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REVIEW ARTICLE

Comparative transcriptional and translational analysis of heme oxygenase expression in response to sulfur mustard

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Abstract

Sulfur mustard (SM) is a potent alkylating agent which reacts with nucleophilic groups on DNA, RNA and proteins. It is capable of inducing cellular toxicity and oxidative stress via production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The accumulation of high amounts of the reactive species causes harmful effects such as DNA damage, lipid peroxidation, protein oxidation, inflammation and apoptosis. Although SM (also known as mustard gas) and its derivatives are rapidly removed from the body, long-term damages are much more serious than the short-term effects and may be correlated with the subsequent changes occurred on the genome. In order to defend against oxidative properties of this toxic molecule, cells trigger several anti-oxidant pathways through up-regulating the corresponding genes. Enzymes like heme oxygenase-1, superoxide dismutase and glutathione-*S*-transferase are the examples of such genes. These enzymes produce anti-oxidant substances that are able to scavenge the reactive species, alleviate their noxious effects and protect the cells. Following SM gas exposure, gene transcription (mRNA levels) of these enzymes are ramped up to help detoxify the cells. Yet, some studies have reported that the up-regulated transcription does not necessarily translate into higher protein expression levels. The exact reason why this phenomenon happens is not clear. Creation of mutations in the genome sequence may lead to protein structure changes. Phosphorylation or other post-translational alterations of proteins upon SM exposure are also considered as possible causes. In addition, alterations in some microRNAs responsible for regulating post-translation events may inhibit the expression of the anti-oxidant proteins in the poisoned cells at translational level.

Keywords

Epigenetic, heme oxygenase, peroxynitrite, post-translational, sulfur mustard

History

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Introduction

Sulfur mustard (SM) [bis-2-(chloroethyl) sulfide] is a hazardous vesicant agent which is known as a potent chemical weapon (1). This agent was first used by military forces during World War I. Recent extensive use of SM by Iraqi military forces during Iraq–Iran war in 1980s victimized over 100 000 civilian and military personnel in Iran (2).

The lipophilic nature of this compound allows its quick penetration into skin and cellular membranes (3). Cytotoxic mechanisms are the main disturbance which cause pathological signs in the exposed tissues. This agent can be converted to cyclic sulfonium intermediates such as sulfoxide and sulfone which subsequently interact with different functional groups, including hydroxyl, carboxylic, thiol and amino groups of various bimolecules. Additionally, atoms of the purine and pyrimidine bases, notably N_1 of adenine and N_7 of guanine are frequently alkylated targets of SM exposure. Crucial alterations in the key macromolecules such as DNA, RNA, proteins (reaction with cysteine, histidine, glutamic acid and aspartic acid), phospholipids and carbohydrates are considered toxic. Accumulation of several different toxic products give rise to clinical complications (4). Enzymes like heme oxygenase-1, superoxide dismutase and glutathione-*S*-transferase are examples of such proteins. These proteins produce anti-oxidant substances that are able to scavenge the reactive species, alleviate their hazardous effects and protect cells. Following SM gas exposure, gene transcription of these enzymes increases and their mRNA levels rise in the exposed cells. In contrast to an elevation of mRNA levels in the cells, some studies reported that protein expression did not occur subsequent to the increased mRNA levels. The exact reason of this phenomena is not understood. In this review, we discussed the probable reasons involved in the absence of protein expression following elevated mRNA levels focusing on heme oxygenase-1.

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SM clinical manifestations

The exposed victims suffer from two categories of complications. The first one includes acute symptoms observed immediately after exposure. The second category is known as late complication which occurs later and is more serious (5,6). Dose, duration, and routes of the encounter with SM are the main factors determining the kind and severity of side effects. Direct contact, inhalation and ingestion are three significant routes by which skin, eyes, respiratory tract and gastrointestinal tract are injured, respectively. Entry of SM via gastrointestinal tract leads to systematic toxicity that influences renal, bone marrow and blood circulation. High-dose ingestion of SM causes suppression of bone marrow and the immune system, leading to anemia, leucopenia, fever and cachexia. The presence of high amounts of these species causes harmful effects such as DNA damage, lipid peroxidation, protein oxidation, inflammation and apoptosis. In order to defend against oxidative properties of this chemical component, cells trigger several anti-oxidant pathways through increasing the corresponding gene expression.

