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Glutathione is required for efficient production of infectious picornavirus virions

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Abstract

Glutathione is an intracellular reducing agent that helps maintain the redox potential of the cell and is important for immune function. The drug L-buthionine sulfoximine (BSO) selectively inhibits glutathione synthesis. Glutathione has been reported to block replication of HIV, HSV-1, and influenza virus, whereas cells treated with BSO exhibit increased replication of Sendai virus. Pre-treatment of HeLa cell monolayers with BSO inhibited replication of CVB3, CVB4, and HRV14 with viral titers reduced by approximately 6, 5, and 3 $\log_{10}$, respectively. The addition of glutathione ethyl ester, but not dithiothreitol or 2-mercaptoethanol, to the culture medium reversed the inhibitory effect of BSO. Viral RNA and protein synthesis were not inhibited by BSO treatment. Fractionation of lysates from CVB3-infected BSO-treated cells on cesium chloride and sucrose gradients revealed that empty capsids but not mature virions were being produced. The levels of the 5S and 14S assembly intermediates, however, were not affected by BSO treatment. These results demonstrate that glutathione is important for production of mature infectious picornavirus virions.
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Keywords: Coxsackievirus B3; Picornavirus; Glutathione; Replication

Introduction

Oxidative stress has been implicated in the etiology of several diseases including diabetes and cardiovascular disease. Recent evidence has demonstrated, however, that reactive oxygen species can act as important secondary messengers in signal transduction pathways (Esposito et al., 2004; Hehner et al., 2000; Pani et al., 2000). The cell has a number of mechanisms available to control oxidative stress. These comprise several enzymes including catalase, glutathione peroxidases, thioredoxin reductase, and superoxide dismutase. The cell also has a number of lower molecular weight molecules that can act as reactive oxygen species (ROS) scavengers, the most important of which is the tripeptide, glutathione (GSH). Glutathione is present at millimolar concentration in the cell (Uhlig and Wendel, 1992), is important in detoxification pathways via glutathione transferases (Meister and Anderson, 1983), and is critical for maintaining the redox potential of the cell. It is now appreciated that the redox potential of the cell can influence the activation of many genes through the induction of transcription factors including NF-κB and AP-1 that are important for immune function (Abate et al., 1990; Schenk et al., 1994), antioxidant defense (Bauer and Bauer, 2002), and induction of apoptosis (Haddad, 2002; Kwon et al., 2003).

Oxidative stress has been implicated as a possible mechanism leading to selection of more virulent viral genotypes. An amyocarditic stain of coxsackievirus B3 (CVB3) passaged through selenium or vitamin E-deficient mice rapidly expresses a myocarditic genotype indicated by specific changes to the viral genome (Beck et al., 1994, 1995). A similar finding was observed when an attenuated strain of influenza A rapidly gained virulence upon passage through selenium deficient mice (Nelson et al., 2001). These results suggest dietary affects on oxidative stress may influence viral disease progression.

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Glutathione levels are also influenced by diet (Jackson et al., 2004; Manhart et al., 2001; Micke et al., 2001; Miller et al., 2002; Roth et al., 2002) and GSH has been shown to influence viral replication. Glutathione levels are depressed in CD4+ T-cells from HIV-1-infected individuals (van der Ven et al., 1998). In vitro, HIV-1 infection of macrophages decreased GSH levels (Garaci et al., 1997b; Staal, 1998) and intracellular GSH levels can predict the prognosis of the disease (Herzenberg et al., 1997). Inhibition of GSH synthesis by buthionine sulfoximine (BSO) increased HIV yield in culture (Garaci et al., 1997b). Influenza virus replication also has been shown to be inhibited by GSH (Cai et al., 2003; Nencioni et al., 2003). Similarly, HSV-1 infection in vitro caused a dramatic decrease in intracellular GSH concentrations and addition of GSH to the culture medium decreased viral replication by greater than 99% (Palamara et al., 1995). Likewise, Sendai virus replication was enhanced by BSO inhibition of GSH synthesis (Macchia et al., 1999). Decreased plasma GSH levels correlated with increased levels of cardiomyocyte apoptosis (Kyto et al., 2004). Few studies, however, have examined the influence of GSH on picornavirus replication. In this study, we examine the effect of GSH on picornavirus replication and demonstrate that BSO inhibits efficient viral replication by blocking formation of mature infectious virions but does not interfere with virus-induced cytopathic effect (CPE).

Results

Effect of BSO on GSH synthesis

BSO is a potent and specific inhibitor of GSH synthesis, inhibiting γ-glutamyl synthase (Griffith and Meister, 1979). A concentration of 0.2 mM has been reported to inhibit synthesis of glutathione (GSH) in cell culture (Hamilos and Wedner, 1985; Wullner et al., 1999). To confirm this, HeLa-H1 or L-cell monolayers were treated with BSO for 24, 48, or 72 h. Cells from BSO-treated or control cell monolayers were then harvested and assayed for total glutathione (GSH+GSSG) content (Baker et al., 1990; Tietze, 1969). BSO treatment reduced total GSH content to undetectable levels by 48 h post-treatment (<1% of control values; data not shown).

