Original Article

Critical role of GSH in Sulfur Mustard-induced Oxidative Stress and Cytotoxicity in Human Skin Fibroblast Cell Line

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Abstract

In this study the role of glutathione (GSH) in sulfur mustard -induced oxidative stress and cytotoxicity, in human skin fibroblast cell line (HF2FF) was evaluated. Sulfur mustard-induced superoxide radical and hydrogen peroxide formation were evaluated by determination of superoxide dismutase and catalase activity in cell lysate. The cytotoxicity of sulfur mustard was estimated by lactate dehydrogenase leakage. The intracellular GSH content was modulated by *N*-acetylcysteine (NAC), a GSH precursor, and buthionine sulfoximine (BSO), a specific GSH synthesis inhibitor. It was found that sulfur mustard exposure led to a dose-and time-dependent decrease in GSH content in HF2FF cells. NAC increased intracellular GSH level and protected the cells against sulfur mustard-induced reactive oxygen species formation and lactate dehydrogenase leakage. In contrast, buthionine sulfoximine pretreatment depleted cellular GSH and enhanced the susceptibility of HF2FF to the cytotoxic effects of sulfur mustard. These results indicated that GSH plays a critical role in protecting HF2FF cell line against sulfur mustar-induced cell injury, most probably through its antioxidant activity.

Keywords: Glutathione; Reactive oxygen species; Sulfur mustard; HF2FF cells; NAC; Buthionine sulfoximine.

Introduction

Sulfur mustard (2, 2-dichlorodiethylsulfide, SM) is an alkylating agent that have been used for many years as chemical weapon (1). SM is highly toxic chemical agent and still remained a threat to both civilians and military personnel (2). It is well recognized that human skin fibroblast cells are a target for SM, and major pathological events have been seen in these cells after SM exposure.

Based on the following evidences: the role of reactive oxygen species (ROS) formation and oxidative stress in sulfur mustard-induced injury have been well established *1*) production of various species of ROS by sulfur mustard in cell-free systems, *2*) in vitro SM-induced oxidative damage such as lipid peroxidation and oxidative DNA breakage, *3*) elevated level of ROS, oxidative damage, and the upregulation of the antioxidant mechanisms under in vivo conditions, and *4*) protective effects of antioxidants against SM-induced cytotoxicity (3).

Under normal circumstances, there is a delicate balance between ROS formation and antioxidant defenses in cells. When the generation of ROS is overwhelming, as in the case of SM exposure,

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or impairment of the antioxidant defense mechanism, an oxidative stress is induced, resulting in cell injury. Glutathione (GSH) (L-γ-glutamyl-L-cysteinylglycine) is among the most important antioxidants in organisms due to its potent antioxidant capacity, close involvement in many cellular functions, and abundance in tissues or cells (4, 5). Its reduced (GSH) and oxidized (glutathione disulfide, GSSG) forms constitute the major thiol redox system in cells, and the redox status is of crucial importance for cellular function (6).

Regarding to the role of glutathione in the protection against oxidative stress and detoxification of xenobiotics, its availability in the reduced form (GSH) may be a key factor in maintenance of the health. It has been established in several different animal models, as well as in human, that a decrease in GSH concentration may be associated with aging and pathogenesis of many diseases (7-11).

In recent years, there are increasing evidences that intracellular redox status affects cellular and molecular events in various cells. Redox status modulates protein activities (12), the ability of certain transcriptional factors to bind to their cognate DNA (13), signal transduction (14), cell necrosis (15), and cell apoptosis (16). It is also associated with cell proliferation (17, 18, 19) and affects growth factor functions (20, 21). However, the role of thiol-disulfide balance in cell proliferation is unresolved.

Oxidative stress is an important pathophysiological stimulus that affects the cellular redox status. However, besides inducing redox shift, oxidative stress can directly affect cell functions such as enzyme activities, cell signaling, and cellular responses (22). The effects of redox changes is often overlaped with those of oxidative stress. For this reason, it is sometimes unclear whether cellular responses elicited by oxidative stress are mediated directly by ROS or by ROS-induced redox imbalance.

The main objective of the present study was to evaluate the role of GSH on SM-induced oxidative stress and cytotoxicity in HF2FF cell line, the principal target cells of SM in the skin. In the present investigation, the intracellular GSH level in HF2FF cells was modulated by NAC, a GSH precursor, and BSO, a specific GSH synthesis inhibitor. The data from these experiments provide direct evidence showing that GSH plays a critical role in sulfur mustard-induced oxidative stress and cell injury.

