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Requirement of Intracellular Free Thiols for Hydrogen Peroxide-Induced Hypertrophy in Cardiomyocytes

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Abstract Reactive oxygen species (ROS) are by-products of aerobic metabolism and are implicated in the pathogenesis of several diseases. H_2O_2 produces oxidative stress and acts as a second messenger in several cell types. We tested whether the effect of H_2O_2 on cellular events could be altered by changes in the intracellular redox status in a cardiomyocyte cell line. Using flow cytometric measurements, we found that adding H_2O_2 induced hypertrophy in control cells in a time-dependent manner. Pre-incubation of the cells with buthionine sulfoximine (BSO), an inhibitor of de novo GSH synthesis, induced increase in the number of cells of small sizes by the addition of H_2O_2 as compared to non-BSO pre-incubated control cells, and exacerbated the decrease in viability. Total thiol and GSH levels in H9c2 cells pre-incubated with BSO were about 75 and 30% of control, respectively, and GSH levels fell to below the limitation of detection after the addition of H_2O_2, although total thiol levels were not markedly decreased. In the cells pre-incubated with BSO, hypertrophy was not observed by the addition of H_2O_2 at any level of concentration. *N*-acetyl-L-cysteine and cysteine not only prevented increase in the number of cells of small sizes caused by H_2O_2 but also induced hypertrophy in cells pre-incubated with BSO. These results suggest that the intracellular free thiol levels determine whether cell death or hypertrophy occurs in cardiomyocytes in the presence of H_2O_2. On the other hand, the hypertrophied cells did not become larger by adding H_2O_2, but had high levels of cellular GSH, suggesting the possibility that the hypertrophied cells have tolerance to oxidative stress. J. Cell. Biochem. 89: 944–955, 2003.

Key words: thiol; H_2O_2; cardiomyocyte; hypertrophy; flow cytometry

Reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H_2O_2), and the hydroxyl radical, are generated under a variety of physiological conditions, and interact with biological materials such as lipids, proteins, and nucleotides. These interactions cause damage to cell organelles and may be involved in the pathogenesis of certain diseases [Halliwell and Gutteridge, 1984; Sakurai and Cederbaum, 1998; Sakurai et al., 2001a]. It has been suggested that oxidative stress would be involved in several cardiopathies such as arrhythmia, heart failure, and hypercardia and ischemia-reperfusion injury [McCord, 1985; Priori et al., 1990]. Cardiomyocyte apoptosis is increased during oxidative stress and during the transition to heart failure in human and animal models of myocardial injury [Fliss and Gattinger, 1996; James et al., 1996; Narula et al., 1996; Aikawa et al., 1997]. Hypertrophy in cardiomyocytes induced by several forms of stress such as hypertension, hemodynamic overload, and oxidative stress may compensate for cardiac dysfunction, such as reduction of the fractional volume or hyposystole [Grossman et al., 1975; Francis et al., 1995; D'Angelo

Abbreviations used: BSO, buthionine sulfoximine; Cys, cysteine; FBS, fetal bovine serum; FS, forward light scatter; SS, side light scatter; GSH, glutathione, reduced form; GPx, glutathione peroxidase; DMEM, Dulbecco's modified Eagle's medium; NAC, *N*-acetyl-L-cysteine; PBS, phosphate-buffered saline; ROS, reactive oxygen species; DCFDA, 2′,7′-dichloro-fluorescein diacetate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; WST, 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt.

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et al., 1997]. In vitro studies using a cultured cardiomyocyte cell line and isolated neonatal rat cardiomyocytes have demonstrated that while H_2O_2 can cause cell death, the surviving cells undergo hypertrophy [Adams et al., 1998; Chen et al., 2000].

Several lines of evidence showed that the oxidative stress could be implicated in a variety of intracellular signaling pathways. For example, H_2O_2 can cause activation of extracellular signal-regulated protein kinase which is a component of mitogen-activated protein kinase [Guyton et al., 1996]; ROS can regulate the activation of stress-activated protein kinases such as JNKs and SAPKs in cardiac myocytes [Laderoute and Webster, 1997], and the expression of G protein $p21^{ras}$ for initiation of signal transduction [Lander et al., 1997]. In addition, H_2O_2 could induce activation of phosphoinositide 3-kinase, which leads to the activation of p70 S6 kinase-1 and the increase of cell size in cardiomyocyte [Tu et al., 2002]. The expression of proto-oncogenes such as *c-fos* and *c-jun* is increased in the stage of experimental cardiac hypertrophy [Brand et al., 1993; Pollack et al., 1997]. However, the explanation of how H_2O_2 modifies the target components, and how the processes of cell death or hypertrophy are established has remained elusive.

There is extensive evidence that intracellular reduced glutathione (GSH) participates in the detoxification reactions of peroxides in cells, either directly or indirectly as a cofactor for enzymes such as glutathione peroxidase (GPx) [Meister, 1988; Sies, 1999; Carmel-Harel and Storz, 2000]. It has been demonstrated that GSH is decreased during ischemia and that ROS interact with the thiol groups of intracellular proteins [Werns et al., 1992; Baines et al., 1997]. Thus, GSH functions as an important cellular defense against ROS, particularly H_2O_2. In addition, intracellular GSH is thought to be one of the main determinants of thiol-disulfide balance, providing the cells with a reduced local environment, and causing a reductive cleavage of disulfide linkages [Sies, 1999; Carmel-Harel and Storz, 2000]. It has also been found that intracellular GSH levels are reduced under apoptotic conditions in hepatoma cells treated with ebselen and in lymphocytes treated with Fas [Banki et al., 1996; Yang et al., 2000]. However, the detailed role of intracellular GSH in modulating oxidative stress in cardiomyocytes is not clear. Pre-incubation with buthionine sulfoximine (BSO), a specific inhibitor of de novo GSH synthesis, induces the depletion of intracellular GSH, and enhances oxidative damage [Chen and Cederbaum, 1998; Sakurai and Cederbaum, 1998]. Therefore, in this study, we have addressed the relationship between H_2O_2, the redox status, and alterations of H9c2 cells. H9c2 cells are a subclone of an original clonal cell line derived from rat heart tissue [Kimes and Brandt, 1976].