Effect of BSO on viral replication

Untreated or BSO-treated cell monolayers were infected with CVB3 at a low MOI (0.5). Untreated cell monolayers exhibited 100% CPE after approximately 18–20 h post-infection, whereas BSO-treated monolayers were normal in appearance (Figs. 1C and D, respectively). Cell lysates derived from untreated monolayers contained high levels of infectious virus (Table 1). In contrast, viral titers in cell lysates derived from BSO-treated monolayers were near the limit of detection (approximately 1.5 $\log_{10}$ $TCID_{50}$/ml; Table 1). These results indicate that viral replication was inhibited by pre-treatment of cells with BSO. Infection of a cell monolayer at a low MOI, however, initially infects only a fraction of the total cells present and requires release of infectious virus to initiate secondary rounds of infection to infect the entire monolayer. Thus, infection at a low MOI could overestimate the degree of inhibition. To more accurately determine the degree of viral replication in cells treated with BSO, monolayers were infected at a high MOI and the infections were terminated after 8–10 h; the approximate length of time for one replication cycle.

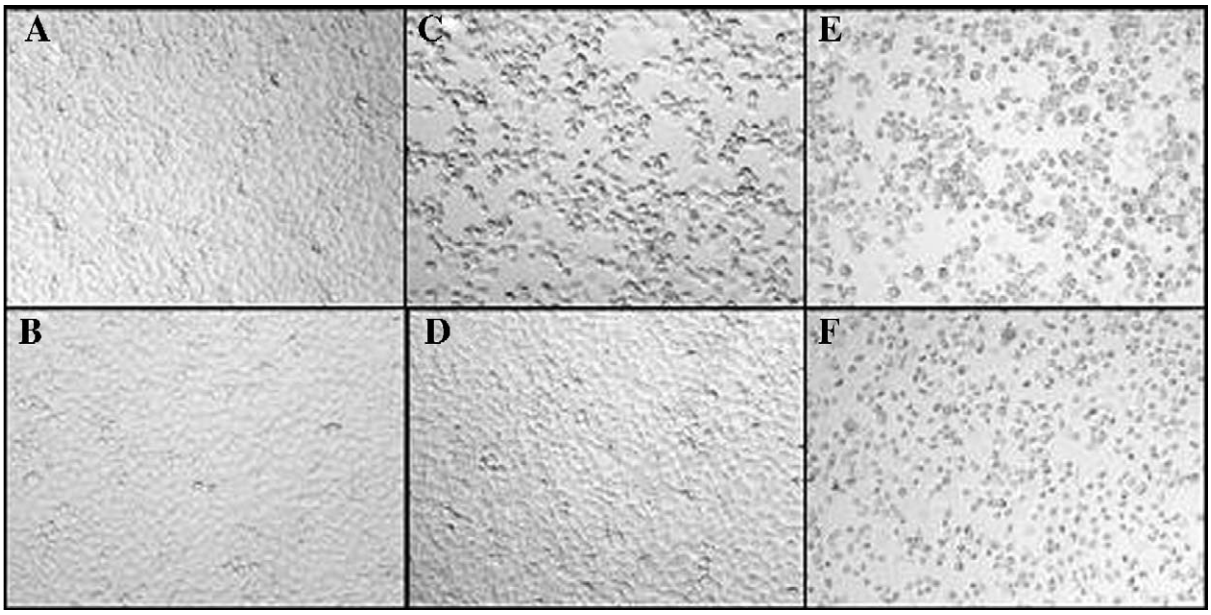

Fig. 1. Effect of MOI and BSO treatment on CVB3-induced CPE in HeLa-H1 cells. Untreated or BSO-treated Hela-H1 cells were infected with CVB3 at a low (0.5) or high (5.0) MOI and the development of CPE monitored. As expected, uninfected control (Panel A) or BSO-treated cells (Panel B) did not exhibit any signs of CPE. CVB3-infected control cells infected at an MOI of 0.5 (Panel C) or 5.0 (Panel E) exhibited CPE as expected but BSO-treated cells infected at a low MOI (0.5) did not exhibit CPE (Panel D). In contrast, BSO-treated cells infected at a high MOI (5.0, Panel F) did exhibit CPE that was indistinguishable from infected control cells.

Table 1
Effect of BSO on production of infectious virus

Virus	MOI	Titer, control[a]	Titer, BSO
CVB3	0.5	9.89±0.24	1.63±0.16
CVB3	5.0	9.53±0.16	3.22±0.23
CVB4	5.0	8.75±0.20	4.08±0.29
HRV14	5.0	7.31±0.19	4.42±0.29

[a] Values are the mean±SD of $\log_{10}$ transformed data from 3 to 6 replicate cultures.

Untreated cell monolayers infected with CVB3 at a high MOI (5.0) exhibited 100% CPE and produced large amounts of infectious virus (Table 1, Fig. 1E). BSO-treated monolayers infected at a high MOI also exhibited 100% CPE but produced about 10^6 less infectious virions than the control cultures (Fig. 1F, Table 1). Thus, whereas viral-induced CPE was unaffected by BSO treatment production of infectious virus was inhibited. All subsequent infections were conducted at high MOIs.

To rule out the possibility that infectious virions were being produced but became inactivated, one-step growth curves were constructed. As the results in Fig. 2 demonstrate, large amounts of infectious virions are produced between 4 and 6 h post-infection in control cells and then virus production plateaus between 6 and 10 h. In contrast, the amount of infectious virions produced by BSO-treated cells remains at lower levels throughout the time course and may reflect residual virus left from the initial infection. Thus, lack of detection of infectious virus at later time points, 8–10 h post-infection, in BSO-treated cells does not appear to result from inactivation of virus produced at earlier time points.

A high titer viral stock solution was treated with BSO at 2.0 mM for 1 h at room temperature to determine if BSO directly effected virus infectivity and replication; a mock treatment served as a control. No effect of BSO treatment on virus infectivity was observed (data not shown). These results indicate BSO is not directly inactivating the virus but is affecting some aspect of viral replication.