Expremintal

Materials
N-acetylcysteine (NAC), 5,5'-dithio-bis (2-nitrobenzoicacid) (DTNB) GSH, GSSG, NADPH+H$^+$, ATP, Lactate dehy drogenase (LDH), Phosphoenol pyrovate (PEP), Pyrovate kinase and bothionie sulfoximine (BSO) were purchased from Sigma chemical co. (st Louis, Mo.USA). SM (purity>98%) was obtained from the ministry of Defense. RPMI 1640 and Fetal calf serum were purchased from life Technologies (Gibco, cergy-pontoise, France), antibiotics (penicillin, stereptomycin), RPMI, HBSS and Trypsin was purchased from Sigma chemical co. (st Louis, Mo.USA).

Methods
Cell culture
HF2FF-cell line was obtained from Iranian Pastur institute .The cells were routinely plated at a density of 10^5 cells/plate and grown in RPMI 1640 (pH 7.4) , supplemented with 10% of FCS and antibiotics (7Unit/ml penicillin, $100\,\mu g$/ml streptomycin) at 37°C in 95% air-5% CO2.

Chemical treatment
Confluent monolayers were used in each assay and all treatments were carried out in HBSS media . NAC was prepared in 1mM concentration, stored at -20°C and BSO (1.8mM) were prepared in distilled water just before use. For GSH depletion, the cells were incubated for 24h with 1mM BSO, as earlier described by R. Drew *et al*.(23). Under these conditions BSO affect neither cell proliferation nor viability, at least during 30h of incubation. SM was stored in isopropanol (30, 100, 180, 300 and 1000 µM) at -20°C . NAC was added to HBSS media with final concentration of 1mM, the cells were rinsed with HBSS 60min post NAC exposure and then RPMI was added. After 24h incubation, the cells treated with SM in HBSS. The final isopropanol concentration was less than 0.1% (v/v).

Determination of intracellular glutathione

The concentration of reduced glutathione was determined according to the Tietze method with slight modifications (24). Briefly, HF2FF cells were cultured at 5×10^5 cells/well in 6-well culture plates for 2 days. At the end of the incubation, cells were washed twice with Ca^{2+}- and Mg^{2+}-free PBS and lysed with 0.1 ml of 3% perchloric acid for 15 min at 4 °C, then centrifuged at $800 \times g$ for 5 min, and supernatant were neutralized with 0.9 ml of 0.1 M sodium phosphate/5mM EDTA buffer, pH7.5 (phosphate/EDTA buffer). The reaction mixture contained 20µl of the neutralized extract, 0.96 ml of phosphate/EDTA buffer, 20 µl of 60 mM 5,5'-dithio-bis(2-nitrobenzoic acid). The increase of absorbance at 412 nm was monitored for 4 min. At each determination, a standard curve of glutathione was prepared.

Catalase activity

Catalase activity was measured by monitoring enzyme-catalyzed decomposition of H_2O_2. Briefly, a solution of H_2O_2 was added to test tubes containing samples, a water blank, and a H_2O_2 standard solution (standard) and incubated for 3min .The enzymatic reaction was terminated by addition of H_2SO_4. $KMnO_4$ (1.4 ml) to each tube, then vortexed and the absorbance of unreacted $KMnO_4$ was recorded at 480 nm. One unit of catalase activity is defined as $k/(0.00693)$, where $k = \log (S_o/S_2) \times (2.3/t)$, S_o is absorbance of standard minus absorbance of blank, S_2 is absorbance of standard minus absorbance of sample and t is time interval (25).

Superoxide dismutase activity

Total SOD activity (Mn-plus Cu, Zn-SOD) was determined as previously described by Hermes-Lima and Storey (26), under the following assay conditions: 5 mmol l^{-1} EDTA, 2.5 mmol l^{-1} MnCl$_2$, 0.25 mmol l^{-1} NADH, 4 mmol l^{-1} 2-mercaptoethanol in 50 mmol l^{-1} potassium phosphate buffer, pH 7.2. One SOD unit is defined as the amount of enzyme that inhibits the superoxide-induced oxidation of NADH (monitored at 340 nm) by 50% (IC$_{50}$). Several 1 ml cuvettes were run for each sample, using increasing amounts of enzyme extract (from 0 to 150 µl), the data were plotted as velocity *versus* amount of enzyme extract, and an IC$_{50}$ value was obtained. Blanks were run in the absence of 2-mercaptoetanol.

