Original research

Protection of HepG2 cells against acrolein toxicity by 2-cyano-3,12-dioxooleana-1,9-dien-28-imidazolide via glutathione-mediated mechanism

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Abstract

Acrolein is an environmental toxicant, mainly found in smoke released from incomplete combustion of organic matter. Several studies showed that exposure to acrolein can lead to liver damage. The mechanisms involved in acrolein-induced hepatocellular toxicity, however, are not completely understood. This study examined the cytotoxic mechanisms of acrolein on HepG2 cells. Acrolein at pathophysiological concentrations was shown to cause apoptotic cell death and an increase in levels of protein carbonyl and thiobarbituric acid reactive acid substances. Acrolein also rapidly depleted intracellular glutathione (GSH), GSH-linked glutathione-*S*-transferases, and aldose reductase, three critical cellular defenses that detoxify reactive aldehydes. Results further showed that depletion of cellular GSH by acrolein preceded the loss of cell viability. To further determine the role of cellular GSH in acrolein-mediated cytotoxicity, buthionine sulfoximine (BSO) was used to inhibit cellular GSH biosynthesis. It was observed that depletion of cellular GSH by BSO led to a marked potentiation of acrolein-mediated cytotoxicity in HepG2 cells. To further assess the contribution of these events to acrolein-induced cytotoxicity, triterpenoid compound 2-cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im) was used for induction of GSH. Induction of GSH by CDDO-Im afforded cytoprotection against acrolein toxicity in HepG2 cells. Furthermore, BSO significantly inhibited CDDO-Im-mediated induction in cellular GSH levels and also reversed cytoprotective effects of CDDO-Im in HepG2 cells. These results suggest that GSH is a predominant mechanism underlying acrolein-induced cytotoxicity as well as CDDO-Im-mediated cytoprotection. This study may provide understanding on the molecular action of acrolein which may be important to develop novel strategies for the prevention of acrolein-mediated toxicity.

Keywords: HepG2 cells, acrolein, 2-cyano-3, 12-dioxooleana-1, 9-dien-28-imidazolide, glutathione-*S*-transferases, aldose reductase, cytoprotection

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Introduction

Acrolein is present in varying concentrations in our natural and artificial environments. Chemically, acrolein is a highly reactive α,β-unsaturated aldehyde, with very strong electrophilic characteristics.[1] It is a byproduct of a chemical process forming acrylate polymers and DL-methionine and is industrially used as herbicides and slimicides.[2] Acrolein is found in foods such as fruits, wine, cheese, and beer at very low concentrations.[3] Environmentally, acrolein naturally exists in food and is released by incomplete combustion of organic matter. Thus, acrolein is found in smoke released from cigarette smoking, the exhaust pipes of internal combustion engines, and in vapors of overheated cooking oil.

Humans are mainly exposed to acrolein via cigarette smoke.[4] A single cigarette can contain 25–50 μg of acrolein and constitutes as one of the many hazardous materials that is a health risk in cigarettes.[1,5] Smokers are exposed to about 40 times more acrolein than concentrations found environmentally. Normal levels of acrolein in the environment are usually at 0.04 to 0.08 ppm but can be present upto 90 ppm in cigarette smoke.[6] Elevated concentrations of acrolein, of

up to 180 μmol/L, have been found in various human tissues.[7–9] The normal concentration of acrolein–protein adducts in the normal human plasma is about 30–50 μmol/L,[7] whereas the plasma concentration of acrolein in patients with chronic renal failure has been reported as high as 180 μmol/L.[9,10]

Various studies have shown that acrolein can also cause injury of the respiratory tract and suppression of the pulmonary host defense. It alters gene regulation, mucociliary transport, and alveolar barrier integrity, which ultimately causes acute lung injury, chronic obstructive pulmonary disease (COPD), and possibly asthma and lung cancer.[11,12] Acrolein is reported to induce oxidative stress thereby causing mitochondrial dysfunction in brain cells.[13] Previous studies showed that acrolein can form acrolein adducts with cellular components, particularly proteins and DNA, which have been detected in plasma of patients with renal failure,[14–17] Alzheimer's disease,[18,19] diabetes,[14,20] and atherosclerosis.[16,21–23] The liver is the principal organ for xenobiotic transformation. Most exogenous chemicals are metabolized and eventually secreted into the liver. Hence, liver cells, or hepatocytes, are highly exposed to significant concentrations of such chemicals, which can result in liver dysfunction, cellular injury and, in extreme cases, organ failure.[24] Acrolein can cause hepatotoxicity *in vitro* and *in vivo*.[25–28] However, the mechanisms involved in acrolein-induced hepatocellular toxicity are not completely understood.

Glutathione (GSH), glutathione-*S*-transferase (GST), and aldose reductase (AR) have been suggested to be involved in the detoxification of 4-hydroxynonenal derived from oxidative processes.[29,30] GSH is the major endogenous antioxidant and has been found in high amounts in stressed cells. GSH is comprised of three amino acids – glutamine, cysteine, and glycine. It forms γ-L-glutamyl-cysteinyl-glycine via two step de novo synthesis. It is naturally present at 0.5–10 mmol/L concentration in cells. In response to oxidative stress, GSH is converted to its oxidative state, glutathione disulfide (GSSG), which regenerates reduced GSH via glutathione reductase and NADPH. About 90% of GSH is present in its reduced form in the cell. While it is found in its disulfide state in its extracellular environment; minute changes in GSH intracellular state can cause very significant effects on redox dependent cell signaling. GSH has also been shown to play a very important role in protecting the cell against cellular damage induced by maintaining reductive environmental toxicants, electrophiles, and variety of other stress inducers.[31,32] Apart from cytosolic environment, GSH also plays a vital role in detoxifying electrophiles via GST conjugation.[33] GSH has been shown to react with organic hydroperoxides to form a less reactive conjugate that can be facilitated by GST.[34] GST was further proposed to be an important defense mechanism against pathogenesis of multiple disease processes.[35] Another line of cellular defense against 4-hydroxynonenal has been thought to be AR. It has been reported that AR has a GSH-binding site serving as a substrate for AR.[36]

Although the above studies suggest that GSH, GST, and AR may play an important role in efficient detoxification of 4-hydroxynonenal, studies on the roles of these cellular factors in acrolein mediated toxicity in hepatocytes have not been reported in the literature. In addition, whether upregulation of GSH, GST, and AR by CDDO-Im, a triterpenoid compound, can ameliorate the toxic effects of acrolein in hepatocytes are still lacking. Our study demonstrates for the first time that acrolein exposure causes rapidly depleted GSH, GST and AR in human HepG2 cells. HepG2 line is a widely used model for studying hepatotoxicity and cytoprotection because they possess different phase I and phase II xenobiotic-metabolizing enzymes.[37] Results further showed that depletion of cellular GSH by acrolein preceded the loss of cell viability suggesting that cellular GSH depletion may be an important event in acrolein-induced cytotoxicity. We further present evidences of upregulation of cellular GSH by CDDO-Im, which in turn afforded protection against acrolein-induced cytotoxicity in HepG2 cells via GSH-mediated mechanism.

