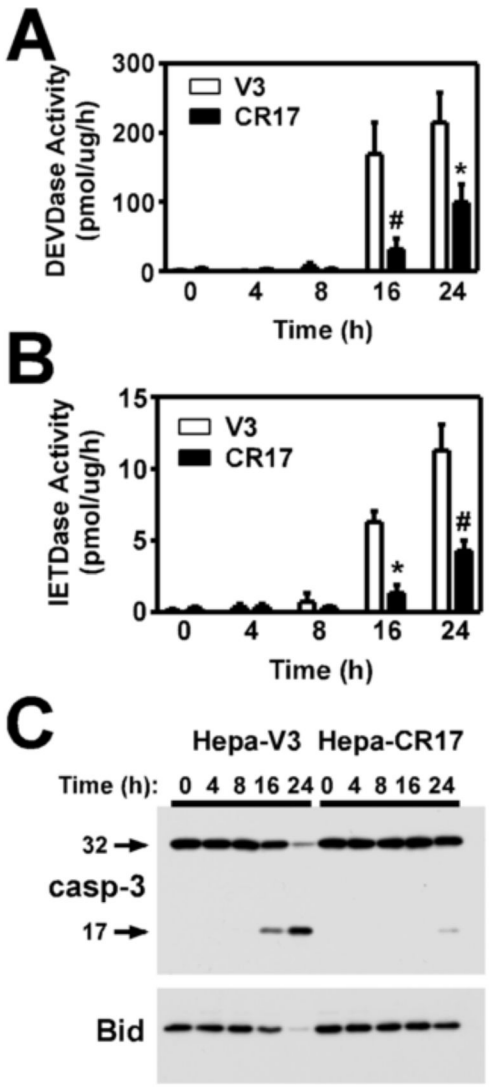

Figure 5. Overexpression of GCL inhibits As3+-induced caspase-3/8 activation

Hepa-V3 and Hepa-CR17 cells were treated with 20 uM As3+ for the time periods indicated. (A) Caspase-3-like activity was measured utilizing the fluorogenic substrate Ac-DEVD-AMC, (B) caspase-8-like activity was measured using Ac-IETD-AMC, and (C) the cleavage and/or processing of pro-caspase-3 and Bid were assessed by immunoblotting. The data presented in A/B are averages +/− SEM of at least three experiments. * $p < 0.01$, # $p < 0.001$, indicates a significant difference compared to As3+-induced caspase activity in Hepa-V3 cells at that time point.

