

Glutathione Depletion by L-Buthionine-S,R-Sulfoximine Induces Apoptosis of Cardiomyocytes through Activation of PKC- δ

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Abstract

In the present study, we investigated the effect of intracellular glutathione (GSH) depletion in heart-derived H9c2 cells and its mechanism. L-buthionine-S,R-sulfoximine (BSO) induced the depletion of cellular GSH, and BSO-induced reactive oxygen species (ROS) production was inhibited by glutathione monoethyl ester (GME). Additionally, GME inhibited BSO-induced caspase-3 activation, annexin V-positive cells, and annexin V-negative/propidium iodide (PI)-positive cells. Treatment with rottlerin completely blocked BSO-induced cell death and ROS generation. BSO-induced GSH depletion caused a translocation of PKC- δ from the cytosol to the membrane fraction, which was inhibited by treatment with GME. From these results, it is suggested that BSO-induced depletion of cellular GSH causes an activation of PKC- δ and, subsequently, generation of ROS, thereby inducing H9c2 cell death.

Key Words: Glutathione, Reactive oxygen species, H9c2, Cell death, PKC- δ

INTRODUCTION

Cardiovascular disease (CVD) continues to be a substantial health-care burden despite recent treatment advances. Oxidative stress has long been regarded as a key pathophysiological mediator that ultimately leads to CVD including atherosclerosis, hypertension, and heart failure (Chen and Keaney, 2012). Reactive oxygen species (ROS), which are involved in normal physiological functions at low concentrations, can have deleterious effects when produced in excess. Over time, ROS may result in a pathological state of imbalance known as oxidative stress. Most therapies that are currently being used may provide some reduction in oxidative stress, but there is no consensus on the clinical outcomes of various antioxidants. Currently, there are no antioxidant therapies that specifically target oxidative stress in patients with heart failure. Therefore, a better understanding of the mechanism of injury induced by oxidative stress related to CVD is needed (Ahmed and Tang, 2012).

Previous studies have pointed out changes in the redox status of glutathione in a failing heart (Damy *et al.*, 2009). Glutathione (GSH) is a tripeptide (γ -glutamylcysteinyl glycine) that plays an important protective role against oxidative stress-induced damage in mammalian tissues (Sevin *et al.*, 2013).

Indeed, it acts as a substrate for scavenging ROS via the enzymes glutathione peroxidase and glutathione reductase. Reduction of cellular GSH, which allows ROS accumulation, has been shown to cause apoptosis in Jurkat T cells (van den Dobbelen *et al.*, 1996) neutrophils (O'Neill *et al.*, 2000), and neural cells (Narasimhan *et al.*, 2011). Moreover, many studies have also examined the relationship between GSH levels and heart disease. Decreases in plasma GSH levels are associated with cardiomyocyte apoptosis (Kytö *et al.*, 2004) and are correlated with the severity of heart failure symptoms in patients (Damy *et al.*, 2009). Hearts depleted of intrinsic GSH suffer from more functional damage inflicted by ischemia/reperfusion than do normal hearts (Blaustein *et al.*, 1989). Recently, it was reported that NOX4 induces GSH levels through regulation of Nrf2 in cardiomyocytes, and triggers the adaptive stress response to protect the heart (Brewer *et al.*, 2011). However, other studies have disputed these findings, showing no significant difference in functional integrity between GSH-depleted and GSH-adequate hearts (Chatham *et al.*, 1988). Therefore, whether or not oxidative stress induced by GSH depletion contributes to the progression of heart disease remains elusive.

Experimental depletion of cellular GSH can be produced by using L-buthionine-S,R-sulfoximine (BSO), a potent and spe-

Open Access <http://dx.doi.org/10.4062/biomolther.2013.065>

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Received Sep 2, 2013 Revised Sep 16, 2013 Accepted Sep 23, 2013

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cific inhibitor of γ -glutamyl-cysteine synthetase (Meister and Anderson, 1983). In this study, we investigated the effects of GSH depletion by BSO on cellular viability and determined the mechanism of cellular damage induced by oxidative stress through GSH depletion in cardiomyocytes.