Effects of SM on upper airway via inhalation are more far more destructive than the effects on lower airway. Disruption of laryngeal and tracheobronchial mucosa, inflammation, necrosis and edema in respiratory tract are common primary pathological features upon SM inhalation (6). Results from several studies showed that late signs can emerge 15–20 years after SM exposure. It is worth bearing in mind that the respiratory tract symptoms are the most common complications (7). Yazdani et al. suggested that the elevated levels of nuclear factor κB1/RelA in the airway wall consequent to SM exposure might cause cell survival (8).

Bronchiolitis obliterans is prevalent in these patients (9). The increased expression of Smad3 and Smad4 resulted from TGF-β signaling and has been reported in SM-exposed patients. This can cause peribronchial fibrosis followed by airway remodeling (10). Major phatophysiological manifestation following SM exposure are summarized in Table 1.

Molecular mechanism of SM

Numerous mechanisms are involved in the SM biological effects in targeted tissues (Table 2). Review of the literature suggests that different mechanisms can be explained in acute and delayed complications. Two theories were stated as a molecular principle of delayed effects of SM, particularly in the pulmonary disorders. The most significant impact is DNA alkylation and cross-linking, which is observed in a low-dose exposure (11). The release of a Cl ion presented in a structure of this compound produce reactive sulfonium ions, which quickly interacts with nucleophil groups on DNA. Because of the presence of two Cl, each agent can simultaneously react with two groups. Inter- and intra-strand reactions are possible (4). This event leads to DNA strand breakage and subsequently activates poly (ADP-ribose) polymerase type 1 (PARP-1). This enzyme catalyzes ADP-ribose units transferring from NAD^+ to various proteins located in the nucleus (12). Depletion of cellular NAD reservoir is found after the extensive injury. Furthermore, activation of this enzyme can stimulate up-regulation of ATM and ATR gene that contribute in DNA repair pathways (13). Based on the repair systems, cells select one of the following fates: cell cycle arrest, terminal differentiation or death via necrosis or apoptosis. The second hazardous mechanism responsible for the delayed effects is oxidative stress. Excessive production of reactive species and disruption of oxidants/anti-oxidants balance in the cell are two significant factors to create oxidative toxicity in the cells. SM triggers both of these effects. In the exposed cells, enhanced production of oxidant species, particularly ROS and a lack or decrease of their detoxificant substantial amounts have been recognized as a main reason (14). To date, various types of ROS are known; the most popular of them is superoxide ($O_2^{\bullet}$), hydroxyl (OH^-) and hydrogen peroxide (H_2O_2). All of them are generated from a partial reduction of oxygen and are strong toxicants (15). In addition, they are highly reactive and chemically they interact with biomolecules including proteins, lipids and nucleic acids (14). It should be noted that infiltration and accumulation of immune cells involved in inflammation, in particular macrophages and neutrophils, takes place in injured tissues. These two types of cells primarily contribute to host defense processes. Production and release of ROS during respiratory burst has been proven as a significant defensive mechanism against chemical-stimulated toxicity, especially SM that was frequently demonstrated in the respiratory system (16–18).

Mitochondria are another potent source of ROS generation through an electron transport pathway. Mitochondria can regulate apoptosis and autophagy by this function. SM directly influences on mitochondrial functions, alters their compounds and elevates ROS production (19). Furthermore, a number of cellular enzymes such as NADPH oxidase and xanthine oxidase also create ROS (14).

Table 1. Acute and late clinical features after sulfur mustard exposure.