The goal of the current report was to determine whether intracellular GSH was necessary for the induction of hypertrophy caused by the addition of H_2O_2. Herein, we tested the effects of an inhibitor of GSH synthesis and of reduced thiol compounds on the alterations caused to cardiomyocytes treated with H_2O_2. H_2O_2 induced the hypertrophy and the cell death in the control and GSH-depleted cells, respectively. Our findings imply that H_2O_2 was necessary but not sufficient as a signaling component for the hypertrophy and that the intracellular free thiol levels determine whether cell death or hypertrophy occurs in cardiomyocytes incubated with H_2O_2.

MATERIALS AND METHODS

Chemicals

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), BSO, and *N*-acetyl-L-cysteine (NAC) were obtained from Sigma Chemical Co., St. Louis, MO. GSH, cysteine (Cys), and H_2O_2 were from Wako Pure Chemical Industries, Ltd., Osaka, Japan. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay kit was from Promega Co., Madison, WI. The 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, sodium salt (WST) assay kit was from Dojindo Laboratories, Kumamoto, Japan. 2′,7′-dichloro-fluorescein diacetate (DCFDA) was from Eastman Kodak Co., Rochester, NY. All other chemicals used in this study were of the highest grade available from commercial suppliers.

Culture and Treatment of Cells

Rat cardiomyocyte H9c2 cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in DMEM containing 10% heat inactivated FBS in an incubator in an atmosphere of 5% CO_2, 95% air at

37°C. The standard treatments of H9c2 cells were as follows. The cells were seeded onto 24-well plates at a concentration of 1×10^5 cells/well or onto 96-well plates at 5×10^4 cells/well for cell proliferation assays. After an overnight pre-incubation with or without 0.1 mM BSO, the cells were incubated with H_2O_2 for various times and concentrations as indicated in the figures. The cell number was determined directly by counting with a hemocytometer.

Flow Cytometry of Cells

Relative changes in cell size were observed using a Coulter ELITE cytofluorometer (Beckman Coulter, Inc., Fullerton, CA) [Crissman et al., 1985; Marrone and Crissman, 1988; Bortner and Cidlowski, 2000]. Following the incubation, the cells (1×10^6) were washed and resuspended in 1 ml of divalent cation-free PBS containing 5 mM of EDTA at room temperature. The forward light scatter (FS) and side light scatter (SS) were recorded for each cell to determine the relative size and cell density, and 5,000 cells were screened. Control H9c2 cells were gated so that normal sizes were assumed to represent 90% of the cell population (region B), and large (region A) and small (region C) sizes were each 5% of the total cell population. The data shown are from a single experiment representative of three other independent experiments with similar results.

Thiol Contents

The experimental conditions were the same as described for the cytotoxicity determination except that the incubation time was 1 h. Following the incubation, the cells (5×10^6) were washed, resuspended in 1.5 ml of phosphate-buffered saline (PBS), and sonicated with an ultrasonic disrupter (model UR-200p, Tomy Seiko Co., Tokyo, Japan) three times for 10-s period at 35 W in ice-water. The total thiol content was measured by using 5,5′-dithiobis(2-nitrobenzoic acid) at 412 nm as described previously [Sakurai et al., 2000, 2001b]. The content of GSH was measured by using a GSH assay kit (Bioxytech GSH-400 kit, OxisResearch, Portland, OR). To remove contamination by protein thiols, the cells (5×10^6) were washed and resuspended in 1 ml of PBS containing 10% trichloroacetic acid. The suspension was centrifuged at 1,750*g* at 4°C for 10 min. The supernatant was extracted three times with four volumes of diethyl ether. To remove the remaining diethyl ether, the aqueous phase was purged with N_2 gas at room temperature. The sample was adjusted to 0.9 ml with a HEPES buffer, pH 7.4, and was assayed for intracellular GSH according to the GSH assay kit protocol. The protein concentration was determined using the Micro BCA kit (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Cytotoxicity Measurement

The proliferation of cells was determined using an MTT or a WST reduction assay [Ishiyama et al., 1996; Sakurai and Cederbaum, 1998; Sakurai et al., 2001a]. The results obtained from the two assays were essentially the same. Briefly, after the incubation with H_2O_2, the medium was removed. For the MTT assay, DMEM (575 μl) containing 10% FBS and a dye solution was added to each of the 24 wells for a 1-h incubation at 37°C. A solubilization/stop solution (500 μl) was added for an additional 4 h of incubation. The absorbance at 570 nm (formation of formazan) and 630 nm (reference) was recorded with a spectrophotometer [Hitachi U-2000, Tokyo, Japan).