Materials and methods

Chemicals and materials

Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin, fetal bovine serum (FBS), and trypsin were purchased from Gibco, Invitrogen. 2-Cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im) was purchased from Toronto Research Chemicals, Inc. GSH, oxidized glutathione (GSSG), yeast-derived glutathione reductase, 1-chloro-2,4-dinitrobenzene (CDNB), *o*-phthalaldehyde (OPT), NADPH, 3 -(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and bovine serum albumin (BSA) were purchased from Calbiochem. Acrolein and hydrogen peroxide were purchased from Sigma. Tissue culture flasks and 24- and 48-well plates were purchased from Corning.

Tissue culture

HepG2 cells were purchased from ATCC. Tissue culture flasks were kept at 37°C in a humidified atmosphere of 5% CO_2. Cells were fed with DMEM media with 10% FBS, 100 μg/mL of penicillin and 100 μg/mL of streptomycin every other day. Cells were sub-cultured once they reached 80% confluence.

Cell extraction preparation

After treatment, cells were collected and resuspended in ice-cold 50 mmol/L KH_2PO_4/K_2HPO_4 buffer containing 2 mmol/L EDTA at pH 7.4. Sonicated cells were centrifuged at 13,000 rpm at 4°C for 10 min. Supernatants were collected from centrifuged tubes and kept on ice for measurement of GSH and phase II enzymes.

Assay for GSH content

Total cellular GSH content was measured using OPT-based fluorometric method that is specific for determination of GSH at pH 8.0.[38] The cell extract (10 μL) was incubated with 12.5 μL of 25% metaphosphoric acid and 37 μL of 0.1 mol/L sodium phosphate buffer containing 5 mmol/L EDTA at pH 8.0 at 4°C for 10 min. The samples were

centrifuged at 13,000 rpm at 4°C for 5 min. The resulting supernatant was incubated with 100 μL of OPT solution and 1.89 mL of 0.1 mol/L sodium phosphate buffer containing 5 mmol/L EDTA for 15 min at room temperature. Fluorescence intensity was then measured at excitation 350 nm and emission 420 nm. Cellular GSH content was calculated using GSH standard curve.

Assay for GST activity

The GST activity was measured using CDNB as a substrate in a freshly prepared final reaction mixture of 0.6 mL.[38] The reaction mix containing GSH, 50 mmol/L CDNB, and 3 mg/mL of bovine serum albumin (BSA) in 0.1 mol/L phosphate buffer at pH 6.5 was added to each cuvette. The reaction was started by adding 10 μL of the sample and the rate of formation of CDNB–GSH conjugate was measured on spectrophotometer at 340 nm for 5 min at 25°C. The cellular GST activity was calculated using an extinction coefficient of 9.6 $mmol/L^{-1}cm^{-1}$ and was expressed as nmol of CDNB–GSH conjugate formed per min per mg of cellular proteins.

AR assay

AR was measured at room temperature by following the oxidation of NADPH on spectrophotometer at 340 nm.[39] Reaction mixture for AR activity was made from 50 mmol/L potassium phosphate with 0.4 mol/L lithium sulfate, and 10 mmol/L of D-glyceraldehyde. To each cuvette 0.945 mL of assay mixture was added followed by 40 μL of the sample. The reaction was started by adding 15 μL of 10 mmol/L NADPH. AR activity was measured at 340 nm at 25°C for 5 min.

Cell injury

Cell injury was measured using MTT assay, HepG2 cells were plated into 48-well plates. On day one, they were treated with cytoprotectant CDDO-Im for 24 h. On the second day, cells were treated with acrolein for 24 h. On day three, all the media were removed and cells were fed with media containing 0.5% FBS and MTT (0.2 mg/mL) following 2-h incubation at 37°C. After 2 h of incubation, all the media were removed and cells were treated with 0.2 mL mixture of dimethyl sulfoxide, isopropanol, and deionized water (1:4:5) to solubilize formazan crystals. The amount of dissolved formazan was measured at 570 nm.[40]

Assay for thiobarbituric acid reactive substances (TBARS)

For the TBARS assay, working standards (25, 50,100, 150 and 200 μL) were added to microcentrifuge tubes and volumes were made up to 200 μL with distilled water. A blank standard contained 200 μL of distilled water. Then, 50 μL of 8.1% SDS, 375 μL of 20% acetic acid, 375 μL of 0.8% TBA, and 150 μL of distilled water were added to all the tubes. All the tubes were incubated at 95°C for 60 min followed by a cooling procedure using tap water. The upper organic level was taken for photometric evaluation at 532 nm.[41,42]

Protein carbonyl (PC) assay

The standards contained 300 μL of DNPH and 300 μL of 2 mol/L HCl added to microcentrifuge tubes as the control. Then 75 μL of each sample was added to the control and standard tubes followed by 1 h incubation in the dark. After that, 375 μL of 20% TCA was added to these tubes followed by 5 min of incubation on ice. Tubes were further centrifuged at 10,000 rpm for 10 min at 4°C. Supernatants were discarded and the above mentioned step was repeated two times. After centrifugation, the supernatant was discarded and pellet was resuspended in 500 μL of ethanol followed by centrifugation at 10,000 rpm for 10 min at 4°C. This step was repeated one more time before the final wash. After the final wash, the protein pellet was resuspended in 500 μL of 6 mol/L guanidine hydrochloride. Tubes were centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was transferred from the sample and control tubes to 96-well plates and absorbance was measured at 370 nm using plate reader.[43]

Lactate dehydrogenase (LDH) release assay

HepG2 cells were grown up to 60% confluence in DMEM medium with 10% FBS in 24-well culture plate. Cells were exposed to treatment in 200 μL non-phenol red DMEM with 0.5% FBS. The medium was then collected into microcentrifuge tubes and was centrifuged for 5 min at 13,000 rcf at 4°C. Supernatant was then collected in another tube and was used for measuring LDH in spectrophotometer at 320 nm.[44]

Flow cytometry for cell death

HepG2 cells, grown upto 85% in 55 cm^2 plates, were treated or untreated with different concentrations of acrolein and 100 nmol/L of CDDO-Im. Afterwards, cells were washed with PBS, trypsinized, and diluted to a concentration just under 5×10^5 cells/mL. A 25 μL sample of cells was added to 475 μL of Guava Viacount Reagent and was incubated in dark for 5 min. Cell viability, apoptosis, and cell death were determined with Guava Easycyte Miniflow Cytometer (Millipore). The Guava Viacount Reagent distinguishes between viable and non-viable cells based on differential permeability of DNA binding dyes in Viacount Reagent. The fluorescence of each dye is resolved operationally to allow quantitative assessment of viable and non-viable cells present in suspension.