MATERIALS AND METHODS

Cell cultures and drug treatment

Heart-derived H9c2 cells were purchased from American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. H9c2 cells were pretreated with glutathione monoethyl ester (GME), protein kinase C (PKC)- ζ inhibitor (Santa Cruz Biotechnology, Santa Cruz, CA, USA), trolox, GF109203X, Go6976, or rottlerin (Sigma-Aldrich, St Louis, MO, USA) for 30 min. Subsequently, the cells were treated with 100 mM BSO (Sigma-Aldrich) in the presence or absence of GME or inhibitor.

Measurements of cellular GSH level

Intracellular GSH were measured using GSH colorimetric detection kit (BioVision, Milpitas, CA, USA). Briefly, cells were lysed in GSH buffer for 10 min. Cell lysates centrifuged at 8,000 *g* for 10 min and supernatants were collected. Supernatants were incubated with reaction buffer for 10 min. After adding of substrate solution, GSH level were monitored at 405 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

Measurements of ROS release

Intracellular ROS were measured fluorometrically using 2',7'-dichlorofluorescein (DCF) diacetate (DA) (Molecular probes, Eugene, OR, USA), which permeates cells easily and hydrolyzes to DCF after interacting with intracellular ROS. Cultured cells were washed twice with HCSS incubated with 10 μ M DCF-DA and 20% Pluronic F-127 for 30 min, and washed with HCSS, as described previously (Lee and Jung, 2012). Subsequently, cells were observed under a confocal microscope (Olympus, Tokyo, Japan). DCF fluorescence intensities were obtained in Fluoview FV300 software. DCF sample values are expressed as percentage relative to the control value.

Flow cytometric analysis for propidium iodide (PI)/annexin V staining

Apoptotic and necrotic cells were detected by double staining with PI and annexin V-FITC using the annexin-V apoptosis detection kit I (BD PharMingen, San Diego, CA, USA) (Lee *et al.*, 2011; Yang *et al.*, 2012). Briefly, cells were detached by trypsin-EDTA treatment, gently resuspended in cold binding buffer to 5×10^5 cells, and incubated with annexin V-FITC and PI at room temperature in the dark. Living cells were defined as those negatively stained for both PI and annexin V. Early apoptotic cells were defined as those staining positive for annexin V and negative for PI. Late apoptotic cells were defined as those staining positively for both. Necrotic cells were defined as those staining only with PI. The fluorescence intensity of the cell surface was measured by flow cytometry (FACS-Vantage, Becton Dickinson, Franklin Lakes, NJ, USA). Values are expressed as the percentage of annexin V-positive cells to total cells counted.

Nuclei staining

Cells were fixed with 4% paraformaldehyde for 10 min. Cell were incubated with Hoechst 33258 for 10 min. All samples were then observed under a confocal microscope (Olympus, Tokyo, Japan).

Measurement of caspase-3 activity

Cells were washed once with ice-cold phosphate-buffered saline and extracted on ice in lysis buffer (10 mM Tris-HCl, 0.32 M sucrose, 10 mM DTT, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 μ g/ml aprotinin, and 10 μ M leupeptin) for 1 h. Lysates were centrifuged at 10,000 *g* for 10 min at 4°C, and 200 μ g of cytosolic extracts were mixed with reaction buffer (100 mM HEPES pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT, and 10 μ M leupeptin) to a final volume of 100 μ l containing 200 μ M of Ac-DEVD-p-Na. Samples were then incubated for 2 h at 37°C, as described previously (Lee and Jung, 2012). The enzyme-catalyzed release of p-nitroanilide was monitored at 405 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