To assay for WST reduction, 10 μl of the cell counting kit solution containing 5 mM of WST and 0.2 mM of 1-methoxy-5-methylphenazinium methylsulfate was added to the incubation medium in each of the 96 wells for a 1- to 4-h incubation at 37°C. The absorbance at 450 nm (reference 620 nm) was recorded with a microplate reader (Labsystems Multiskan Bichromatic, Helsinki, Finland).

Determination of Intracellular Peroxides

Intracellular peroxide levels were assessed using DCFDA. Cells pre-incubated with or without 0.1 mM of BSO were incubated with 0, 0.025, or 0.25 mM of H_2O_2 for 30 min at 37°C. Following incubation, the cells were treated with 0.01 mM of DCFDA for 15 min at room temperature. Micrographs were taken with the same exposure time (12 s) for all conditions.

Data Analysis

Data are represented as mean ± SD and were statistically analyzed by the Student's *t*-test for pair data. Differences are considered statistically significant at $P < 0.05$.

RESULTS

Effect of Thiol on Changes of H9c2 Cell Size After Addition of H_2O_2

During the study of cell death induced by H_2O_2, flow cytometry analyses showed that in control H9c2 cells (pre-incubated without BSO), H_2O_2 elicited an increase in the FS value (an indicator of cell size), whereas no change was observed in the SS value (an indicator of cell density) [Crissman et al., 1985; Marrone and Crissman, 1988; Bortner and Cidlowski, 2000]. As shown in Figure 1A, 24 h after the addition of H_2O_2 (0.25 mM), the control cells showed a small increase (region A, 8.3%) and decrease (region C, 15%) in the FS value compared to the cells before the treatment with H_2O_2 (5%, respectively). At 72 h, 27% of the cells were in region A, and there was a slight increase of cells in region C. In contrast, as shown in Figure 1B, H_2O_2 caused a slight decrease in the cell number in region A (2.6%) and a marked increase in region C (58%) at 72 h in the GSH-depleted cells which were pre-incubated with 0.1 mM of BSO overnight. In the presence of catalase, no significant changes that the increase and decrease of cell sizes were induced by the addition of H_2O_2 were observed in the cells pre-incubated with or without BSO, respectively (data not shown). These results indicate that H_2O_2 induces the hypertrophy with control cells increasing in cell size and maintaining the same density, and the decrease in the size of cells pre-incubated with BSO. Although the data were not shown, a small decrease in the viability of the control cells including cells of small size was observed after 24-h exposure to H_2O_2 (0.25 mM) judging from the results of the WST assay. However, when the cells were pre-incubated with 0.1 mM of BSO, the viability was markedly decreased in the presence of H_2O_2 at a concentration of 0.25 mM and decrease in viability was observed within 2 h after the addition of H_2O_2.

We next investigated intracellular GSH levels in H9c2 cells treated with BSO, H_2O_2, or both (Fig. 2). The total thiol content of control

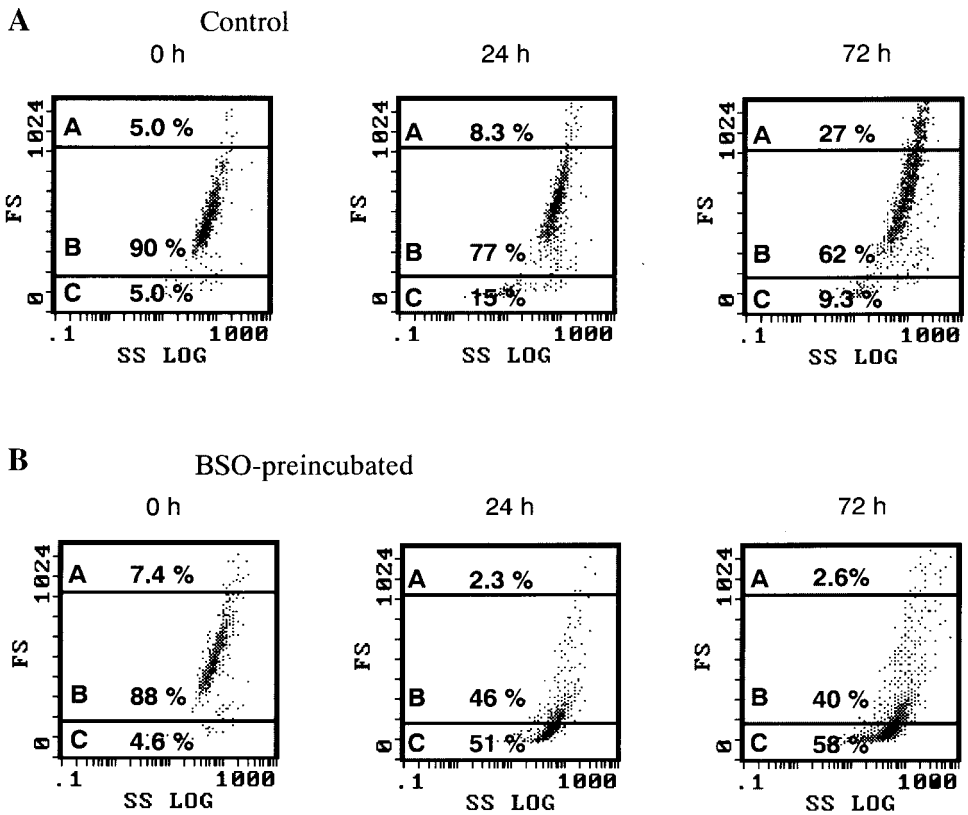

Fig. 1. Effect of pre-incubation with BSO on changes of cell size after addition of H_2O_2. The H9c2 cells pre-incubated without (**A**) or with (**B**) 0.1 mM of BSO were incubated with 0.25 mM of H_2O_2 for 24 and 72 h. Relative change of cell size were observed by flow cytometry as described in Materials and Methods. Similar results were observed in three other independent experiments using three different preparations of cells.