Real-time PCR analysis

The synthesis of GSH from its constituent amino acids involves the action of two ATP-dependent enzymes, γ-glutamyl cysteine ligase (GCL) and GSH synthase. GCL, the rate limiting enzyme in the overall pathway, is a heterodimer composed of catalytic (GCLC) and modulatory (GCLM) subunit. GCLC retains all the catalytic activity and GCLM improves the catalytic efficiency. To study the effects of CDDO-Im on the cellular levels of m-RNA for GSH, HepG2 cells were incubated with 100 nmol/L of CDDO-Im for 1, 3, 6, and 24 h. Total RNA from HepG2 cells were isolated using Trizol reagent. One microgram of

RNA from each sample was reverse transcribed to cDNA. The reaction mixture in each well contained 5 μL of distill autoclaved H_2O, 10 μL of cyber green, and 2 μL of forward and reverse primer. The primers used in quantitative real-time PCR were GCLC (forward, 5′-ACCATCATCA ATGGGAAGGA-3′; reverse, 5′-GCGATAAACTCCCTC ATCCA-3′) and GCLM (forward, 5′-CTCCCTCTCGGGT CTCTCTC-3′; reverse, 5′-ATCATGAAGCTCCTCGCTGT-3′). The mean qualities of GCLC and GCLM were normalized based on the mean of control gene GAPDH.[45]

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using GraphPad Prism software (La Jolla, CA). All data are expressed as mean ± SEM from at-least three different experiments. Significant treatment differences were subjected to Tukey's multiple comparison tests. A value of $P < 0.05$ was considered different.

Results

Acrolein-induced cytotoxicity involves apoptosis, protein damage, and lipid peroxidation in HepG2 cells

The cellular toxicity of different concentrations of acrolein on HepG2 cells was assessed by MTT and LDH release. HepG2 cells were treated with 40, 80, and 120 μmol/L of acrolein for 24 h in order to obtain a dose-dependent response for acrolein exposure. Figure 1a shows the relationship between different concentrations of acrolein and the cell survival rate. Figure 1b shows LDH release with different concentrations of acrolein. Incubation of cells with various concentrations of acrolein for 24 h caused a significant decrease in cell viability (Figure 1a) and increase in release of LDH (Figure 1b). To examine the mechanism of cell death caused by acrolein exposure in HepG2 cells, the apoptotic and necrotic cell death were investigated by flow cytometric analysis (Figure 1c). As shown in Figure 1c, cells exposed to acrolein had increased shift of cells towards apoptosis and late apoptosis/necrosis compared to control (Figure 1c). To examine the cellular damage caused by acrolein, the amount of protein and lipid damage caused by various concentrations of acrolein in HepG2 cells was studied. For measuring protein damage and lipid peroxidation, HepG2 cells were incubated with 80 and 120 μmol/L concentrations of acrolein for 24 h. Panels d and e of Figure 1 show the amount of TBARS, a marker of lipid peroxidation, and panel b shows PC levels, a marker of protein damage, caused by acrolein in HepG2 cells. As shown in Figure 1d–e, significant increases in TBARS and PC levels were observed in cells treated with acrolein in a concentration-dependent manner.

Depletion of intracellular GST and AR by acrolein

To investigate the effects of acrolein on intracellular levels of AR and GST in HepG2 cells, cells were treated with 120 μmol/L acrolein at various time points followed by measurement of the activities of cellular GST and AR. As shown in Figure 2a and b, a striking decrease in intracellular levels of GST and AR was observed after 2 h incubation with 120 μmol/L of acrolein in HepG2 cells.

GSH depletion by acrolein in HepG2 cells

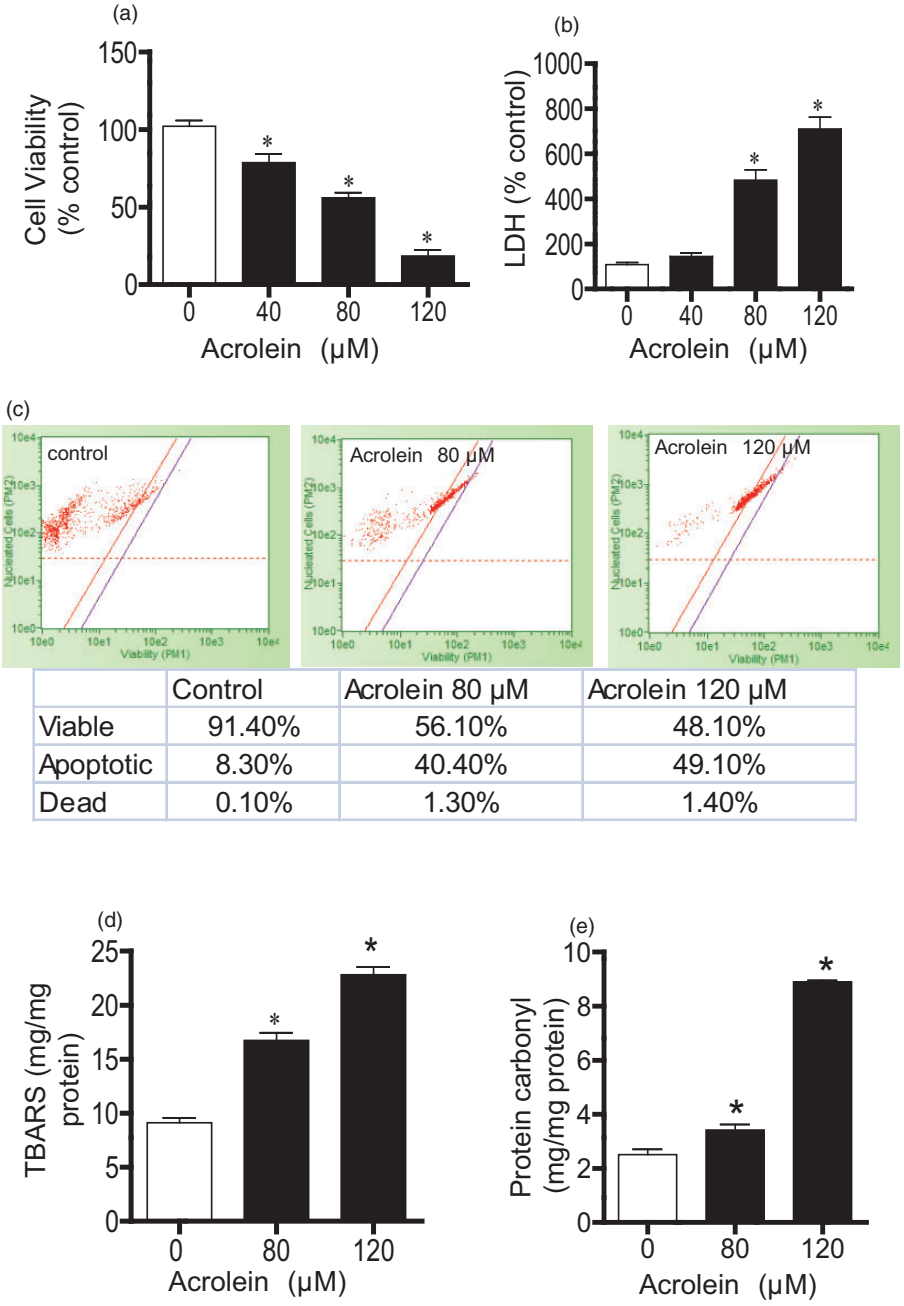
HepG2 cells were incubated with 80 and 120 μmol/L of acrolein for 24 h. As shown in panel d of Figure 2, incubation of cells with 80 and 120 μmol/L of acrolein resulted in a significant depletion of cellular GSH. Panel c of Figure 2 depicts the time-dependent decrease in GSH content in HepG2 cells treated with acrolein (120 μmol/L). Significant decrease in cellular GSH content can be observed after 2 h incubation with 120 μmol/L of acrolein which preceded the decrease of cell viability as indicated by release of LDH to the culture media (Figure 2d,e). Both decrease in GSH content and release of LDH were time dependent (Figure 2).