Effected tissue	Acute complication	Late complication
Eye	Conjunctivitis, corneal and eyelid edema, photophobia, belpharospasm, irritation	Ulcerative keratitis, corneal ulcerative disease, late conjuctivitis
Skin	Itching, erythema, blister formation, hypo and hyper-pigmentation, face and neck redness	Poikiloderma, dermatitis, skin cancer
Respiratory system	Acute inflammation in air ways, inflammatory exudates, pseudomembrane formation in the tracheobronchial tree, respiratory edema, ulceration and necrosis,	Chronic bronchitis, chronic obstructive pulmonary disease, asthma and bronchiolitis obliterans, lung fibrosis, cough, dyspnea, excessive sputum production and hemoptosis
Gastrointestinal tract	Vomiting, nausea, constipation, lesser bloody diarrhea	–
Neuropsychiatric	headache, anxiety, fear of the future, restlessness, confusion, and lethargy	Anxiety, depression, personality disorders, convulsions, psychosis

Table 2. Molecular mechanism contributed in SM cellular toxicity.

Effects	Affected pathways
DNA damage	–DNA bases alkylation, PARP activation, cellular NAD and ATP depletion → cell death –Up-regulation of the ATM and ATR genes –Activation of the cytochrome P450 isoforms, xenobiotic response elements, growth arrest proteins and c-*fos* –↑Synthesis and release of plasminogen activator
Oxidative stress	↑ROS, NOS and $ONOO^-$, lipid peroxidation and membrane damage Reduced Glutathione depletion, ↓superoxide dismutase, catalase, glutathione peroxidase, and glutathione-*S*-transferase, heme oxygenase-1activity Induce metalloproteinase and collagenase, destroy extracellular structure
Cell death	Stimulate FAS/FASL system ↑ Sphingomyelinases activity by TNF-α and ↑Ceramide Trigger caspase-8 and caspase-9-mediated pathway ↑Ca+ level by phospholipase C activation
Inflammation	↑Expression and activation of IL-1α, IL-1β, IL-6, IL-8, and TNF-α ↑Phagocytic activity of macrophage and neutrophil located in injured tissues ↑p38 as a member of MAP kinase family
Cytoskeleton disturbance	Alkylated and cross-linked keratin filaments, perturbing the intermediate filament network and actin filament → cell morphology
Cell membrane damage	Inactivates Akt pathway, calpain activation by Ca2+ influx, cell lysis

On the other hand, an alkylating property of SM decreases the formation of glutathione in the cell through direct interaction with its tail. Reduced glutathione is a functional anti-oxidant that scavenges reactive oxygen species (ROS) and introduces a potent anti-oxidant located in lung with the ability to neutralize endogenous and exogenous toxic components. Depletion of this substance causes the rise of ROS in the cells. SM can induce secretion of inflammatory mediators from various cell types such as epithelial cells, endothelial cells, macrophage, neutrophils, etc. This event accompanies escalated reactive oxygen and nitrogen species (14). Another effect of SM is the elevation of NO by stimulation of inducible type of nitric oxide synthesis. As described above, active macrophage and neutrophil cells as well as exposed epithelial cells are capable of expressing this enzyme. Nitrite, nitrogen dioxide, nitrosonium cations nitronium, nitrosoperoxycarbonate anion, nitroxyland nitryl chloride are examples of reactive NOS that lead structural modifications in vital macromolecules and emerge cellular toxicity (14). High intracellular amounts of ROS and NOS enhance peroxynitrite ($ONOO^-$) production. This compound is a potent nitrosating agent which reacts with the key biomolecules mentioned above (20). Following deleterious effects of peroxynitrite, PARP and repairing enzymes are activated and consequently stimulate apoptosis or necrosis pathways (15).