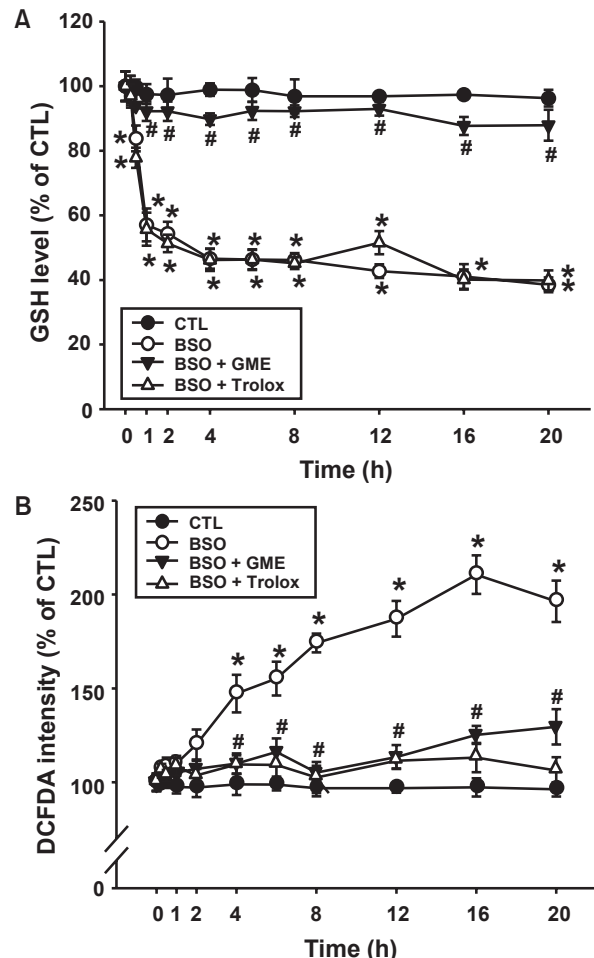


Fig. 1. Effect of glutathione monoethyl ester (GME) on BSO-induced ROS generation. H9c2 cells were incubated with BSO (10 mM) in the presence or absence of GME (300 μ M) or trolox (10 μ M) for an indicated time. (A) Glutathione (GSH) level. (B) DCF-DA intensity. Data are expressed as mean \pm S.E.M. ($n=3$). * $p<0.05$ vs. 0 h. # $p<0.05$ vs. BSO.

Lactate dehydrogenase (LDH) assay

Cell death was analyzed by measuring LDH release into medium as described previously (Lee and Jung, 2012). Briefly, 24 h after BSO treatment, 25 μ l medium was collected from each well and mixed with 100 μ l NADH solution (0.03% β -NAD [reduced form of the disodium salt] in phosphate buffer) and 25 μ l pyruvate solution (22.7 mM pyruvic acid in phosphate buffer) at room temperature. NADH consumption was followed for 2 min at 340 nm. The percent LDH was calculated from the maximum LDH release (100%) induced by lysing cells with 0.1% Triton X-100.

Western blot

Protein expression of PKC- δ was measured using western blot analysis as previously described (Lee *et al.*, 2012). Cells were lysed using lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 1% NP-40, 150 mmol/L NaCl, 0.25% Na-deoxycholate, 2 mmol/L EDTA, 1 mmol/L NaF, 1 mmol/L Na₃VO₄, 1 mmol/L PMSF, 10 μ g/ml of aprotinin, and 10 μ mol/L leupeptin). Lysates were subsequently centrifuged at 14,000 rpm for 15 min and supernatants were collected. Then, equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reacted with anti-PKC- δ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

and anti- β -actin antibody (Sigma-Aldrich). After probing with horseradish peroxidase (HRP)-conjugated secondary antibody, proteins were visualized using LAS 1000 (Fuji Photo Film, Tokyo, Japan).

Statistical analysis

All data are expressed as mean \pm S.E.M. Numerical data were compared using the Student's *t*-test for paired observations between two groups. A *p* value < 0.05 was considered significant.

RESULTS

Depletion of GSH induced ROS generation

To determine the effect of GSH depletion on BSO-induced ROS generation, we used GME to maintain the intracellular GSH concentration in the presence of BSO (Torres *et al.*, 1997). The GSH level in H9c2 cells decreased by treatment with 10 mM BSO and was approximately 80% at 0.5 h, 57% at 1 h, 46% at 4 h, and 43% at 12 h after treatment (Fig. 1A). The decrease in cellular GSH levels by BSO was restored to control levels by GME (300 μ M). However, trolox (10 μ M), an ROS scavenger, had no influence on the cellular GSH level.