Effects of BSO pretreatment on acrolein-induced cytotoxicity

As shown in Figure 3b, incubation of HepG2 cells with 25, 50, and 100 μmol/L of buthionine sulfoximine (BSO) for 24 h causes significant depletion in cellular GHS levels without altering cell viability (Figure 3a). To determine if depletion of cellular GSH by BSO could potentiate acrolein-induced toxicity, HepG2 cells were pretreated with 100 μmol/L BSO for 24 h followed by treatment with various concentrations of acrolein for another 24 h. As shown in Figure 3c, the observed MTT assay results indicate that pretreatment with 100 μmol/L of BSO potentiated acrolein-induced toxicity in HepG2 cells.

Cytoprotective effects of CDDO-Im on acrolein-mediated cytotoxicity

Our data shows that exposure to acrolein results in decrease in cellular GSH content and depletion of intracellular GST and AR activities (Figure 2a,b). Triterpenoids are steroid-like compounds derived from plant extracts. CDDO-Im is a synthetic triterpenoid designed to be more potent to various types of oxidative stress.[46] However, there are no studies involving its protective capabilities against acrolein-mediated injury in HepG2 cells. To investigate the cytoprotective effects of CDDO-Im on acrolein-mediated cytotoxicity, HepG2 cells were tested in response to acrolein exposure for 24 h with or without a 24-h pretreatment of 100 nmol/L CDDO-Im. As shown in Figure 4a, control cells showed a decrease in cell viability when treated with acrolein at concentrations of 40, 80, and 120 μmol/L for 24 h. Conversely, the cells pretreated with 100 nmol/L CDDO-Im for 24 h showed a significant concentration-dependent increase in cell viability when treated with acrolein under the same conditions, as determined by the MTT assay. Flow cytometric analysis was further employed to examine the cytoprotective effects of CDDO-Im. As shown in Figure 4b, cells pretreated with CDDO-Im (100 nmol/L) reduced the number of cells moving towards apoptosis compared to cells only treated with acrolein. This further supports results that CDDO-Im provides cytoprotection to HepG2 cells against acrolein-mediated toxicity.



	Control	Acrolein 80 μM	Acrolein 120 μM
Viable	91.40%	56.10%	48.10%
Apoptotic	8.30%	40.40%	49.10%
Dead	0.10%	1.30%	1.40%

Figure 1 Acrolein-induced cytotoxicity, apoptosis, protein damage, and lipid peroxidation in HepG2 cells. Panel a shows acrolein-induced cytotoxicity in HepG2 cells measured by MTT assay. The cells were treated with different concentrations of acrolein for 24 h. Panel b depicts the increase in LDH levels in concentration-dependent manner after acrolein treatment for 24 h. Panel c represents FACS profile of viable and apoptotic cells treated with indicated concentrations of acrolein for 24 h. Viable cells are located on left side of each panel, apoptotic cells between the two lines, and dead cells on the right side. Percentage of viable, apoptotic, and dead cells were quantified by flow cytometer. Panel d shows increase in TBARS (μg/mg protein) levels in HepG2 cells treated with 80 and 120 μmol/L concentrations of acrolein for 24 h. Panel e depicts increase in protein carbonyl levels in HepG2 cells. Values are mean ± SD of three independent experiments. *Indicates statistical difference of $P < 0.05$ from the respective control group. (A color version of this figure is available in the online journal.)

Effects of CDDO-Im treatment on GSH content and GST, AR activity

Results show that acrolein significantly decreased intracellular levels of GSH, GST, and AR (Figure 2a,b). Thus, it was further determined whether intracellular GSH and the activities of GST and AR are altered by CDDO-Im. Results showed that incubation of HepG2 cells with 100 and 250 nmol/L of CDDO-Im for 24 h resulted in dramatic increase in the intracellular GSH content in a concentration-dependent manner without affecting the activities of GST and AR (Figure 5a–c). Notably, incubation of HepG2 cells with CDDO-Im at a concentration as low as 10 nmol/L leads to a 20–30% increase in cellular GSH content. However, the activities of GST and AR were not altered by these doses of CDDO-Im (Figure 5a,b). GSH is known to be present in mitochondria of mammalian cells, and the mitochondria are major targets of ROS generated by reactive aldehydes such as acrolein. For these reasons, GSH