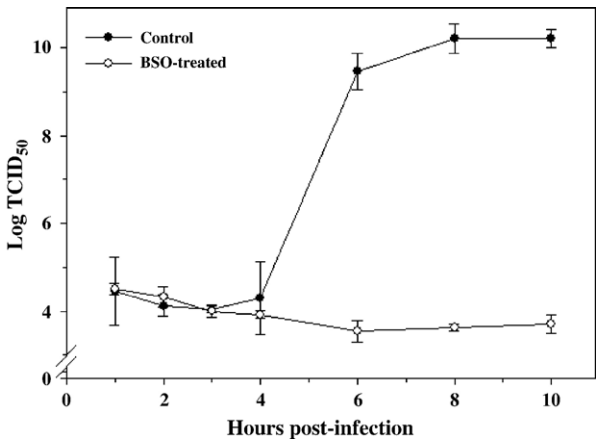

Fig. 2. One-step CVB3 growth curve in control or BSO-treated cells. Control or BSO-treated cells were infected with CVB3 at an MOI of 5.0 and replicate cultures were harvested at 1, 2, 3, 4, 6, 8, or 10 h post-infection. The amount of cell-associated virus was quantified. —●—control cells; —○—BSO-treated cells.

The effect of BSO on the replication of CVB4 and human rhinovirus 14 (HRV14) was tested to determine if BSO had a general effect on picornavirus replication. Replication of both CVB4 and HRV14 was inhibited by pre-treatment of cells with BSO by approximately 4 and 3 logs, respectively (Table 1). These results suggest that BSO inhibits replication of picornaviruses, but results of others (Garaci et al., 1997b; Macchia et al., 1999) indicate that BSO does not inhibit replication of all RNA viruses.

The results suggest that GSH may be necessary for production of infectious CVB3 because BSO is a known specific inhibitor of GSH synthesis. This was tested by adding GSH ethyl ester (GEE) to BSO-treated cultures. GEE is a GSH derivative that, unlike GSH, can be readily transported across the cell membrane and then converted to GSH via action of an intracellular esterase (Anderson et al., 1985; Wellner et al., 1984). Cell cultures were pre-treated with BSO and then incubated with media containing BSO (0.2 mM) and varying concentrations of GEE (0.5–10.0 mM) for approximately 15 h prior to infection with CVB3. Addition of GEE to the culture medium increased the amount of infectious virus produced in a dose-dependent manner with 2 mM GEE restoring infectious virus production to near control levels (Fig. 3). Thus, GSH appears to be necessary for efficient production of infectious picornaviruses and BSO inhibits production of infectious virus by inhibiting GSH production.

Because GSH helps maintain the redox potential of the cell and can serve as a reducing agent, the ability of other reducing agents to act as surrogates for GSH was determined. Cells were pre-treated with BSO, infected with CVB3, and then 5 mM GEE, 2-mercaptoethanol or DTT was added to the culture medium during and after the 1-h virus absorption period. Neither 2-mercaptoethanol nor DTT

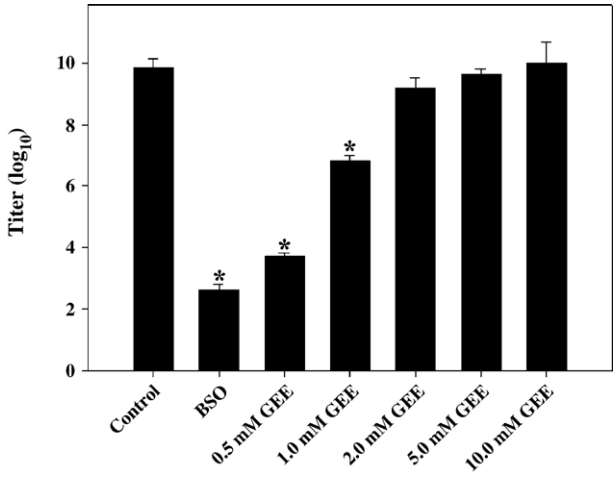

Fig. 3. Reversal of BSO-induced inhibition of infectious virion production by glutathione ethyl ester (GEE). Control or BSO (0.2 mM)-treated HeLa cell monolayers were co-incubated overnight with varying concentrations of GEE, infected at an MOI of 5.0 with CVB3, and harvested 8–10 h later. The viral titers of the resulting supernatants were measured by $TCID_{50}$. Results are the mean±SD of $\log_{10}$ transformed data from triplicate samples. Values were compared using Kruskal–Wallis ANOVA on Ranks followed by Dunnett's multiple comparison procedure. An asterisk (*) indicates that the treatment group was significantly different from the control group; $p<0.05$.

restored viral replication, whereas 5 mM GEE restored viral replication to near control levels (data not shown).

Effect of BSO on viral RNA synthesis

To determine if BSO was inhibiting viral RNA production, control or BSO-treated HeLa cell monolayers were infected at an MOI of approximately 5.0 and total RNA isolated 1, 2, 4, and 8 h post-infection to determine if the BSO-induced block in production of infectious virus was due to lack of production of positive strand RNA. Real-time PCR was used to measure the relative amounts of viral RNA produced in the control versus BSO-treated CVB3-infected monolayers. The amount of viral RNA isolated from control and BSO-treated cells was not different between the 1 and 2 h time points; however, the amount of viral RNA increased logarithmically between 2 and 8 h (Fig. 4) but was not different between control and BSO-treated cultures. Therefore, BSO had no effect on viral RNA production.