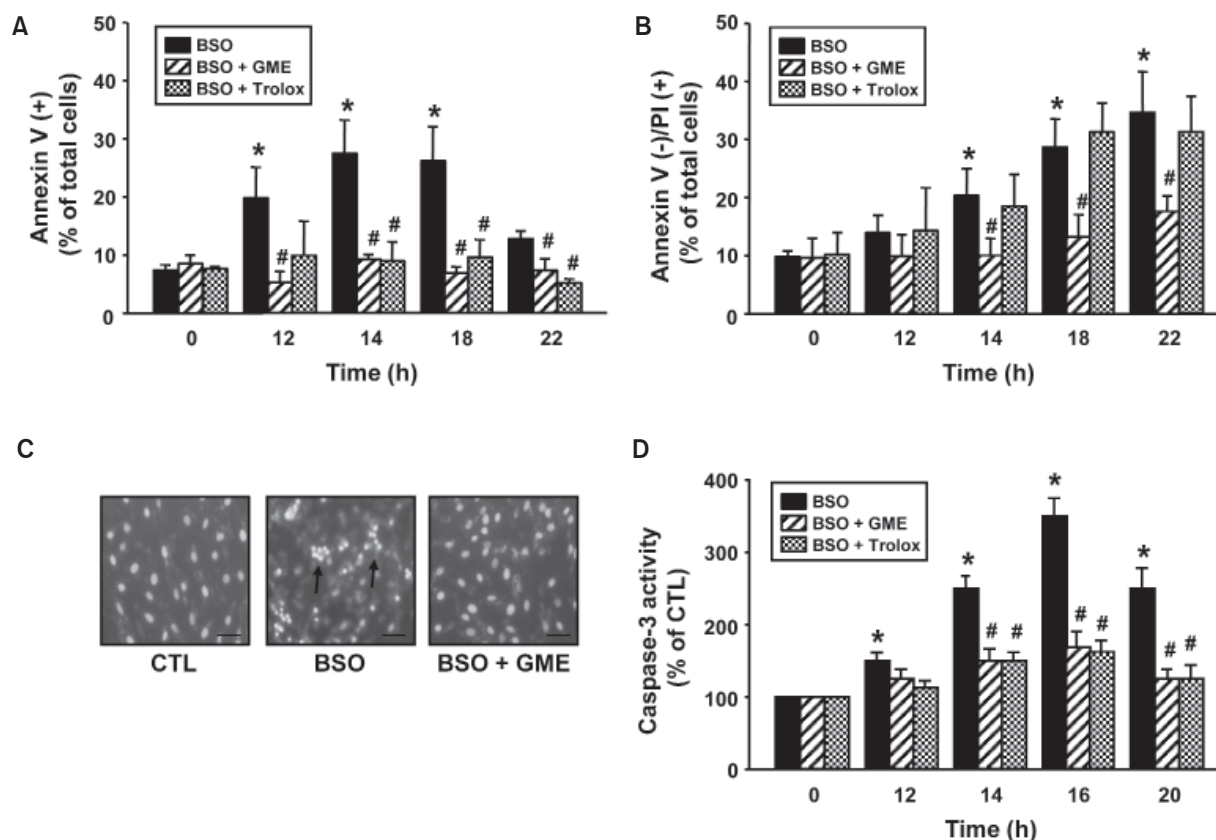


Fig. 2. Effect of glutathione monoethyl ester (GME) on BSO-induced cell death. H9c2 cells were incubated with BSO (10 mM) in the presence or absence of GME (300 μ M) or trolox (10 μ M) for indicated times. (A, B) FACS analysis of cells after Annexin V-PI staining. The percentages of apoptotic [Annexin-V (+)] (A) and necrotic [Annexin-V(-)/PI (+)] (B) cells were analyzed. (C) Staining of cells with Hoechst 33342. Arrows indicate fragmented nuclei and condensed chromatin. Scale bar=10 μ m. (D) Caspase-3 activity. Data are expressed as mean \pm S.E.M. (n=3). **p*<0.05 vs. 0 h. #*p*<0.05 vs. BSO.