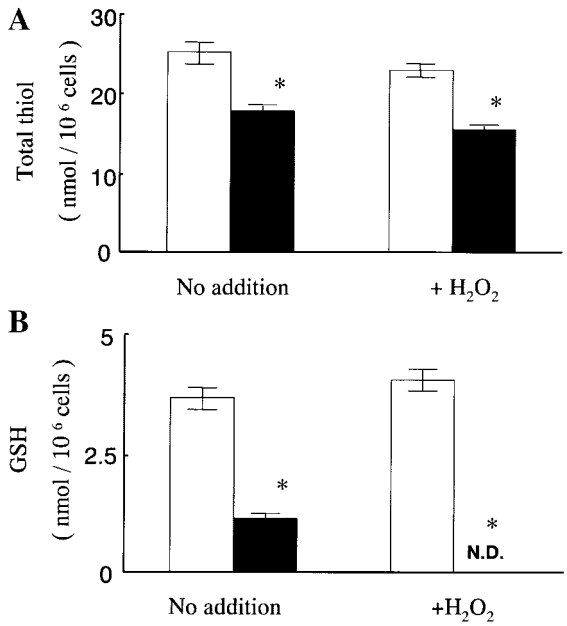

Fig. 2. Alteration of intracellular thiol levels after addition of H_2O_2. After the pre-incubation with (black bars) or without (white bars) 0.1 mM of BSO, H9c2 cells were incubated with or without 0.25 mM H_2O_2 for 1 h. Total thiol (**A**) and GSH (**B**) contents were measured by colorimetric assay as described in Materials and Methods. Each value is the mean ± SD of triplicate experiments. $^*P < 0.05$ compared with control cells (pre-incubated without BSO). ND, not detectable.

H9c2 cells was approximately 25 nmol/10^6 cells; this was slightly higher than the content for cells incubated with 0.25 mM of H_2O_2, although there was not a statistically significant difference. In the case of BSO-pre-incubated cells, the levels of total thiol were about 75% of control cells pre-incubated without BSO after incubation with or without 0.25 mM of H_2O_2, respectively. The intracellular GSH content was about 4 nmol/10^6 cells, and incubation with 0.25 mM of H_2O_2 had no significant influence on the GSH levels in the control cells. Pre-incubation with BSO caused a decrease in the GSH levels to one-third in H9c2 cells, and adding H_2O_2 to the cells caused a further fall in the GSH content below the limits of detection. These results suggest that a deficiency in intracellular GSH increases the sensitivity of the H9c2 cells to the cytotoxic action of H_2O_2.

In order to characterize the role of intracellular thiol in the initiation of hypertrophy, we studied the effect of H_2O_2 at various concentrations on the H9c2 cells pre-incubated with BSO. After 6-h exposure of H_2O_2 at 0.025 mM or less to H9c2 cells pre-incubated with BSO did not cause an inhibition of MTT reduction ability (Fig. 3A). When the incubation time was prolonged to 72 h, the increase in MTT reduction in the cells was similar to the control without BSO, indicating the proliferation of those cells. At 72 h after the addition of H_2O_2 at a low concentration (0.025 mM), the control cells showed a small increase in the FS value (region A, 9.2%, Fig. 3C), and the BSO-pre-incubated cells showed a lesser extent of increase in the values (region A, 7.7%, Fig. 3C) compared to the control (region A, 5.0%, Fig. 1A) and BSO-pre-incubated cells (region A, 7.4%, Fig. 1B) before treatment with H_2O_2, respectively. These results suggest that H_2O_2 alone cannot induce hypertrophy in the GSH-depleted H9c2 cells.

Production of Peroxides in H9c2 Cells Treated With H_2O_2

Figure 4 shows the generation of peroxides in H9c2 cells incubated with H_2O_2 using a specific fluorescent probe, DCFDA. In control and BSO-pre-incubated cells, DCFDA green fluorescence was scarcely observed in cells incubated without H_2O_2 for 30 min. After incubation with 0.25 mM of H_2O_2 for 30 min, the fluorescence significantly increased in cells pre-incubated with or without BSO. Since the trace transition metals such as iron is necessary for increase of DCFDA fluorescence in cells and H_2O_2 cannot oxidize DCFDA directly [Royall and Ischiropoulos, 1993; Tampo et al., 2003], H_2O_2 might induce the generation of peroxides in the cells. In contrast, after incubation with H_2O_2 at a low concentration (0.025 mM), a little fluorescence was observed in both the cells pre-incubated with or without BSO. These results indicate that there is not a clear difference between control and GSH-depleted cells in the generation of peroxide in cells treated with H_2O_2.