Lactate dehydrogenase (LDH) activity

For LDH assay, 2.5×10^4 cells/well in 96-well or 5×10^5 cells/well in 6-well culture plates were cultured for 2 days. At the end of the incubation with SM, culture medium was removed and centrifuged at $800 \times g$ for 10 min to obtain cell-free supernatant. The activity of LDH in the supernatant was determined using Vassault and colleagues method (27). Because NAC could affect LDH activity measured with this method, the absorbance value of RPMI containing NAC (0, 0.01, 0.1 or 1 mM) at 490 nm was subtracted from each value of the supernatant. The results were expressed as percentage of the maximum amount of LDH released from samples that had been treated with 1% Triton X-100. LDH leakage (in percent) was calculated as (LDH activity in medium/total LDH activity)×100.

Statistical analysis

Data were analysed using 2-way analysis of variance. Subsequent to this, groups were compared with each other using Dunnett's test. Significant differences between groups were assumed if the P value was <0.05.

Results

Figure 1 shows the dose-dependent decrease in viability when cells were treated with sulfur mustard for 1 h. With the highest concentration of sulfur mustard (1000 µM), the viability was approximately 5-20% of the control value. Sulfur mustard -induced decline in intracellular GSH level was also time dependent (Figure 2). The GSH concentration in the control cells remained at a constant level (~3.31 µmol/mg protein), whereas the GSH level in the cells treated with sulfur mustard (100 µM) decreased dramatically from 1 h onward (Figure 2) . In the present study, HF2FF cells were pretreated with 0.1 mM NAC and 1 mM BSO for 12 h before exposure to sulfur mustard. Compared with the control cells, NAC significantly enhanced the intracellular GSH content of HF2FF cells, whereas BSO pretreatment markedly
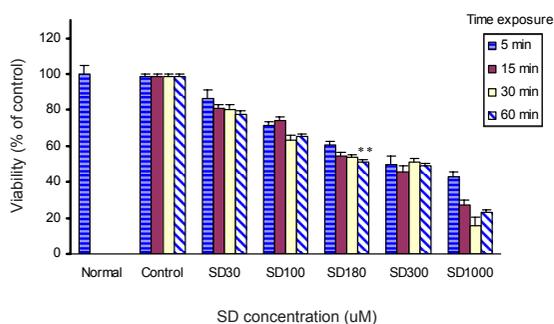
Figure 1. Effect of sulfur mustard exposure on cellular viabillity in human skin fibroblast cells (HF2FF). In dose-response study cells were treated with different doses (from 0 to $1000\,\mu M$) of sulfur mustard and different times (from 0 to 60 min). Data are means±SD; *n=6* experiments.
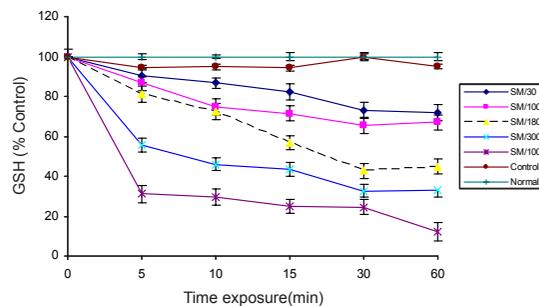 * *P* < 0.05 compared with control group.

Figure 2. Effect of sulfur mustard exposure on intracellular glutathione (GSH) in human skin fibroblast cells (HF2FF). In dose-response study cells were treated with different doses of sulfur mustard for 1 h. Data are means±SD; *n=6* experiments.
 * *P<0.05* compared with control group.