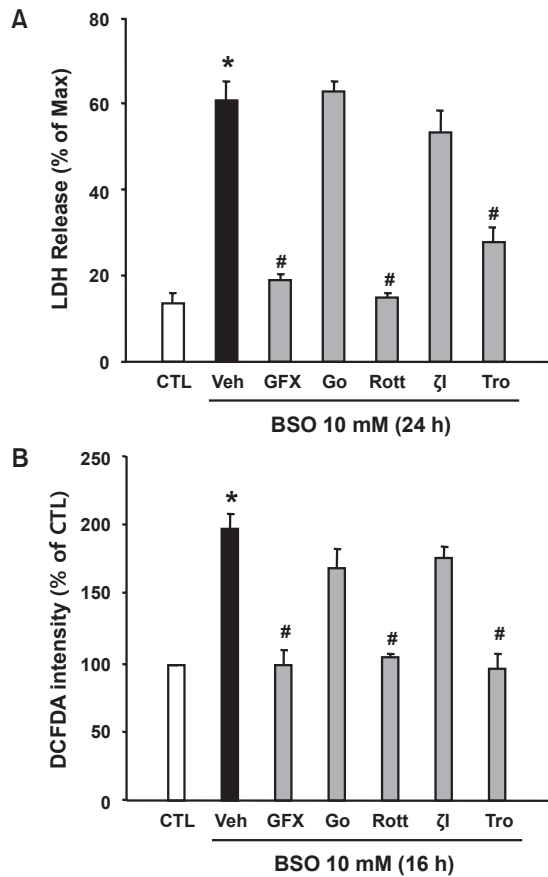


Fig. 3. Effect of PKC inhibitors on BSO-induced cell death and ROS production. H9c2 cells were incubated with BSO (10 mM) in the presence or absence of PKC inhibitors (10 μ M) or trolox (10 μ M). (A) Lactate dehydrogenase (LDH) release. Percent LDH was calculated from the maximum LDH release (100%) induced by 0.1% Triton X-100. (B) DCF-DA intensity. Data are expressed as mean \pm S.E.M. (n=3). * p <0.05 vs. 0 h. # p <0.05 vs. BSO. GFX: GF109203X; Go: Go6976; Rott: rottlerin; ζ I: PKC- ζ inhibitor; Tro: trolox.

As shown in Fig. 1B, ROS production was significantly increased by BSO. BSO-induced ROS generation was dramatically decreased by GME or trolox at each time point. These results suggest that BSO induced ROS generation through the depletion of cellular GSH levels.

Depletion of GSH induced cell death of cardiomyocytes

We examined the effect of GME on BSO-induced cell death. BSO remarkably increased annexinV-positive cells to 19.8% at 12 h, 27.5% at 14 h, and 26.8% at 18 h, indicating that BSO induce apoptosis of H9c2 cells (Fig. 2A). The BSO-induced apoptosis was blocked by treatment with GME (5.3% at 12 h, 9.3% at 14 h, and 6.9% at 18 h). BSO also increased annexinV-negative/PI-positive cells to 20.3% at 14 h, 28.7% at 18 h, and 34.7% at 22 h, indicating induction of necrosis of H9c2 cells (Fig. 2B). Treatment with GME inhibited the BSO-induced necrosis (10% at 14 h, 13.3% at 18 h, and 17.6%). Treatment with trolox blocked only BSO-induced apoptosis but not BSO-induced necrosis. Consistently, BSO increased the frequency of apoptotic cells with fragmented nuclei and