Defensive anti-oxidant mechanisms against SM

As mentioned above, two kinds of complications emerge after SM, early and delayed manifestations. Because of higher frequency of long-term complications in respiratory system, it is possible that exposed cells in this area are able to survive enduring alterations caused by delayed manifestations. Defense against oxidative stress is one of the mechanisms that is triggered in exposed cells especially in lung and airways. Complex defense mechanisms are deployed to combat oxidative stress, and to neutralize ROS and RNS. Different series of enzymatic and non-enzymatic system are activated to protect cell injuries (21). Non-enzymatic ones are substances with low-molecular weight that can scavenge free radicals located in intra- and extra-cellular environment. These agents with different concentration and efficiency are located in various anatomic and subcellular regions. Glutathione, ascorbic acid, tocopherol, urate, bilirubin, and lipoic acid are examples of such agents. Proteins with thiol groups such as albumin and transferrin can neutralize free radicals (22).

Enzymatic anti-oxidants are other agents that contribute to remove oxidant compounds and limit their consequent damage. The most efficient enzyme interfering with oxidative damage is superoxide dismutase (SOD). These enzymes form a family containing three isoforms, cytosolic (Cu-Zn), mitochondrial (Mn) and extracellular (Cu-Zn) enzymes. They are able to convert potent superoxide radicals to H_2O_2 and O_2. The extracellular isoform is expressed in the epithelial of the respiratory system in high amounts and plays a significant role in the airway disorders. In addition, elevation of SOD expression was reported after SM exposure. Since NOS competes with SOD for binding to ROS, SOD can intercept oxidative effects of superoxide radicals only before this attachment. Also, alkylating influences proteins subsequent to SM exposure. This causes attenuation of enzyme activity and inhibition of SOD. Furthermore, the mutagenic characteristic of SM causes mutations in the SOD gene, which consequently causes decreased gene expression or enzyme activity (23).

Although H_2O_2 produced by SOD is less harmful than superoxide radicals, it can still produce oxidative stress. Catalase and glutathione-*S*-transferase are the primary cellular barriers to diminish H_2O_2. Glutathione-*S*-transferase involves in charging GSH redox system and reduces GSSG (15).

Another efficient enzyme in anti-oxidant defense of the respiratory system is heme oxygenase (HO). Heme oxygenases are classified in three groups: HO-1, HO-2 and HO-3. They belong to microsomal enzyme. HO-1 is inducible which is expressed upon high-level response to oxidative stress. This enzyme is capable to eliminate heme groups from the cells and convert them to biliverdin, iron and CO. Heme removal