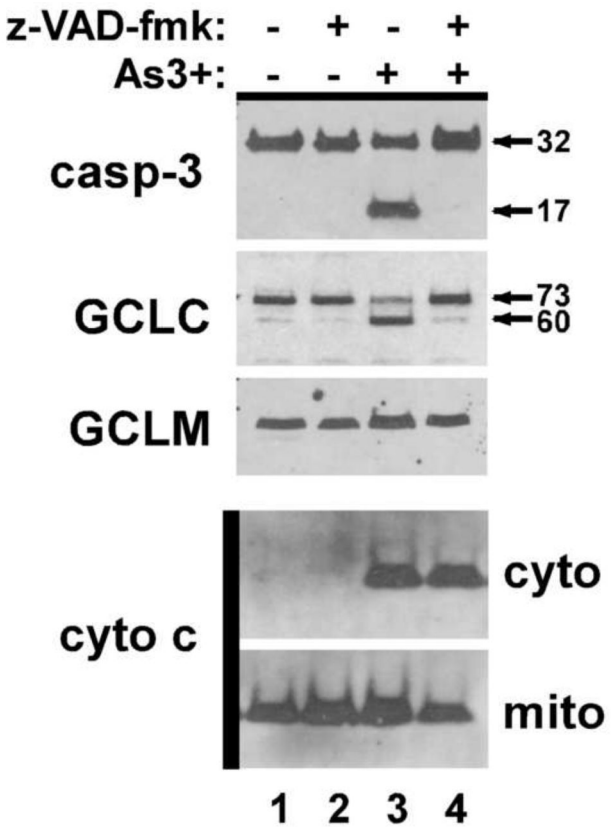

Figure 6. Caspase inhibition does not prevent As3+-induced cytochrome c release

Parental Hepa-1c1c7 cells were pretreated with 50 uM zVAD-fmk for 1 h prior to treatment with 20 uM As3+ for 24 h as indicated. (top panels) Whole cell extracts were prepared and caspase-3, GCLC, and GCLM expression was assessed by immunoblotting. (bottom panels) Cells were harvested and fractionated as described in the Methods section and cytochrome c expression in the cytosolic (cyto) and mitochondrial (mito) fractions was assessed by immunoblotting.

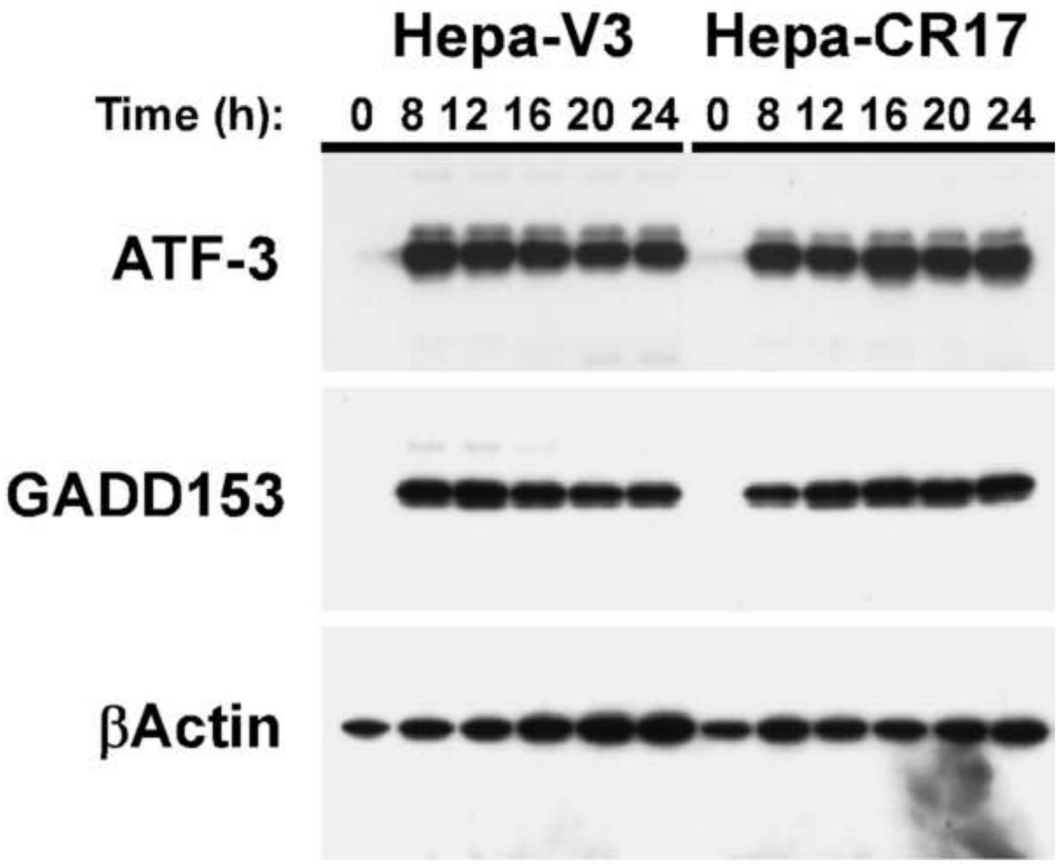

Figure 7. Overexpression of GCL does not abolish As3+-induced protein expression

Hepa-V3 and Hepa-CR17 cells were treated with 20 uM As3+ for the time periods indicated. Whole cell extracts were prepared and ATF-3, GADD153, and βActin expression were analyzed by immunoblotting.