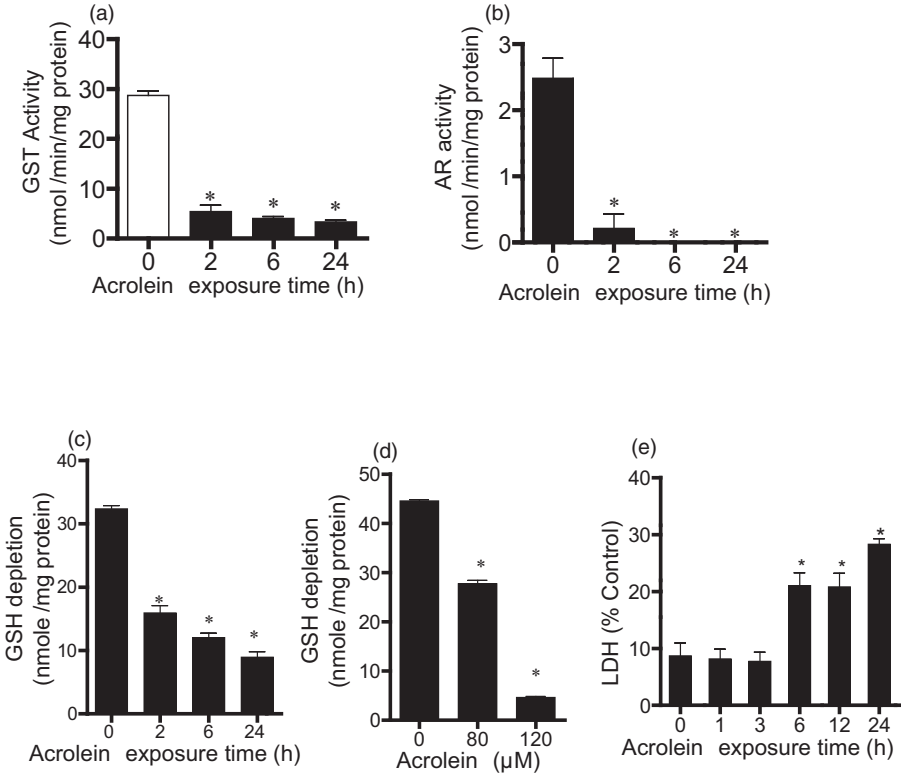


Figure 2 Depletion of intracellular glutathione-*S*-transferases (GST), aldose reductase (AR), and glutathione (GSH) by acrolein. Cells were incubated with acrolein for the indicated time points, followed by measurement of cellular levels of GST, AR, GSH, and LDH activity in the culture media. Values represent means ± SEM from three separate experiments. *Indicates statistical difference ($P < 0.05$) between control and acrolein treatment

levels from mitochondria isolated from HepG2 cells after CDDO-Im treatment for 24 h were examined. Figure 5d shows significant increase in GSH content of mitochondria on 100 nmol/L CDDO-Im treatment.

As intracellular GSH content was significantly induced by CDDO-Im, it was next determined if CDDO-Im treatment could also result in increased levels of mRNA for GCLC, which encodes for the peptide GCL, using a highly sensitive and specific real-time PCR assay. As shown in Figure 5e, the mRNA levels for GCLC were induced. A maximum 2.72-fold elevation of the GCLC mRNA level was reached after incubation of HepG2 cells with CDDO-Im (100 nmol/L) for 6 h.

Effects of BSO co-treatment on CDDO-Im-mediated cytoprotection against acrolein-induced cytotoxicity

Figure 6a shows a decrease in GSH levels on co-treatment of HepG2 cells with CDDO-Im and BSO, which demonstrates that BSO prevented induction in cellular GSH content by CDDO-Im. In Figure 6b, cell viability was measured using MTT assay, a comparative study in GSH induction in presence of acrolein. As shown in Figure 6b, incubation of cells treated with 120 μmol/L of acrolein for 24 h showed a significant decrease in cell viability compared to cells pretreated with 100 nmol/L of CDDO-Im for 24 h followed by acrolein treatment (120 μmol/L) for 24 h. Incubation of HepG2 cells with 100 μmol/L BSO followed by 80 μmol/L acrolein exposure resulted in a significant decrease in cell viability. Similarly, cells pretreated with CDDO-Im (100 nmol/L) and BSO (100 μmol/L) followed by 80 μmol/L acrolein treatment showed a significant decrease in cell viability, indicating a reduced cytoprotective effect of CDDO-Im. These results demonstrate that GSH played a predominant role in CDDO-Im-mediated cytoprotection against acrolein toxicity.

Effects of CDDO-Im and BSO co-treatment on lipid peroxidation and protein damage

Cells were pre-treated with and without CDDO-Im (100 nmol/L) and BSO (100 μmol/L) for 24 h followed by treatment with 120 μmol/L of acrolein for another 24 h. A significant increase in TBARS and PC levels in cells treated with 120 μmol/L acrolein compared to control (Figure 6c,d) was observed. Pretreatment of HepG2 cells with 100 nmol/L of CDDO-Im affords significant protection against acrolein-mediated lipid peroxidation (Figure 6c) and protein damage (Figure 6d). When cells were pretreated with 100 μmol/L of BSO in addition to CDDO-Im, the TBARS and PC levels were dramatically elevated. These results further confirm the role of GSH in CDDO-Im-mediated cytoprotection against acrolein-induced toxicity.

Discussion

Acrolein is a highly reactive α, β-unsaturated aldehyde ubiquitously found in the natural and artificial environment. It is also found naturally in food and water and, therefore,

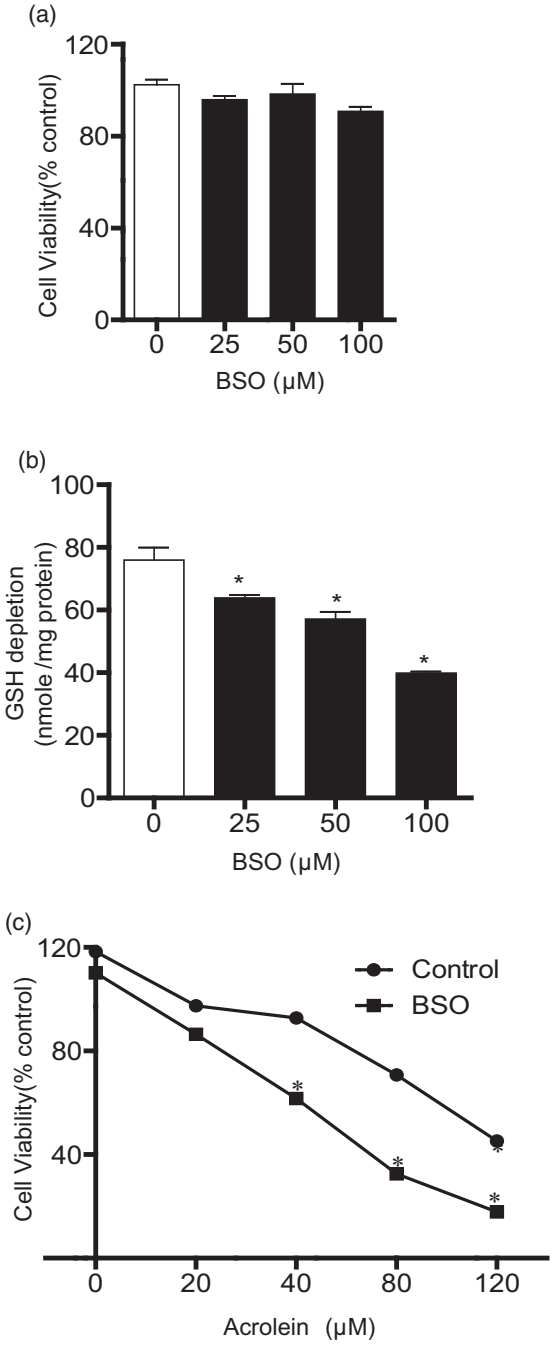


Figure 3 Effects of buthionine sulfoximine (BSO) on cellular GSH and acrolein-induced cytotoxicity in HepG2 cells. Panel a depicts cell cytotoxicity in presence of various BSO concentrations, measured by MTT. Panel b depicts the effects of BSO pretreatment on cellular GSH levels. In panel c, cells were incubated with or without 100 μmol/L of BSO for 24 h, followed by incubation with the indicated concentrations of acrolein for another 24 h. Cell viability was then measured using MTT assay. All values represent mean ± SEM from at least three different experiments. *Significantly different from the respective control group