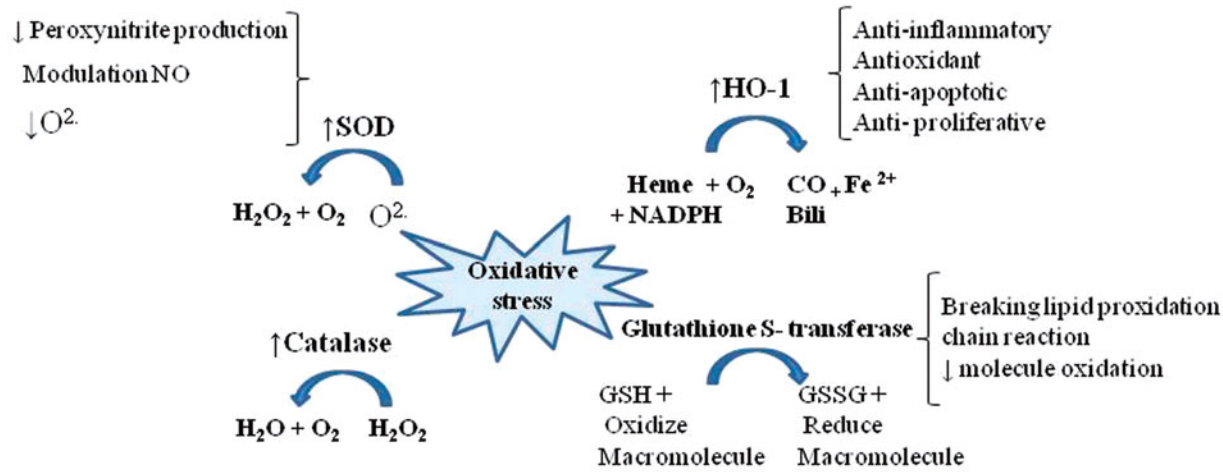

Figure 1. Main cellular enzymatic defense mechanisms against oxidative stress in lung. SOD, superoxide dismutase; HO-1, heme oxygenase 1; Bili, bilirubin.

causes attenuation of inflammatory reactions and oxidative stress (24). Figure 1 summarized the main enzymatic mechanisms for defending against oxidative stress in lungs.

Heme oxygenase-1

Heme oxygenase is a microsomal enzyme that is involved in the primary line of intracellular defense against oxidative stress. Heme oxygenase-1 and -2 are two known isoforms which are encoded with two different genes. Heme oxygenase-1, a 32-kDa protein, expresses as an inducible form following various stimuli other than heme molecules such as glutathione depletors, UV irradiation, endotoxin, heat shock, nitric oxide, heavy metals, cytokines produced inflammation and ROS. Heme oxygenase-2 is a constitutive isoform with a 63-kDa molecular weight. Both isoforms can degrade heme molecules as a powerful intracellular oxidant and produce iron, biliverdin and CO (25). Heme oxygenase exerts cytoprotective functions by end products resulted from heme cleavage. In the next step, biliverdin is converted to bilirubin by biliverdin reductase (26). Bilirubin plays as a strong endogenous anti-oxidant and is able to remove ROS from cells and eliminates its products (27). Released iron contributes to ferritin synthesis and provides cellular iron homeostasis. In addition, CO stimulates guanylyl cyclase which leads to attenuated leukocyte adhesion, suppressed muscle cell proliferation and blocked platelet aggregation. Furthermore, these products are capable of alleviating inflammatory reactions (28).

It is reported that heme oxygenase-1 up-regulation occurs in numerous respiratory system disorders, including asthma, chronic obstructive pulmonary disease (COPD), bronchiolitis obliterans and acute respiratory distress syndrome (ARDS). Recent evidence confirms that heme oxygenase-1 plays a significant role in pathogenesis of COPD. Elevated mRNA level of heme oxygenase-1 reported in patients with asthma compared to normal controls. This event may avoid disease progression and tissue remodeling. On the other hand, reduced expression of this enzyme accompanies emphysema (24).

As described before, glutathione depletion and ROS generation are cytotoxic complications after SM exposure. In this case, to protect against cellular oxidative damage, heme oxygenase-1 is over expressed. Despite the over expression of heme oxygenase-1 at mRNA level, no alteration is observed at protein level (25). Loss of heme oxygenase-1 protein in bronchial epithelial cells of exposed individuals causes lack of functional protein therefore increase at mRNA level is inefficient to protect cells from oxidative injuries. The discrepancy between mRNA and protein levels also reported in other studies performed on expression patterns of neutrophil gelatinase-associated lipocalin (29) and metallothionein-/A (30) in exposed individuals. This event may cause enhance chronic inflammation and tissue obstacle, airway loss and fibrosis. The main reason for the inability of this type of cells to synthesize functional protein is still unknown, but some probabilities are suggested such as changes in microRNAs expression, translation regulation, epigenetic events and nitrosation of protein consequent to peroxynitrite as a secondary complication of SM. In the following sections, we attempt to discuss the above mechanism in detail.