Effect of Thiol Compounds on H_2O_2-Induced Change of Cell Size

As shown in Figure 5, the thiol compounds NAC and Cys not only prevented the increase in cell number in region C but also elicited an increase of cells in region A in the BSO-pre-incubated H9c2 cells, 72 h after the addition of 0.25 mM of H_2O_2. GSH also prevented the increase of cells in region C, but scarcely affected the increase of cells in region A. The different effects of GSH, NAC, and Cys on the increase in cell number in region A may reflect

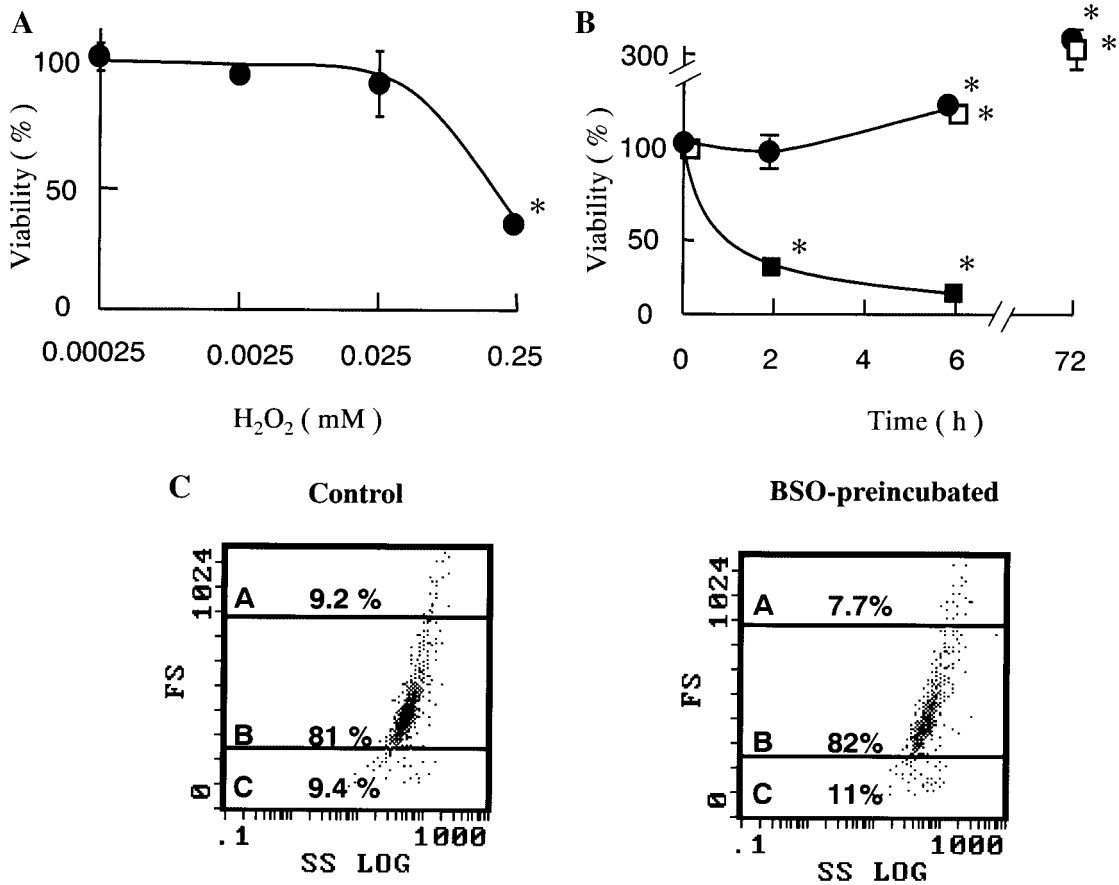

Fig. 3. Effect of low concentration of H_2O_2 on BSO-pre-incubated cells. H9c2 cells were pre-incubated with (●, ■) or without (□) 0.1 mM of BSO overnight at 37°C. **A**: After the addition of various concentrations of H_2O_2 to the culture medium, the cells were incubated for 6 h. **B**: H9c2 cells were incubated with 0.025 (●, □) or 0.25 (■) mM of H_2O_2 for the indicated times. Each point represents the mean ± SD of triplicate experiments. **C**: Cells were incubated with 0.025 mM H_2O_2 for 72 h. Other conditions were the same as those described in Figure 1. Similar results were observed in three other independent experiments using three different preparations of cells. $^*P < 0.05$ compared with control cells.

the different permeability of the plasma membrane to these compounds [Anderson and Meister, 1987; Meier and Issels, 1995; Sies, 1999]. The thiol compounds used in this study had no effect on the size of cells incubated without H_2O_2. In the presence of catalase, no cytotoxicity of H_2O_2 was observed in the cells under any conditions (data not shown). These results suggest that intracellular free thiols are involved in the hypertrophy in H9c2 cells induced by the external addition of H_2O_2. As shown in Figure 5B, NAC, Cys, and GSH completely prevented H_2O_2-cytotoxicity in GSH-depleted cells. However, GSSG, an oxidized form of GSH, had no clear influence on the viability and the changes in cell size induced by H_2O_2. These results suggest that reduced-thiols protect GSH-depleted cells against H_2O_2-induced cytotoxicity.