human exposure to this toxicant is very common. Reported or calculated levels of acrolein in humans vary greatly. The normal serum levels of acrolein in humans are estimated around 50 μmol/L.[7] However, acrolein concentrations in fluid lining the respiratory tract of smokers have been reported at levels as high as 80 μmol/L.[47] Due to its solubility in water and ability to easily cross cell membranes, elevated concentrations of acrolein of upto 180 μmol/L have been found in various human tissues.[7,8,10] The accumulated acrolein in patients with chronic renal failure has been reported to be equivalent to 180 μmol/L, causing damage to cellular macromolecules.[10]

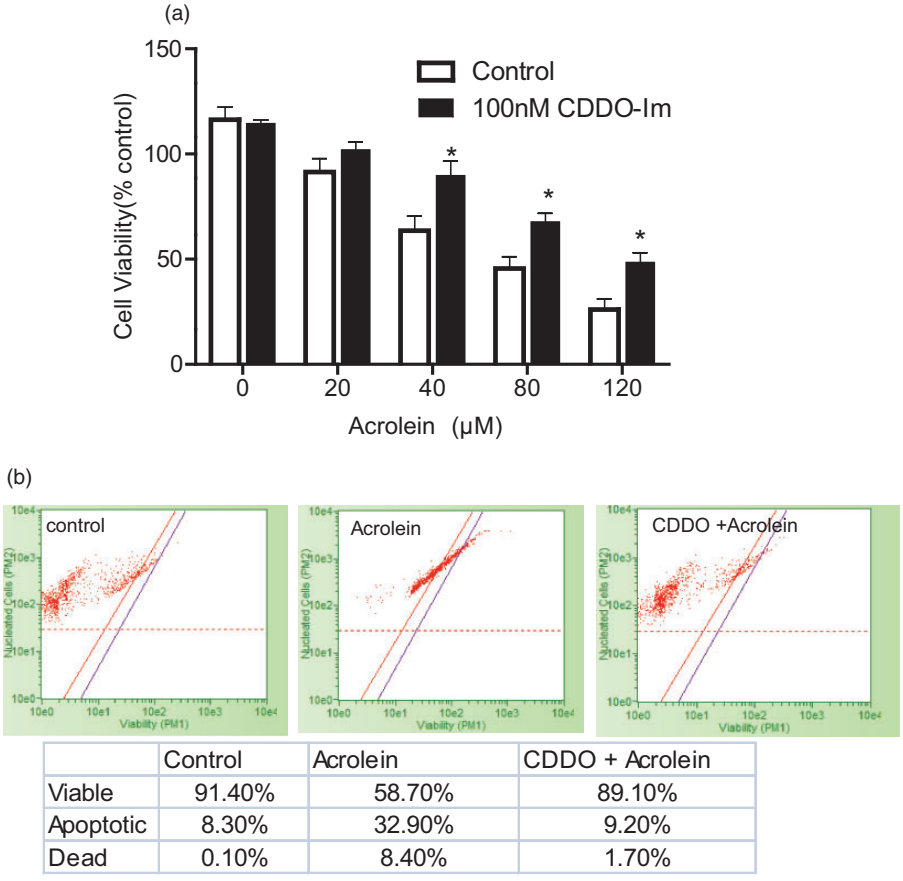
Since humans can be exposed to acrolein from the external environment as well as by endogenous generation, tissue-specific and localized levels of acrolein in the human body are more likely to be even higher than 180 μmol/L. Notably, liver is the major biotransformation organ, and phase I enzymes are most highly concentrated in the hepatocytes. Thus, compared to other organs of the body, liver is more susceptible to elevated levels of acrolein and tissue injury from the toxic effects of acrolein-mediated oxidative stress. It is important to note that the acrolein concentrations used in this *in vitro* study (40–120 μmol/L) are greater than expected in normal conditions, but are in a range anticipated in the liver under pathological conditions.

Many clinical studies have linked cigarette smoking to hepatotoxicity, where smoking causes increase in liver fibrosis and hepatocellular carcinoma. A major source of acrolein is the smoke released from incomplete combustion of organic matter. Studies show that exposure to acrolein can lead to the liver damage and heavy cigarette smokers are repeatedly exposed to high concentrations of acrolein, contributing to severe liver problems.[48] However, the mechanisms involved in acrolein-induced hepatocellular toxicity are not completely understood.

This study examines the toxic effects and cytotoxic mechanisms of acrolein on HepG2 cells. The HepG2 cell line is widely used for studying hepatotoxicity/cytoprotection due to the high amount of phase I and phase II drug metabolizing enzymes in its cells. Results show dramatic decrease of intracellular GSH, GSH-linked phase II enzyme GST, and phase II enzyme AR. This suggests that acrolein exerts its toxic effects in HepG2 cells by depleting phase II detoxifying system. Among them, GSH is a major intracellular non-protein antioxidant that plays an important role in attenuating the oxidative pathophysiology.

Many of the previous studies have suggested that depletion in cellular GSH levels leads to oxidative damage causing various types of liver disorders.[32] GSH has been suggested to directly react with acrolein to form a less reactive conjugate. The marked depletion of cellular GSH by acrolein in a concentration- and time-dependent fashion is also in agreement with the previous report that GSH may be a first line of cellular defense against acrolein-induced toxicity.[49] Results further show that depletion of cellular GSH by acrolein preceded the loss of cell viability suggesting that cellular GSH depletion may be an important event in acrolein-induced cytotoxicity.

To further investigate the involvement of GSH depletion in acrolein-induced cytotoxicity, BSO was used to deplete cellular GSH in HepG2 cells. BSO is a potent inhibitor of GSH without affecting cell viability. Results show that depletion of cellular GSH by BSO resulted in a dramatic potentiation of acrolein-induced cytotoxicity. This further confirms that GSH plays a critical role in the detoxification of acrolein in HepG2 cells.