Disturbance between mRNA and protein expression

Since SM and its derivatives are excreted shortly after exposure (31), long-term complications must be generated after stable alterations in the cellular genome. Mutations created in the genome and epigenetic alterations are these types of events. In the cells, SM is converted to cyclic intermediates which have a potency to react with the electron-rich structures. In addition to generation of DNA adducts, these intermediates can form cross-linking in intra- and inter-strands of DNA molecules. N7 of guanine moieties is a most frequent site where SM attacks them (32). N7-[2-[(2-hydroxyethyl)thio]ethyl]-guanine, bis[2-(guanin-7-yl)ethyl] sulfide, *N3*-[2-[(2-hydroxyethyl)thio]ethyl]-adenine, and *O6*-[2-[(2-hydroxyethyl)thio]ethyl]-guanine and its 2′-deoxyguanosine derivative are common adducts (33). Depurination process causes the release of modified bases and breaks DNA strands. Furthermore, the frequency of chromosome aberrations (34) and sister chromatid exchange (35) increases after SM poisoning. Previous studies demonstrated that SM and its derivatives are able to stimulate mutations in the genome.

This finding has been obtained from studies done in Drosophila (36), *Neurospora crassa* (37) and mouse lymphocyte cells (38). Also, mutations in p53, particularly G→A transition, and hypoxanthine phosphoribosyl transferase has been reported in the exposed individuals (39). Moreover, it was demonstrated that r-RNA coding regions were more susceptible to mutations induced by SM (40,41). On the other hand, unknown mutations occurred in presence of SM may originate to truncate conformation of proteins (25).

Truncated transcription consequent to alkylating promoter regions of gene plus the inability of RNA polymerase to bind to promoter are another destructive effects of SM (42).

Epigenetic events are another possible cause of long-term effects. This pheromone includes inheritable changes in gene expression emerging without any alterations in the gene sequence. Two major mechanisms, DNA methylation and histone modification, are involved in epigenetic changes that are exerted by three enzymes named histone acetyl transferase (HAT), histone deacetylase (HDACs) and DNA methyltransferase (DNMTs). Kunak et al. suggested that it is possible to activate HDAC and to inhibit DNMT after SM poisoning. This prevents transcription of useful genes of anti-oxidant enzymes and anti-inflammatory proteins (43).

Inefficiency of HO-1 mRNA translation may correspond to the difference between mRNA and protein levels. Moreover, post-transcriptional regulation may also determine the amount of protein expression (44). MicroRNAs are novel regulators and their expression is altered due to SM. MicroRNAs are endogenous conserved RNAs with ~23 nucleotides that bind to complementary sites on the target mRNA transcript commonly causing translational repression and gene silencing. So far there are no studies on the role and the pattern of microRNAs expression following SM exposure, therefore further assessments are suggested to clarify this subject (45).

Another probability that may explain the difference between the level of mRNA and protein expression is the stimulation of oxidative protein decomposition via peroxynitrite compounds. They are more toxic than NO and superoxide. Numerous biological molecules are their target substrates (15,20). They can bind to unsaturated lipid acids and low-molecular weight anti-oxidants such as glutathione, α- tocopherol and ascorbic acid (46,47). Nitrosation of tyrosine, tryptophan, cystein and methionine, the elevation of protein carbonyl levels and protein fragmentations are the alterations described following SM exposure. All of them may lead to a change in hydrophobicity properties of protein surface, which results in disruption of secondary and tertiary structures (47–49). These injuries act as a signal to trigger the proteolytic removal of the damaged protein via the proteasome. To date, proteasome is known as a cellular proteolytic system contributed in the elimination of oxidized proteins. It is a barrel-like particle with a catalytic core (50). Grune et al. reported that peroxynitrite-treated proteins had intensive susceptibility to degradation by the proteasome (51). In the physiological conditions, this function plays a significant role in the turnover of oxidatively damaged proteins. It should be noted that the environment of respiratory system, particularly in the lung of exposed patents contains various oxidative substances. The sources of these substances were explained in the section on ''SM Clinical Manifestations.'' Therefore, lack of anti-oxidant proteins due to degradation induced by these substances may be a probable outcome.

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Declaration of interest

The authors declare that they have no conflict of interest.

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