decreased the GSH level in HF2FF cells (Figure 3). Although the subsequent sulfur mustard exposure significantly reduced the GSH level, those cells with NAC pretreatment contained a higher level of GSH compared with the cells treated with sulfur mustard only. On the other hand, BSO pretreatment further enhanced the extent of GSH depletion caused by sulfur mustard exposure because the cells with both BSO and sulfur mustard treatment displayed the lowest GSH level among all these groups. The viability of these three groups of HF2FFs were exactly match with GSH levels (Table 1). Table-1 shows the different effects of NAC and BSO on sulfur mustard-induced cytotoxicity as measured by the percentage of LDH leakage. After 24h of incubation, LDH leakage in the control, control (180μM/SM), BSO (180μM/SM), and NAC(180μM/SM)cells was 11.5±0.85, 71.7±4.72, 78.31±5.96, and   49.34±3.32%, respectively. These data   suggest that, NAC

pretreatment significantly reduced the LDH leakage induced by sulfur mustard, whereas the cells pretreated with BSO showed a marked increase in LDH leakage compared with those cells exposed to sulfur mustard only. In this study, SOD activity was used for the determination of $O_2\cdot$ formation and catalase activity was used for measurement of intracellular H_2O_2 formation. The results are summarized in Table 2. As shown in Table 2, NAC pretreatment did not change the SOD and catalase activity in sulfur mustard-exposed HF2FFcells than the control cells. In contrast, BSO pretreatment significantly enhanced the SOD and catalase activity in sulfur mustard-treated HF2FF cells (Table 2).

Discussion

The involvement of ROS and oxidative damage in sulfur mustard-induced cytotoxicity and fibrosis has been extensively studied. It is

Table 1. Effects of NAC and BSO on LDH and GSH in sulfur mustard-exposed HF2FF cells. Cells were pretreated with NAC (0.1 mM) or BSO (1 mM) for 1 h and then treated with 180 μM SM for 1 h. Data are means±SD; *n=6* experiments.

	% LDH leackage	GSH(**μ**M/mg protein)
Normal	11.5±0.85	0.331±0.12
NAC	12.2±1.1	0.744±023*
BSO	11.9±0.85	0.15±0.1*
Control (180μM/SM)	71.7±4.72*	0.17±0.14*
BSO (180μM /SM)	78.31±5.96*	0.11±0.09*
NAC (180μM/SM)	49.34±3.32*	0.24±0.11*

 * *P<0.05* compared with control group; LDH: Lactate dehydrogenase; GSH: Glutathione; NAC: N-acetylcysteine; BSO: Buthionine Sulfoximine.
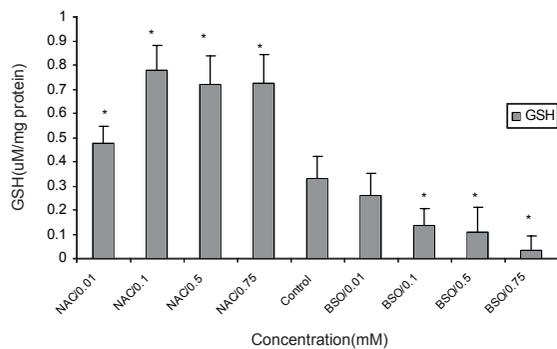
Figure 3. Effects of *N*-acetylcysteine (NAC) and buthionine suloximine (BSO) on intracellular GSH changes in HF2FFs. Cells were pretreated with NAC (0.1 mM) or BSO (1 mM) for 12 h. Data are means±SD; *n*=6 experiments.
 * *P*<0.05 compared with control group.

well known that GSH is the major intracellular antioxidant with multiple biological functions. One of its most important functions is protection against oxidative damage caused by ROS through enzymatic and nonenzymatic reactions (28). Therefore, it will be of interest to evaluate the effect of GSH on sulfur mustard toxicity. The present study was thus undertaken to assess the role of intracellular GSH on sulfur mustard-induced oxidative stress, cytotoxicity in primary cultured HF2FF cells and evaluation of intracellular GSH level on cell protection. The intracellular GSH level was modulated with NAC, a GSH precursor, and BSO, a specific GSH synthesis inhibitor. The results obtained show that NAC was able to increase the intracellular GSH content in HF2FF cell and suppress sulfur mustard-induced ROS formation and LDH leakage. In contrast, BSO pretreatment led to intracellular GSH depletion and enhanced the susceptibility of HF2FFs to sulfur mustard-induced oxidative stress and cell injury. Therefore, the present study provides

convincing evidence supporting the notion that GSH plays an important role in sulfur mustard-induced oxidative stress and cytotoxicity in HF2FF cells.