Effect of H_2O_2 on Hypertrophied Cells

Figure 6 shows the effect of H_2O_2 on cell size and the intracellular GSH levels in H_2O_2-treated cells for 72 h including 22% of hypertrophied cells. When the cells containing hypertrophied cells were incubated with 0.25 mM of H_2O_2 for an additional 72 h, no increase or decrease in cell number in region A (20%) was observed compared to the cells containing hypertrophied cells (22%) with additional incubation without H_2O_2. The number of cells in region C slightly increased, although there was not a significant difference. These results suggest that the hypertrophied cells are less sensitive to H_2O_2 compared to the control cells. The level of GSH in hypertrophied cells incubated with 0.25 mM of H_2O_2 for 72 h was approximately 10 nmol/10^6 cells, and was twice

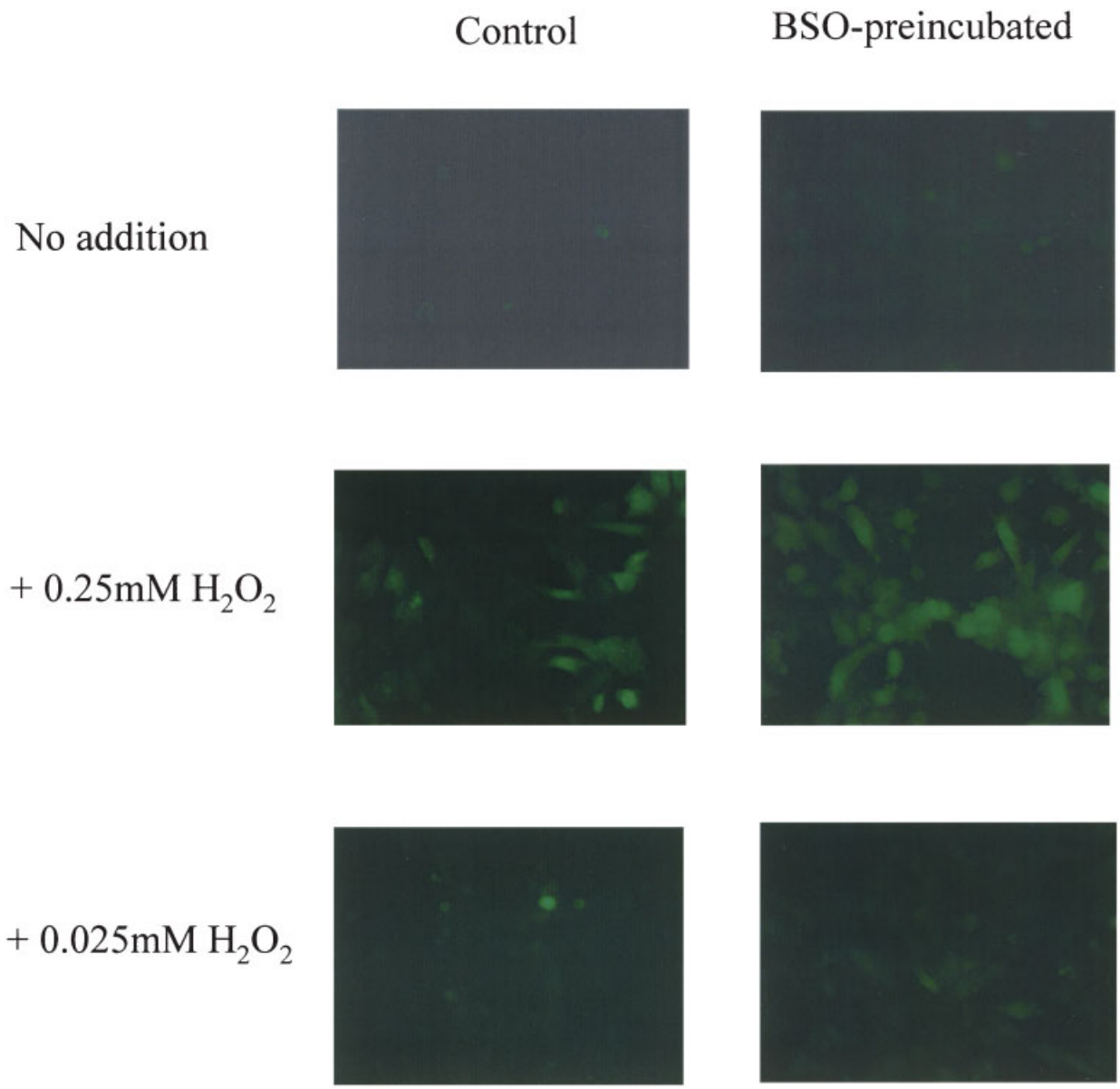

Fig. 4. Generation of intracellular peroxides after addition of H_2O_2. After the pre-incubation with or without 0.1 mM of BSO, the cells were incubated with or without H_2O_2 for 30 min. Then, cells were treated with 0.01 mM of DCFDA for 15 min. Similar results were observed in three other independent experiments using three different preparations of cells.

as much compared to the level of control cells incubated without H_2O_2 for 72 h.

DISCUSSION

The present study demonstrates that adding H_2O_2 elicits the hypertrophy and the decrease in cell sizes in H9c2 cells pre-incubated without and with BSO, respectively. BSO can inhibit de novo synthesis of GSH, which is one of the important intracellular components involved in the cell defense system against oxidative stress. H_2O_2 could potentially act as a signaling element by activating subsequent steps in a signal transduction cascade. Indeed, numerous studies have implicated H_2O_2 as a participant in the initiation of multiple-signal transduction cascades, including the stress- and mitogen-activated protein kinases, the nuclear transcription factors c-Jun and NF-κB, and other signaling systems [Rao, 2000; Chen et al., 2001; Lee and Koh, 2003]. Although H9c2 cell using