Guanidine hydrochloride is a known inhibitor of the picornavirus RNA polymerase (Caliguiri and Tamm, 1968; Wimmer et al., 1993). Because BSO does not inhibit viral RNA synthesis, treatment of cells with 200 μg/ml GuHCl should inhibit viral RNA production equally in control and BSO-treated cells. This was confirmed by measuring viral RNA production in the presence and absence of 200 μg/ml GuHCl and 0.2 mM BSO. Viral RNA production was virtually abolished in both control and BSO-treated cells by treatment with GuHCl (data not shown). Thus, the BSO block must be occurring at a replication step that occurs after viral RNA synthesis.

Effect of BSO on viral protein synthesis

^{35}S-labeled proteins from cell lysates of uninfected or CVB3-infected control or BSO-treated HeLa-H1 cell monolayers were separated on SDS–polyacrylamide gels. The

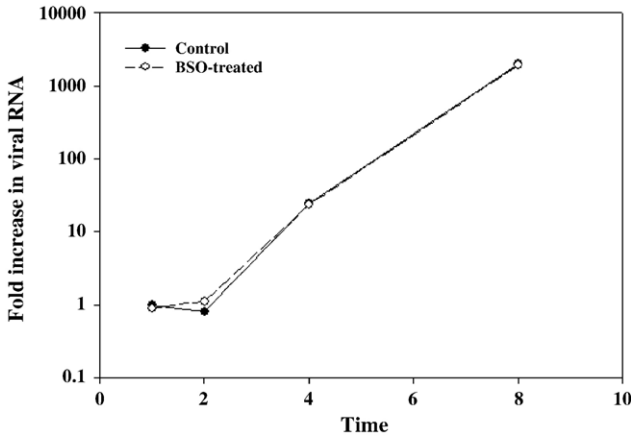

Fig. 4. Effect of BSO treatment on viral RNA synthesis. Control or BSO-treated cells were infected with CVB3 at an MOI of 5.0. RNA was isolated from the cells at 1, 2, 4, or 8 h after infection, the relative amounts of viral RNA produced measured by real-time PCR as described in Materials and methods. Viral RNA levels in control (—●—) or BSO-treated (—○—) cells.

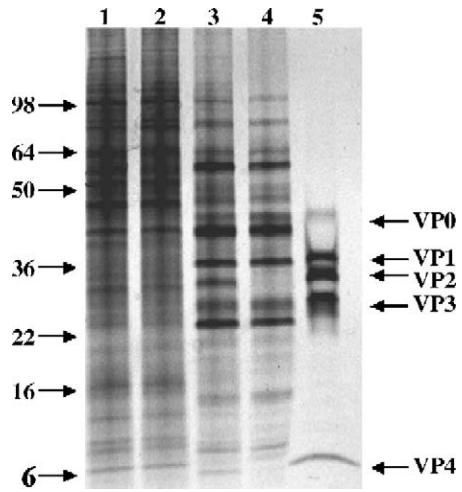

Fig. 5. Effect of BSO on viral protein synthesis. Control and BSO-treated cells were infected with CVB3 at an MOI of 5.0 and metabolically labeled with Tran-^{35}S-Label as described in Materials and methods. Uninfected control and BSO-treated cells were also metabolically labeled in an identical fashion. Equal amounts of cell lysate were subjected to SDS–PAGE on 10–20% gradient gels and subjected to autoradiography. Lane 1—uninfected control cells; Lane 2—uninfected BSO-treated cells; Lane 3—infected control cells; Lane 4—infected BSO-treated cells; and Lane 5—purified mature virions.

number and intensity of labeled proteins observed in both control and BSO uninfected cells is comparable (Fig. 5, Lanes 1 and 2, respectively). Thus, BSO treatment alone does not alter protein synthesis in the cells to any appreciable extent. Similarly, the number and intensity of bands appearing in lysates from CVB3-infected cells was very similar for control and BSO-treated cells, suggesting that viral protein synthesis was not significantly altered by BSO treatment (Fig. 5, Lanes 3 and 4, respectively). The only clear difference was the presence of VP2 and VP4 in infected control cultures that were not present in BSO-treated cultures. One possible explanation for the lack of infectious virus and the absence of VP2 and VP4 was that only empty capsids were being produced in BSO-treated cells.

To determine if BSO-treated cells only produced empty capsids, the effect of BSO treatment on production of intact infectious virions was evaluated by purifying virus from control and BSO-treated cultures over CsCl gradients. The virus preparation from control cells produced two prominent bands on the CsCl gradient, the lower band in Fig. 6A, which is known to contain infectious RNA-containing virions, and an upper band, which contains empty capsid (Minor, 1985). In contrast, the virus preparation from BSO-treated cells only yielded one band corresponding in location to the upper band in the gradient from control virus preparation. SDS–PAGE analysis of the lower band isolated from the control virus gradient contained viral proteins VP1–VP4, as would be expected for mature RNA-containing virons (Fig. 6B, Lane 1). The upper band from the control virus gradient contained viral proteins VP0, VP1, and VP3, but no VP2 or VP4, which are formed by cleavage within VP0 to produce mature virions (Fig. 6B, Lane 2), and is consistent with the presence of empty capsids or other assembly