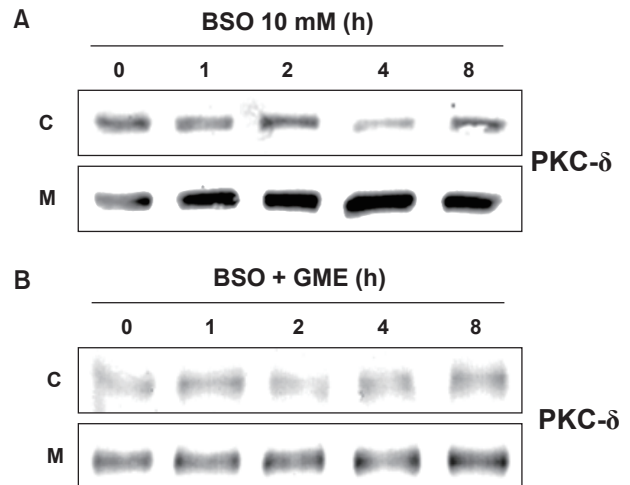


Fig. 4. Effect of glutathione monoethyl ester (GME) on BSO-induced translocation of PKC- δ . (A, B) Western blot analysis of PKC- δ . H9c2 cells were incubated with BSO (10 mM) in the presence or absence of GME (300 μ M) for indicated times. Blots are representative images (n=3). C: cytosol fraction; M: membrane fraction.

condensed chromatin compared with control cells (Fig. 2C). Treatment with GME completely protected cells from morphological changes by BSO. Next, we examined the effect of GME on the activation of caspase-3, which plays an important role in apoptotic cell death. As shown in Fig. 2D, caspase-3 activity was significantly increased to 250% at 14 h, and reached its maximal level (350%) at 16 h after BSO treatment. This BSO-induced caspase-3 activity was dramatically inhibited by GME or trolox. These results suggest that BSO induced apoptosis and necrosis through the depletion of cellular GSH levels.

PKC- δ contributed to BSO-induced cell death and ROS production

We further examined the effect of PKC isotypes on BSO-induced cell death. As shown in Fig. 3A, LDH release was remarkably increased by BSO compared to that observed in the control condition. This BSO-induced LDH release (61%) was decreased by the general PKC inhibitor (GF109203X, 18%) or the PKC- δ inhibitor (rottlerin, 15%). However, the conventional PKC inhibitor (Go6983) or the PKC- ζ inhibitor did not inhibit BSO-induced LDH release. Next, we determined the effect of various PKC inhibitors on ROS production (Fig. 3B). Consistent with LDH release, BSO-induced ROS generation (189%) was blocked by the GF109203X (103%) and the rottlerin (112%). These results suggest that PKC- δ plays a role in BSO-induced ROS production and cell death.

Depletion of GSH induced activation of PKC- δ

We determined the effect of GME on BSO-induced activation of PKC- δ . As shown in Fig. 4A, PKC- δ began to translocate to the membrane fraction from the cytosol fraction at 1 h after BSO treatment. Treatment with GME inhibited this BSO-induced translocation of PKC- δ (Fig. 4B). These results suggest that depletion of cellular GSH levels mediates BSO-induced PKC- δ activation.

DISCUSSION

In the present study, using a heart-derived H9c2 cell line, we observed that BSO-induced GSH depletion was associated with: 1) increased apoptosis, 2) increased ROS production, 3) increased caspase-3 activity, and 4) increased PKC- δ activity. These results support our hypothesis that BSO-induced GSH depletion induces cardiomyocyte apoptosis through PKC- δ activation.

In patients with chronic heart failure, it is reported that increased oxidative stress is associated with reduced left ventricular function and is correlated with the severity of the disease (Belch *et al.*, 1991; Mallat *et al.*, 1998; Maack *et al.*, 2003). Furthermore, cell culture and animal studies have suggested that ROS may be important mediators of cardiac hypertrophy and the development of contractile dysfunction (Dhalla *et al.*, 2000; Maack *et al.*, 2003). Ischemia results in impaired antioxidant defense, and subsequent reperfusion results in an increased concentration of ROS (Molyneux *et al.*, 2002). Depletion of cellular antioxidants such as GSH allows for the generation of significant quantities of ROS, which have been suggested to act as signals for the induction of apoptosis (Armstrong *et al.*, 2002). Many models have been proposed to explain the induction of GSH depletion. Depletion of GSH may be induced by GSH synthesis inhibitors (e.g., BSO), GSH-S-transferase-dependent thiol alkylation agents (e.g., diethyl maleate, phorone, etc.), peroxides (e.g., tert-butyl hydroperoxide, H₂O₂, etc.), and non-enzymatic GSH-reacting agents (e.g., diazene and diamide). We chose to use the GSH synthesis inhibitor BSO because of its specificity and because the block in GSH synthesis can be overcome by supplying the cell with GME (Torres *et al.*, 1997).