Previous studies showed that the concentrations of antioxidant molecules, including GSH, decreased in different tissues after exposure of sulfur mustard (15). On the other hand, there was a maximal decrease in intracellular GSH in HF2FF cells 1 h after sulfur mustard exposure, which coincides with the dramatic increase in LDH leakage at the same time points after sulfur mustard treatment. It is believed that there are three possible mechanisms accounting for the decline in cellular GSH in response to sulfur mustard exposure: *1*) via GSH peroxidase (GPx) reaction, *2*) via the GSH transferase reaction, and *3*) via GSH efflux (5, 14). GPx mainly catalyzes the direct reaction of GSH with ROS such as H_2O_2 and hydroxyl radical, resulting in the formation of GSSG.

The close resemblance of the time course of GSH with that of LDH leakage tends to suggest that GSH efflux is an important mechanism accounting for sulfur mustard-induced intracellular GSH depletion in HF2FFs. The availability of cysteine is one of the rate-limiting factors in GSH synthesis, and NAC acts as a precursor of GSH to facilitate GSH synthesis (20). In this study, NAC pretreatment was able to increase the intracellular GSH level and inhibit sulfur mustard-induced LDH leakage, which is consistent with an earlier study showing that the addition of NAC was responsible for a decrease in silica-induced cytotoxicity in cultured pulmonary endothelial cells. Furthermore, the present study also examined the effects of NAC on sulfur mustard-induced ROS

Table 2. Effects of NAC and BSO on SOD and catalase activity in sulfur mustard-exposed HF2FF cells. Cells were pretreated with NAC (0.1 mM) or BSO (1 mM) for 1 h and then treated with 180 μM SM for 1 h. Data are means±SD; *n*=6 experiments.

	SOD (i.u/mg protein)	Catalase (i.u/mg protein)
Normal	56.8±11.92	12.3±2.11
BSO	63.32±10.44	13.92±1.98
NAC	55.87±11.36	12.02±1.15
Control (180 μM/SM)	87.61±24.2*	14.56±3.46*
BSO (180μM /SM)	89.3±20.62*	15.23±4.23*
aNAC (180μM/SM)	52.40±12.75	11.93±3.89

 * *P*<0.05 compared with control group; SOD: Superoxide dismutase; BSO: Buthionine Sulfoximine; NAC: N-acetylcysteine.

formation. It is interesting to note that NAC pretreatment significantly suppressed catalase activity. The differential effects of NAC on different species of ROS were also observed in an earlier study by Aruoma and his co-workers (2). They demonstrated that NAC is a potent scavenger of H_2O_2 and hydroxyl radicals, whereas the scavenging activity of NAC against $O_2\cdot$ was found to be rather weak. At present, the antioxidant property of NAC has been well characterized, and it is known that NAC acts as a precursor of GSH to facilitate intracellular GSH synthesis (2, 20). Therefore, the inhibitory effects of NAC against sulfur mustard-induced ROS formation and LDH leakage clearly indicate the important role of GSH in protection against sulfur mustard toxicity in HF2FF cells. Some earlier studies (18, 19) in animals and humans used aerosolized GSH to enhance pulmonary GSH content and to counteract the imbalance of oxidant-antioxidant in idiopathic pulmonary fibrosis. BSO is a specific inhibitor of glutamylcysteine synthetase, the key enzyme in intracellular GSH synthesis. It has been widely used for depleting intracellular GSH in various cells and tissues (1, 23). In the present study, BSO pretreatment led to a reduction in GSH content in control cells and a further decline in GSH level in sulfur mustard-treated HF2FF cells. Accordingly, the cells pretreated with BSO became more susceptible to the toxic effects of sulfur mustard as shown by the significantly increased LDH leakage. Results from the present study also showed that BSO-pretreated HF2FF cells generated a higher level of ROS in both the control and sulfur mustard-exposed cells, indicating that GSH mainly acts as an ROS scavenger to protect against sulfur mustard-induced cell injury. The aggravating effect of BSO on sulfur mustard-induced oxidative stress, cytotoxicity, and genotoxicity further supports the notion that GSH plays a critical role in the toxicity of sulfur mustard (22). It seems that sulfur mustard-induced ROS formation led to the depletion of GSH and impairment of the antioxidant system, which in turn, exacerbates the oxidative damage in sulfur mustard-exposed cells.

In summary, the present study evaluated the critical role of GSH in sulfur mustard-induced oxidative stress and cytotoxicity in cultured HF2FF cells. It is believed that the understanding of the importance of GSH will be beneficial to the development of preventive or therapeutic agents in control of sulfur mustard-induced injuries in human skin.

Acknowledgement

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