	Control	Acrolein	CDDO + Acrolein
Viable	91.40%	58.70%	89.10%
Apoptotic	8.30%	32.90%	9.20%
Dead	0.10%	8.40%	1.70%

Figure 4 CDDO-Im protects against acrolein-mediated cytotoxicity as assessed by MTT and flow cytometric analysis. The cells were pretreated with 100 nmol/L of CDDO-Im for 24 h, followed by incubation with different concentrations of acrolein for another 24 h. Cell viability was then measured using MTT assay (Panel a). Panel b represents FACS profile of viable and apoptotic HepG2 cells. Viable cells are located on left side of each panel, apoptotic cells between the two lines, and dead cells on the right side. Percentage of viable, apoptotic, and dead cells were quantified by flow cytometer. Values represent mean ± SEM with at least three different experiments. *Indicates difference ($P \leq 0.05$) from the respective control group. (A color version of this figure is available in the online journal.)

Apoptosis and necrosis are two mutually exclusive ways of cell death. Most toxicants, if not all, have been shown to induce apoptosis at low doses and are capable of producing necrosis at higher doses. The study further examined the type of cell death caused by acrolein in HepG2 cells by utilizing flow cytometry. Our results show that acrolein-induced apoptosis in a concentration-dependent manner indicating that the apoptosis may, at least in part, be associated with early GSH depletion and acrolein cytotoxicity.

Due to its high reactivity, acrolein toxicity has been suggested to result from covalent interactions of acrolein with critical target molecules such as lipids, proteins, and DNA leading to altered target cell function.[50] Interestingly, acrolein is both an initiator and an end-product of lipid peroxidation. It has been suggested that acrolein causes the alteration of target molecules via oxidative stress, generation of excess reactive oxygen species, and alteration of endogenous antioxidants, such as depletion in GSH level in target cells.[50] In experiments, treatment of HepG2 cells with acrolein resulted in an accumulation of cellular MDA, an indicator of lipid peroxidation measured by TBARS assay. As lipid peroxidation is one of the major outcomes of oxidative stress-mediated injury that directly damages lipid membranes, this result indicated that acrolein toxicity could be due to generation of the lipid peroxidation.

In additional to lipid peroxidation, acrolein toxicity may be attributed to the covalent binding to side-chain amine groups (i.e. lysine, arginine, proline or histidine) of protein into carbonyls. The results of PC increase by acrolein as shown in our data clearly indicate that cellular proteins are modified, which is correlated with the increase in acrolein toxicity. It has been suggested that acute early depletion of GSH, GST, and AR may cause an increase in oxidative stress leading to an abrupt onset of initiation of lipid peroxidation and protein damage.[50]

Our results demonstrated that CDDO-Im pretreatment protects cells against acrolein-induced cytotoxicity. Incubation with low nanomolar concentration of CDDO-Im results in a significant increase in cellular GSH levels suggesting an important role played by GSH in acrolein toxicity. However, the same CDDO-Im treatment of HepG2 cells under present experimental condition did not induce intracellular AR and GST levels, indicating that these two aldehyde-detoxifying enzymes in HepG2 cells might be regulated via distinct signaling pathways. In this regard, the nuclear factor E2-related factor 2 (Nrf2), its

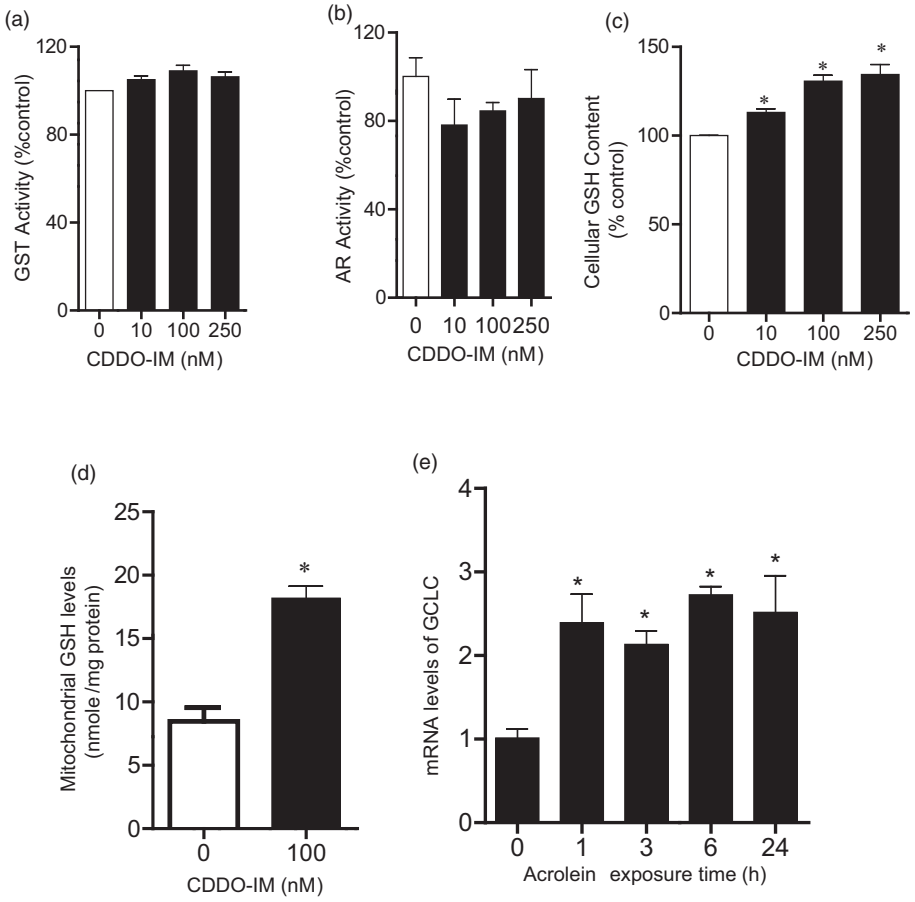


Figure 5 Effect of CDDO-Im treatment on GST, AR, and GSH contents in HepG2 cells. Panels a–c represent change in cellular levels of GST, AR, and GSH treated with or without indicated concentrations of CDDO-Im for 24 h in HepG2 cells. Panel d represents induction of mitochondrial GSH by CDDO-Im in HepG2 cells. The cells were incubated with 100 nmol/L of CDDO-Im for 24 h and mitochondria was then isolated and mitochondrial GSH levels were measured as described in ''Materials and methods'' session. Panel e represents real-time PCR analysis of mRNA levels of GCLC measured by real-time PCR and normalized to GAPDH. Values represent mean ± SEM with at least three different experiments. *Indicates difference ($P \leq 0.05$) from the respective control group

binding to Kelch-like ECH-associated protein 1 (Keap-1), and the antioxidant response element (ARE) have been reported to be critical involved in the expressions of a number of cytoprotective genes in various cells.[51,52] Studies are currently underway in our laboratory to determine if the Nrf2/Keap-1/ARE signaling pathway is responsible for CDDO-Im-mediated cytoprotection against acrolein-induced toxicity in HepG2 cells. GCLC, one of the subunits of GCL, is responsible for catalytic activity in GCL enzyme during GSH biosynthesis. The marked elevation of GCLC mRNA levels measured by real-time PCR indicated that induction of GSH by CDDO-Im in HepG2 cells appeared to occur via increased transcription of one of its subunit genes.