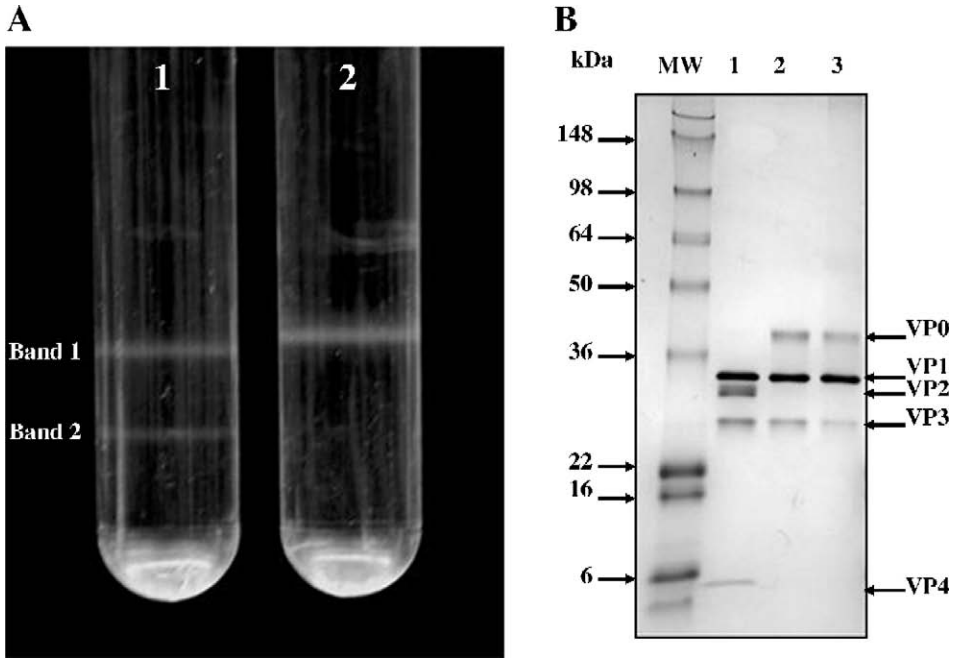

Fig. 6. Effect BSO on virion assembly. (A) CVB3 was propagated in untreated or 0.2 mM BSO-treated HeLa-H1 cells and banded on 5–40% CsCl gradients. Gradient 1—CVB3 propagated on control HeLa-H1 cells; Gradient 2—CVB3 propagated on BSO-treated HeLa-H1 cells. (B) The two bands obtained from gradient 1 (Control) and the single band obtained from gradient 2 (BSO) were isolated and examined by SDS–PAGE. MW, molecular weight markers; Lane 1, control virus sample, band 2; Lane 2, control virus sample, band 1; Lane 3, BSO virus sample. The arrows located on the right-hand side of panel A indicate the location of viral coat proteins VP0–VP4.

intermediates. The only band obtained from the gradient purified BSO viral preparation, which migrated to a position equivalent to the upper band of the control viral preparation, produced a protein banding pattern identical to that of the upper band from the gradient purified control virus preparation (Fig. 6B, Lane 3). Thus, in BSO-treated cells the production of mature virions is inhibited.

Analysis of assembly intermediates

To determine if the lack of mature virions in BSO-treated cells was due to an effect on the levels of assembly intermediates, metabolically labeled viral proteins were separated on sucrose gradients. Both the 5S and 14S assembly intermediates were observed at similar concentrations in control and BSO-treated cells (Fig. 7A). In contrast, CVB3-infected BSO-treated cells produced a very large 75S empty capsid peak, corresponding to the upper band observed on the CsCl gradients, whereas a much smaller 75S peak was observed in lysates from CVB3-infected control cells (Fig. 7B). Furthermore, whereas a 150S mature virion peak was observed in gradients from CVB3-infected control cells, no 150S peak was seen in lysates derived from infected BSO-treated cells, again in agreement with results obtained from the CsCl gradients. No other peaks of intermediate size were observed. The composition of the 5S, 14S, 75S, and 150S peaks were confirmed by analysis of peak fractions by SDS–PAGE and the results are shown in Fig. 8 and are consistent with each of the various assembly intermediates and mature virions. Therefore, these results indicate that BSO treatment of cells results in increased

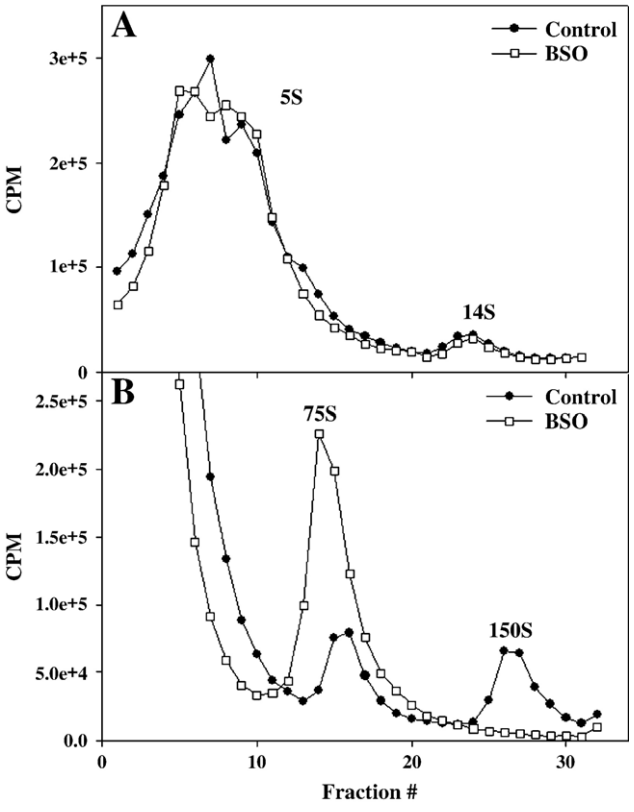

Fig. 7. Effect of BSO treatment on the levels of assembly intermediates. Control or BSO-treated cells were metabolically labeled as described in Materials and methods. Equal volumes of extract were fractionated over 5–20% (A) or 15–30% (B) sucrose gradients. Gradients were fractionated from the top and aliquots counted by liquid scintillation.