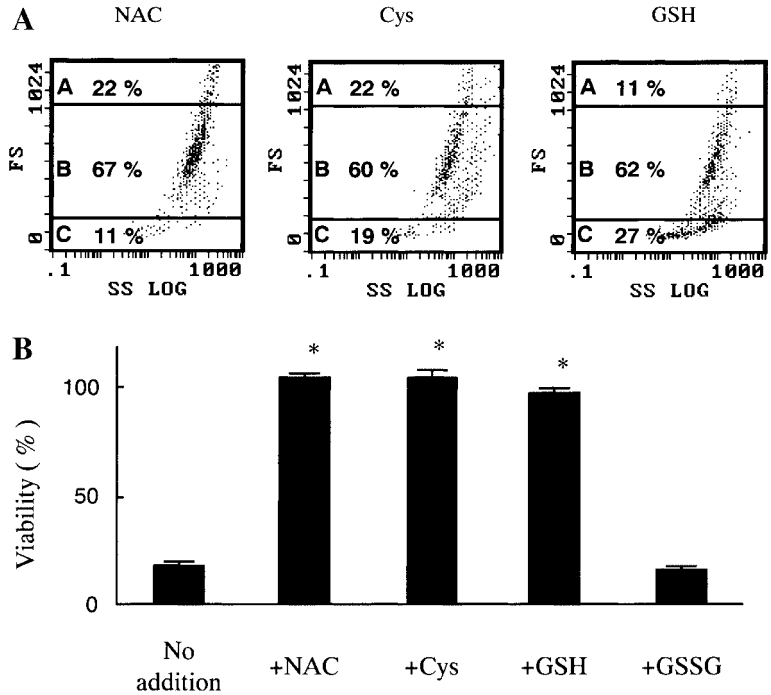

Fig. 5. Effect of thiol compounds on H_2O_2-induced changes of cell size. NAC (2 mM), Cys (2 mM), and GSH (2 mM) were added to the culture medium 1 h before adding H_2O_2. **A**: The cells pre-incubated with BSO were incubated with 0.25 mM of H_2O_2 for 72 h. Similar results were observed in three other independent experiments using three different preparations of cells. **B**: BSO-pre-incubated cells were incubated with 0.25 mM of H_2O_2 for 6 h. The viability of cells was determined by the MTT assay as described in Materials and Methods. Each point represents the mean ± SD of triplicate experiments. $^*P < 0.05$ compared with control cells incubated without H_2O_2 for 72 h.

the present study is a cardiomyocyte cell line derived from rat ventricles, it has been demonstrated that the cells as well as the primary cardiomyocytes, which maintain many characteristics of heart cells developed the hypertrophy in response to H_2O_2 [Chen et al., 2000]. These data suggest that the concentration of H_2O_2, which is partly controlled by intracellular GSH levels, can influence the activation of such pathways in heart cells in vivo.

Hypertrophy in control H9c2 cells (pre-incubated without BSO) was seen after adding 0.25 mM of H_2O_2. It is widely accepted that ROS as well as H_2O_2 participate in producing oxidative stress and acting as a second messenger leading to apoptosis, proliferation, and cell cycle regulation in several cell types [Brumell et al., 1996; Napoli et al., 2001]. The present study demonstrated that there is not a clear difference between control and GSH-depleted cells in the generation of peroxide after the addition of H_2O_2 (0.25 mM). Therefore, it is not likely that the peroxide in cells seems to have a direct relationship with the induction of hypertrophy. Chen et al. showed that while H_2O_2 could cause cell death, the surviving H9c2 cells underwent hypertrophy [Chen et al., 2000]. Several workers have demonstrated that GSH depletion induced by BSO could cause cell death that seems to be apoptosis in several cells [Pereira and Oliveira, 1997; Higuchi and Matsukawa, 1999]. These findings suggest that a certain concentration of H_2O_2 may be coupled to a signal transduction mechanism in apoptosis, which could trigger a functional response. Identification of apoptosis in H9c2 cells treated with H_2O_2 under the present conditions including the pre-treatment with BSO and thiol compounds will be a subject of future studies. In contrast, treatment with H_2O_2 could induce the activation of phosphoinositide 3-kinase, which leads to p70 S6 kinase-1 activation and enlargement of cell size in cardiomyocytes [Tu et al., 2002]. To keep the level of H_2O_2 low and to convert it into inert molecules like H_2O and O_2, cells employ antioxidant enzymes such as catalase and GSH peroxidase. The latter might decompose, adding H_2O_2 to H9c2 cells, because the depletion of GSH leads to the decrease in the size of cells treated with H_2O_2 and the activity of catalase in these cells would be significantly lower as compared with other cells [Teoh et al.,