To further provide additional and more direct evidence for the involvement of GSH in CDDO-Im-mediated protection against acrolein detoxification process, HepG2 cells were treated with CDDO-Im in presence or absence of BSO followed by acrolein exposure. In this particular experiment, HepG2 cells were treated with BSO to reverse the effect of GSH elevation caused by CDDO-Im. Co-treatment with BSO prevented the GSH induction caused by CDDO-Im and reversed the cytoprotective effects of CDDO-Im. This observation strongly indicates that elevation of cellular GSH content by CDDO-Im pretreatment provided significant protection against acrolein-mediated cytotoxicity in HepG2 cells. Data further showed that co-treatment with BSO also completely reversed CDDO-Im-mediated cytoprotective effects on acrolein-induced lipid peroxidation and protein damage in HepG2 cells.

Overall, these results demonstrated that induction of cellular GSH, while not GST or AR, is a primary mechanism underlying CDDO-Im-mediated protection against acrolein toxicity in HepG2 cells. This study for the first time suggests that induction of non-protein antioxidant GSH by synthetic triterpenoid CDDO-Im could be a novel strategy against acrolein-mediated cytotoxicity. These results further suggest that CDDO-Im may be a promising agent to provide protection against electrophilic injuries causing liver disorders. CDDO-Im is a lipophilic compound and hence should easily be able to cross the cell membrane. Although the cytoprotective effects of CDDO-Im against acrolein-mediated hepatotoxicity are largely unexplored, several synthetic analogs of CDDO as an anti-inflammatory agent for rheumatoid arthritis are currently under clinical investigation.[46,53,54]

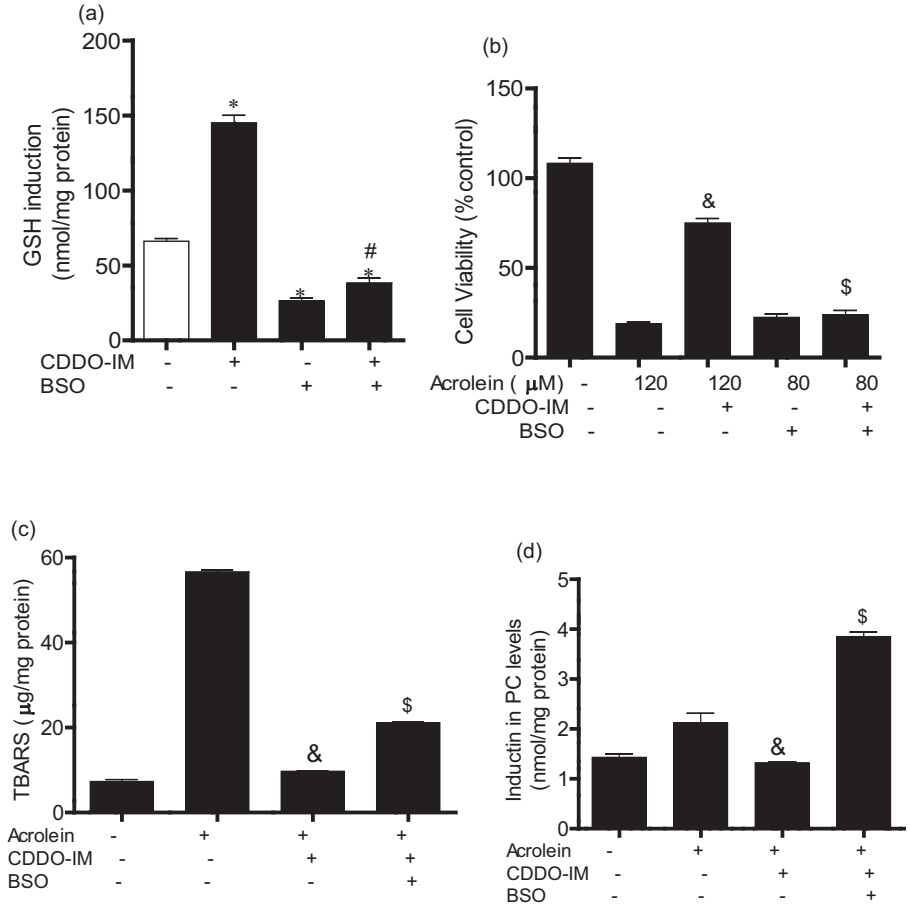


Figure 6 BSO prevents CDDO-Im-mediated cytoprotection against acrolein-induced cytotoxicity, lipid peroxidation, and protein damage in HepG2 cells. In panel a, HepG2 cells were incubated with 100 nmol/L CDDO-Im, 100 μmol/L BSO, and 100 nmol/L CDDO-Im + 100 μmol/L BSO for 24 h followed by intracellular GSH measurement. In panel b–d, cells were incubated with 100 nmol/L of CDDO-Im, 100 μmol/L of BSO, or 100 nmol/L CDDO-Im + 100 μmol/L of BSO for 24 h. The medium was removed and cells were then cultured in presence and absence of indicated concentrations of acrolein in panel b or 120 μmol/L acrolein in panel c and d for 24 h, followed by measurement of MTT assay for cell viability (panel b), protein carbonyl (panel c), and TBARS (panel d). Values represent means ± SEM from at least three separate experiments. In panel a, (*) significantly different from control; (#) significantly different from CDDO-Im treatment only. In panels b–d, (&) significantly different from acrolein treatment only; ($) significantly different from 120 μmol/L acrolein + CDDO-Im group

In summary, this study demonstrates that exposure to acrolein results in a rapid depletion of GSH, non-protein phase II enzyme and causes increase in apoptosis, lipid peroxidation, and protein carbonylation. Furthermore, the endogenous antioxidant GSH can be induced by CDDO-Im, and the CDDO-Im-mediated elevated GSH appears to afford a marked protection against acrolein toxicity suggesting that GSH plays a predominant role in CDDO-Im-mediated protection against acrolein-induced toxicity in HepG2 cells. This study may provide understanding on the molecular action of acrolein, which is important to develop novel strategies for the prevention of acrolein-mediated toxicity.

Author contributions: HZ, RYL and ZJ designed the experiments and contributed data interpretation. HS, AMS, CS, EASB and PN performed experiments. HS and ZJ analyzed data and wrote the paper.

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