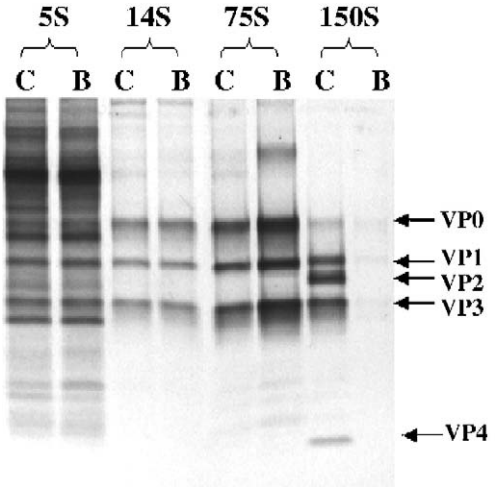

Fig. 8. Protein composition of assembly intermediates. Aliquots from the peak fractions corresponding to the 5S, 14S, and 75S assembly intermediates, the 150S mature virion peak, and the corresponding fraction from the BSO 15–30% gradient were subjected to SDS–PAGE and autoradiography. The letters B and C denote fractions derived from BSO-treated and control cultures, respectively.

levels of empty capsids at the expense of RNA-containing mature virions.

Discussion

The results presented here demonstrate that GSH is required for efficient production of infectious virions because viral replication was inhibited by BSO, a specific GSH synthesis inhibitor. Further evidence that the inhibition of viral replication is GSH specific was demonstrated by the reversal of inhibition by GSH ethyl ester, a GSH analog that can be transported across the cell membrane and then converted into free GSH (Anderson et al., 1985; Wellner et al., 1984). The reversal was dose dependent with approximately 2 mM GEE required to fully recover infectivity. Other sulfhydryl reagents such as 2-mercaptoethanol and dithiothreitol were not effective at restoring production of infectious virions. The reasons for this are not clear as both 2-mercaptoethanol and dithiothreitol are able to cross the plasma membrane. 2-Mercaptoethanol crosses as a mixed disulfide (Ishii et al., 1981) whereas dithiothreitol (DTT) can freely cross the membrane itself (Lauriault and O'Brien, 1991). Both DTT and 2-mercaptoethanol have been reported to protect cell line from BSO-induced cell death (Chen et al., 1987; Wang et al., 2006), suggesting that they can substitute for GSH in some intracellular functions.

The results presented here for picornaviruses contrast with those obtained with HIV and Sendai virus where BSO treatment increased virus replication (Garaci et al., 1997a; Macchia et al., 1999). Increased HIV replication likely occurs via reactive oxygen species activation of NF-κB but a mechanism of action on Sendai virus replication is not known. Influenza replication is inhibited by GSH and is enhanced when GSH levels are reduced (Cai et al., 2003). Inhibition of influenza infection by GSH appears to occur via GSH-dependent inhibition of apoptosis (Cai et al., 2003) which may slow virus release.

Production of infectious virus was not due to lack of viral RNA synthesis. Real-time PCR measurement of RNA production indicated that equivalent levels of viral RNA were being produced in control and BSO-treated cells that were equally inhibited by GuHCl. Viral protein synthesis also was unaffected by BSO treatment as evidenced by the comparable production of radiolabeled viral proteins in control and BSO-treated cells. This indicates that processing of the viral polyprotein P1, by viral proteases 2A and 3C (or 3CD), is occurring normally or the distribution of viral protein products would be altered. In addition, because viral RNA production is unaffected by BSO treatment the viral polymerase, 3D, and VPg, both required for viral RNA synthesis, must be functional.

Assembly of infectious virions, however, does appear to be inhibited in BSO-treated cells. Cell lysates from CVB3-infected BSO-treated cells purified over CsCl gradients did not yield a band corresponding to mature infectious particles but only a band corresponding to empty capsids. Further analysis of infected control or BSO-treated cell lysates by fractionation on sucrose gradients revealed that BSO treatment did not alter the concentration of the 5S and 14S assembly intermediates but produced higher levels of the 75S empty capsids at the expense of mature 150S virion production. Nor was a 110S peak observed as was seen in hydantoin-treated cultures (Vance et al., 1997), a drug that also inhibited replication but did not interfere with viral RNA synthesis or translation. Previous studies have linked the production of large amounts of empty capsids with decreased viral RNA synthesis, arguing for a tight coupling between viral RNA synthesis and RNA packaging (Baltimore et al., 1966; Caliguiri and Compans, 1973; Hellen and Wimmer, 1995) and that empty capsids may be dead-end particles (Verlinden et al., 2000). The results presented here suggest that viral RNA synthesis can be decoupled from the production of mature RNA-containing virions and that BSO treatment produces a defect in infectious virion assembly. This suggests that glutathione itself or a glutathione-dependent process is required for efficient encapsidation of viral RNA to produce infectious virions. Glutathione is a potent intracellular reductant and lack of glutathione may promote a pro-oxidant environment within the cell. Thus, it may be that a reducing environment is needed for a critical step in production of mature RNA-containing virions although two other sulfhydryl reducing agents, 2-mecaptoethanol and dithiothreitol, could not substitute for glutathione, indicating the specificity of the glutathione effect.