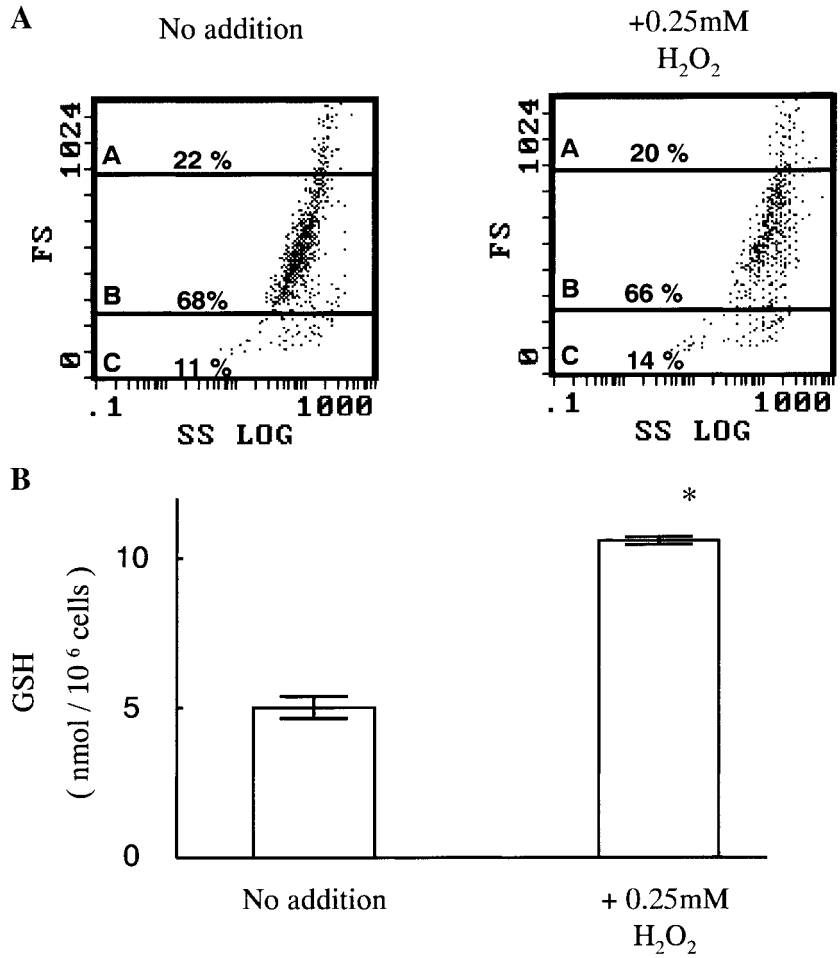

Fig. 6. Effect of H_2O_2 on cell size and GSH levels in cells containing hypertrophied cells. **A**: After incubation with 0.25 mM of H_2O_2 for 72 h, the H9c2 cells containing hypertrophied cells were washed with the medium once and then incubated with or without 0.25 mM of H_2O_2 for additional 72 h. Similar results were observed in three other independent experiments using three different preparations of cells. **B**: The cells were incubated with or without 0.25 mM of H_2O_2 for 72 h. GSH levels were measured by colorimetric assay as described in Materials and Methods. Each value is the mean ± SD of triplicate experiments. $^*P < 0.05$ compared with control cells incubated without H_2O_2 for 72 h.

1992; Teiger et al., 1996]. These results suggest that the cells may respond to a low concentration of H_2O_2 as a mediator in the signal transduction pathway for hypertrophy.

Total thiol and GSH levels were scarcely changed by adding H_2O_2 to control cells under conditions that induced hypertrophy, while GSH levels but not total thiol content were reduced below the limits of measurement under the conditions that induced the decrease in the size of cells pre-incubated with BSO. There are no observations of hypertrophy caused by a variation in intracellular thiol levels, which is brought about by the addition of thiol compounds and/or oxidants to cells incubated without H_2O_2. Furthermore, H_2O_2 per se at any concentration could not induce hypertrophy in GSH-depleted cells. These results suggest the requirement for intracellular GSH rather than for protein thiols in the process of H_2O_2-induced hypertrophy in cardiomyocytes. The intracellular redox state is a consequence of the balance between the levels of oxidizing and reducing equivalents. Particularly, the ratio of GSH to GSSG in cells would represent an overall redox environment. Recently, it was proposed that the intracellular redox environment is involved in the signaling to regulate many aspects of cell fate [Be Beleser et al., 1999; Filomeni et al., 2002; Kim et al., 2002]. We, therefore, speculated that GSH might directly participate in triggering the signal transduction or initiating the process leading to hypertrophy by H_2O_2 rather than the regulation of H_2O_2

concentration in the cells. However, we cannot rule out that the metabolism of thiol compounds by H_2O_2 is directly involved in the signal transduction mechanism for hypertrophy. The role of GSH in hypertrophy will be the subject of future studies.

NAC and Cys not only prevented the decrease in the size of cells followed by death but also elicited hypertrophy in GSH-depleted cells after the addition of H_2O_2, suggesting that thiol compounds can substitute for GSH in the process of H_2O_2-induced hypertrophy. Detoxification of H_2O_2 would be dependent upon GPx activity in control H9c2 cells, because GSH per se reacts poorly with H_2O_2. NAC and Cys as well as GSH can act as reducing substances for GPx, which can catalyze the decomposition of H_2O_2 and organic peroxides [Roveri et al., 1994; Saito et al., 1999]. However, the external addition of GSH did not induce hypertrophy in GSH-depleted cells in the present study. This may be due to the fact that NAC and Cys can be transported across the plasma membrane, but GSH cannot [Anderson and Meister, 1987; Meier and Issels, 1995; Sies, 1999]. We were able to restore the hypertrophy induced by H_2O_2 in GSH-depleted cells by adding thiol compounds, which can cross the cell membrane. These findings may imply an essential role for intracellular free thiol-groups in the signal transduction mechanism as well as the defense system of H9c2 cells.

When H_2O_2-treated cells containing 22% of hypertrophied cells were incubated with H_2O_2, neither a further increase in cell size nor cell death was observed. The intracellular GSH level in the cells containing hypertrophied cells was approximately twice as compared to the level of cells incubated without H_2O_2, suggesting that the hypertrophied cells can have a resistance against oxidative stress. It seems likely that the process of hypertrophy is an adaptation to oxidative stress rather than the damages. Further studies are needed to characterize the hypertrophied cells.

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