Results of previous studies showed that BSO-induced GSH depletion caused the induction of apoptotic and necrotic cell death in a variety of cell types (Rees *et al.*, 1995; Korystov *et al.*, 1996). In cardiomyocytes, mitochondrial GSH depletion was shown to modulate apoptosis in short-term diabetic rat hearts (Ghosh *et al.*, 2005). However, the mechanism by which the depletion of GSH modulates cell death is still unclear. Our results showed that the BSO-induced depletion of cellular GSH levels significantly increased ROS generation and apoptotic cell death in H9c2 cells (Fig. 1, 2). Apoptosis is generally associated with the activation of caspase cascades (Logue and Martin, 2008) and with the Bcl-2 protein family (Gross *et al.*, 1999). In addition, apoptosis is accompanied by signs of mitochondrial dysfunction, including the loss of mitochondrial membrane potential and the release of cytochrome c (Gottlieb *et al.*, 2003). One of the main consequences following mitochondrial cytochrome c release is the activation of caspase-3. Caspase-3 is an effector caspase with a wide spectrum of substrates, and plays a critical role in characteristic apoptotic changes (Chang and Yang, 2000). Our results showed that caspase-3 activity increased under conditions of BSO-induced GSH depletion (Fig. 2D). Thus, we clearly demonstrated that BSO-induced GSH depletion significantly increases apoptosis by the mitochondrial pathway through ROS generation.

It has been demonstrated that PKCs are one of the most important mediators of ischemia-induced changes. The PKC family is comprised of several serine/threonine protein kinases, and has been implicated as an intracellular mediator that is important in the regulation of growth, differentiation,

cell death, and neurotransmission. The PKC subfamilies include conventional PKC (PKC- α , - β 1, - β 2, and - γ), novel PKC (PKC- δ , - ϵ , - η , - θ , and - ι), and atypical PKC (PKC- ζ , - μ , and - λ). Different isoforms may perform distinct functions, as suggested by their differential pattern of localization, differences in their activation conditions, and some differences in substrate specificity. PKC- α , - β , - δ , - ϵ , and - ζ subtypes are known to be predominant isoforms in the rat heart (Doble *et al.*, 2000; Kim *et al.*, 2004). In previous study, we reported that the role of PKC- δ under hypoxia-induced apoptosis (Kim *et al.*, 2004). In rat cardiomyocytes, it is also addressed the role of PKC- δ in cellular redox balance and apoptosis in various experimental conditions. Indeed, PKC- δ was cleaved by caspase-3 under doxorubicin-induced oxidative stress (Lai *et al.*, 2011) and PKC- δ regulated apoptosis by H₂O₂-induced oxidative stress (Cieslak and Lazou, 2007). However, its role in GSH depletion-induced cardiomyocyte apoptosis has not been elucidated. Our results showed that PKC- δ was activated by the BSO-induced depletion of cellular GSH, and rottlerin, a PKC- δ inhibitor, significantly inhibited BSO-induced ROS production and cell death (Fig. 3, 4). This indicates that PKC- δ regulates the ROS generation and cell death induced by GSH depletion.

In summary, our results showed that BSO-induced depletion of cellular GSH induces the translocation of PKC- δ and, subsequently, the generation of ROS, which appears to enhance H9c2 cell death.

ACKNOWLEDGMENTS

This work was supported by a grant from the Next-Generation BioGreen 21 Program (No.PJ009074), Rural Development Administration, Republic of Korea. This research was supported by the Bio & Medical Technology Development Program (2011-0019397) of the National Research Foundation (NRF) funded by the Korean government (MEST) and industry Technology Development Program (111093-3), Ministry for Food, Agriculture, Forestry, and Fisheries, Republic of Korea.

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