Although BSO substantially reduced infectious virion production in HeLa cells in vitro, it did not interfere with induction of viral-induced CPE. This became apparent, however, only in cultures infected at a high MOI where all of the cells are infected during the virus adsorption step. The cell sheet remained intact in cultures infected at a low MOI due to lack of secondary rounds of infection. A study by Mikami et al. (2004) reported that BSO inhibited Echovirus 9-induced CPE in green monkey kidney cells. These investigators, however, only infected cells at a very low MOI of approximately 0.01, thus initially infecting only a very small percentage of the cells under conditions in which secondary rounds of infection would be inhibited.

The ability of CVB3 to induce CPE in BSO-treated cells in the absence of infectious virion production is likely due to the fact that viral protein synthesis is not altered by BSO treatment. Induction of CPE has been shown to be dependent on productive infections and treatment with drugs such as GuHCl, which inhibits viral RNA synthesis and therefore viral protein synthesis, favor the apoptotic pathway (Agol et al., 1998, 2000; Rasilainen et al., 2004; Tolskaya et al., 1995). Viral proteins with pro and anti-apoptotic properties have been identified including VP2, 2A, 2B, 2BC, and 3A (Buenz and Howe, 2006; Goldstaub et al., 2000; Henke et al., 2001; Salako et al., 2006). In productive infections, when viral protein expression is high, especially in the later stages of the viral replication cycle, CPE is favored over apoptosis (Agol et al., 2000; Romanova et al., 2005). Therefore, it seems likely that the CPE observed in BSO-treated cells is due to the normal synthesis and function of viral proteins even though mature virions are not produced.

The results presented here indicate that GSH is necessary for efficient production of infectious virions. Viral RNA and protein synthesis are not affected by BSO treatment, but proper encapsidation of viral RNA and the maturation cleavage step that is characteristic of mature infectious virions production is affected. These results also indicate that induction of viral-induced CPE is not dependent on efficient production of infectious virions.

Materials and methods

Cells and virus

Hela-H1 cells were grown as monolayers in minimal essential medium (MEM, Gibco cat. # 11090-081) supplemented with L-glutamine to 2 mM, non-essential amino acids to 0.1 mM, penicillin-streptomycin to 100 U/ml, and 5% fetal bovine serum (Complete medium). Stocks of coxsackievirus B3/0 (CVB3), an amyocarditic CVB3 strain (Chapman et al., 1994), coxsackievirus B4 (CVB4), and human rhinovirus 14 (HRV14) were prepared in Hela-H1 cells (Sherry and Rueckert, 1985).

Infectivity assays

For CVB3, CVB4, and HRV14, viral titers in tissue culture supernatants were assayed on HeLa-H1 cells by tissue culture infectious dose $(TCID)_{50}$. One hundred microliters of 10-fold serial dilutions of samples was mixed with 100 μL of cell suspension containing 1.0E+04 cells in 96-well tissue culture plates. Six replicates of each dilution were plated and allowed to incubate for three days at 37 °C and 5% CO_2. Wells were visually scored for virus growth and the $TCID_{50}$ calculated (Lynn, 1992).

Cell treatments

Hela-H1 or L-cells were propagated for a minimum of 48 h in the presence of 0.2 mM L-buthionine-*S*,*R*-sulfoximine (BSO) prior to use in experiments, when GSH levels had been reduced to undetectable levels (Baker et al., 1990; Tietze, 1969). Cells were infected either with a low multiplicity of infection (MOI), 0.5, or a high MOI, 5.0–10.0, for 1 h. In all experiments, the cells were washed extensively after the virus absorption step to remove unbound virus. Control and BSO-treated cell cultures infected at a low MOI were allowed to progress for approximately 16–20 h; a time when cultures without BSO treatment were at 100% CPE. In some experiments, glutathione ethyl ester (GEE) was added to BSO-treated cultures at 0.5, 1.0, 2.0 5.0, or 10 mM in addition to BSO at 0.2 mM and incubated overnight prior to and during subsequent infection with CVB3. In other experiments, BSO-treated cultures were also treated with GEE, 2-mercaptoethanol, or dithiothreitol at 5 mM during and after the initial 1-h virus absorption step. For the one-step growth curve, replicate cultures were infected at an MOI of 5 and at 1, 2, 3, 4, 6, 8, or 10 h post-infection, the growth medium removed and the amount of cell associated virus was quantified as described above.

Quantitation of viral RNA

Control or BSO-treated (0.2 mM) cell monolayers were infected with CVB3 at an MOI of 5 and incubated for 1, 2, 4, and 8 h post-infection. The cell monolayers were washed with PBS to remove residual tissue culture medium and the RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions (Solano-Aguilar et al., 2002), and then treated with RNase free DNase. RNA integrity, quantity, and detection of genomic DNA was determined using the Agilent Bioanalyzer 2100 and RNA 6000 Labchip kit (Agilent Technologies, Palo Alto, CA). cDNA was synthesized using Improm-II Reverse Transcriptase System (Promega, Madison, WI) and random primers. Twenty-five nanograms of cDNA/reaction was then used for PCR amplification using a commercially available kit and amplification was measured on an ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA). The probes were 5′ TET (tetrachloro-6-carboxyfluoroscein) labeled and 3′ BHQ1 (Black Hole 1) labeled and were synthesized by Biosource International (Camarillo, CA). VIC- and TAMRA (6-carboxy-tetramethyl-rhodamine)-labeled internal oligonucleotide probe and primers specific for the 18S RNA ribosomal subunit were purchased from Applied Biosystems (Foster City, CA). Fluorescence signals measured during amplification were processed post-amplification and were regarded as positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. This level is defined as the threshold cycle (Ct). Viral RNA levels were normalized to 18S RNA. The Ct value for 18S ribosomal subunit was subtracted from the Ct value for each message to normalize for eukaryotic RNA content. This value is defined as ΔCt. To evaluate the effects of treatment, the mean of $\Delta Ct_{treatment}$ was subtracted from the mean of $\Delta Ct_{control}$. This value is defined as ΔΔCt. The relative fold increase or decrease was then calculated as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). To assess the affect of guanidine hydrochloride (GuHCl) treatment on

viral RNA production, media containing 200 μg/ml GuHCl was added after the initial 1 h virus absorption step. One set of control and BSO-treated cultures was harvested after the virus absorption and washing steps to determine the baseline viral RNA levels prior to GuHCl treatment. Viral RNA was isolated from the remaining cultures at 8 h post-infection. Total RNA from one set of cultures was harvested immediately after the virus absorption and washing steps to serve as a baseline for viral RNA production prior to GuHCl treatment.

Metabolic labeling of viral proteins

HeLa-H1 monolayers ± BSO (4.0×10^6 cells) were infected at an MOI of 5.0. After a 1-h absorption period, cells were fed with complete medium. At 3.5 h post-infection, the monolayers were washed with D–PBS and then Dulbecco's modified Eagle medium (DMEM, Invitrogen cat. # 21013-024) without methionine or cysteine (cystine) but supplemented with L-glutamine to 2 mM, non-essential amino acids to 0.1 mM, penicillin-streptomycin to 100 U/ml was added. At 4 h post-infection, 30 μCi/ml of Trans-^{35}S-Label (>1000 Ci/mmol, MP Biomedicals, Inc.) was added. At 6 h post-infection, the media were removed, the monolayers washed one time with D–PBS, and the cells lysed by addition of 10 mM Tris–HCL, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$ containing 0.1% NP-40 and frozen at −80 °C. After thawing, the cellular debris was removed by centrifugation for 3 min at 16,000×*g*. Equal volumes of cell lysate were subjected to SDS–PAGE on 10–20% gels. The resulting gels were dried and exposed to Biomax MR-1 film (Kodak).

Purification of virus

Untreated or BSO-treated HeLa-H1 monolayers were infected with CVB3 at an MOI of approximately 5.0 and incubated 8–10 h. When CPE became apparent, the medium was removed and the plates frozen. Virus was released from the cells by 3 rounds of freeze/thaw and the resulting cell lysate was clarified by centrifugation. The clarified supernatant was DNase treated, and *N*-lauryl-sarcosine added to 1.0%. The resulting supernatant was overlaid onto a 10-mM Tris, pH 7.4, 30% sucrose cushion and centrifuged at 110,000×*g* for 130 min in a Ti70 rotor. The resulting pellets were dissolved in 10 mM Tris, 0.15 M NaCl, pH 7.4. The concentrated virus was then layered onto 5–40% CsCl (wt/wt) gradients and centrifuged in an SW41 rotor at 100,000 × *g* for 7 h. The gradients were fractionated. Fractions containing the upper and lower bands from the gradients were diluted with 10 mM Tris, 0.15 M NaCl, pH 7.4, and concentrated by ultracentrifugation. The resulting pellets were redissolved in 10 mM Tris, 0.15 M NaCl, pH 7.4, and the protein concentration of the samples was determined using the BCA Protein Assay (Pierce, Chicago, IL).

Analysis of assembly intermediates by sucrose gradient fractionation

HeLa-H1 monolayers ± BSO ($8–12 \times 10^7$ cells) were infected at an MOI of 5.0 and metabolically labeled as described above using 10 μCi/ml of label. One milliliter of the resulting supernatants was layered on top of linear 5–20% (wt/vol) or 15–30% (wt/vol) sucrose gradients. Gradients (15–30% gradients) were centrifuged at 38,000 rpm in a Beckman SW41 rotor for 2.5 h at 4 °C or 37,000 rpm for 17 h (5–20% gradients). Gradients were fractionated from the top and the radioactivity in each fraction (approximately 350 μl) was quantified by liquid scintillation. Samples from the peak fractions were analyzed by SDS–PAGE and autoradiography. Samples for SDS–PAGE were mixed with a loading buffer to give a final concentration of 68.5 mM Tris pH 6.8, SDS and 2-mercaptoethanol of 2%, and 10% glycerol and were heated at 100 °C for 5 min. Equal volumes of lysates or peak fractions were analyzed.

Total glutathione determination

Cell monolayers were washed with D–PBS without Mg or Ca to remove residual medium and treated with a trypsin/EDTA solution to liberate the cells. Cells were then collected by centrifugation and resuspended in D–PBS without Mg or Ca. An aliquot was taken to determine the cell concentration. Remaining cells were collected by centrifugation and resuspended in 50 μl of D–PBS and 200 μl of 5% sulfosalicylic acid added. The samples were centrifuged to remove cellular debris and the resulting supernatant stored at −80 °C until assayed. Glutathione is stable for 12 months in samples prepared in 5% sulfosalicylic acid and stored at −80 °C (Roberts and Francetic, 1993). Samples were assayed for GSH using a modification of the Tietze (1969) recycling method adapted for use in 96-well plates (Baker et al., 1990).

Statistical analyses

Data were analyzed by one or two-way ANOVA with a post hoc multiple comparisons analysis using the Student–Neuman–Keuls or Dunnett's method. Data were transformed as necessary to pass the normality and equal variance tests for statistical analysis or an ANOVA was performed on Ranks. A p value of less than 0.05 was considered significant.

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