Amino Acids (2012) 42:1669–1683
DOI 10.1007/s00726-011-0869-3

ORIGINAL ARTICLE

Modulation of mercury-induced mitochondria-dependent apoptosis by glycine in hepatocytes

Pabitra Bikash Pal · Sankhadeep Pal · Joydeep Das · Parames C. Sil

Received: 7 December 2010 / Accepted: 18 February 2011 / Published online: 4 March 2011

Abstract Mercury (Hg) is one of the universal environmental pollutants and is responsible for various organ pathophysiology including oxidative stress-induced hepatic disorders. In the present study, we aimed to explore the protective role of glycine in Hg-induced cytotoxicity and cell death in murine hepatocytes. Exposure of mercury (20 μM), in the form $HgCl_2$ for 1 h, significantly enhanced the ALT and ALP leakage, increased reactive oxygen species production, reduced cell viability and distorted the antioxidant status of hepatocytes. Flow cytometric analyses shows that Hg-induced apoptotic death in hepatocytes. Mechanism of this pathophysiology involves reduced mitochondrial membrane potential, variations in Bcl-2/Bad proteins, activation of caspases and cleavage of PARP protein. In addition, Hg distinctly increased NF-κB phosphorylation in association with IKKα phosphorylation and IκBα degradation. Concurrent treatment with glycine (45 mM), however, reduced Hg-induced oxidative stress, attenuated the changes in NF-κB phosphorylation and protects hepatocytes from Hg-induced apoptotic death. Hg also distinctly increased the phosphorylation of p38, JNK and ERK mitogen-activated protein kinase (MAPKs). Glycine treatment suppressed these apoptotic events, signifying its protective role in Hg-induced hepatocyte apoptosis as referred by reduction of p38, JNK and ERK MAPK signaling pathways. Results suggest that glycine can modulate Hg-induced oxidative stress and apoptosis in hepatocytes probably because of its antioxidant activity and functioning via mitochondria-dependent pathways and could be a beneficial agent in oxidative stress-mediated liver diseases.

Keywords Mercury · Oxidative stress · Reactive oxygen species · NF-κB · MAPKinases · Apoptosis · Glycine · Antioxidant

Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine aminotranferase
CAT	Catalase
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
GSH	Glutathione
GSSG	Glutathione disulfide
GPx	Glutathione peroxidase
GST	Glutathione S-transferase
GR	Glutathione reductase
$HgCl_2$	Mercuric chloride
NF-κB	Nuclear factor kappa B
ROS	Reactive oxygen species
SOD	Superoxide dismutase
PARP	Poly ADP ribose polymerase
MAPK	Mitogen-activated protein kinase
ERK	Extracellular-signal-regulated kinase

Introduction

Mercury is a broad environmental and industrial pollutant, which induces various changes in the tissues of both human and animals. Human are exposed to methyl mercury through diet, mainly because of fish consumption (Coccine

P. B. Pal · S. Pal · J. Das · P. C. Sil (✉)
Division of Molecular Medicine, Bose Institute,
P-1/12, CIT Scheme VII M, Kolkata 700054,
West Bengal, India
e-mail: parames@bosemain.boseinst.ac.in;
parames_95@yahoo.co.in

et al. 2000). In nature mercury (Hg) exists in a wide variety of physical and chemical states, each of which has specific properties for target organ. Exposure of Hg vapour and organic Hg compounds specifically damages the central nervous system (CNS), where as the kidney, liver and gastrointestinal tract is the target organ for inorganic Hg compounds. A number of studies on mercury toxicity showed that mercuric chloride ($HgCl_2$) generates oxygen free radicals. Hg(II) has a great affinity for sulfhydril (−SH) groups of biomolecules, thus depleting intracellular thiols including reduced glutathione (GSH) (Hansen et al., 2006). Covalent binding to −SH by Hg(II) changes protein conformation and generates adducts with proteins by the modification of side chains. Hg promotes free radical formation via thiol complexation and finally leads to change shape and activity of proteins (Addaya et al. 1984). It imposes a loss of antioxidant activities in glutathione peroxidise (GPx) glutathione reductase (GR), catalase (CAT), glutathione-S-transferase (GST) and superoxide dismutase (SOD). Generation of reactive oxygen species (ROS) in the cytoplasm of cells may increase the mitochondrial hydrogen peroxide production and lipid peroxidation of mitochondrial membrane, resulting in a loss of membrane potential and finally cellular necrosis or apoptosis (Kaur et al. 2006; Jezek and Hlavata 2005).

Antioxidants have been found beneficial to diminish drug and toxin-induced oxidative damages (Chang et al. 2007; Das et al. 2009a, b, 2010a, b; Ghosh et al. 2008a, b, 2010; Manna et al. 2007a, b, 2008a, b, 2009a, b, 2010; Sinha et al. 2007a, b, 2008; Sarkar et al. 2006, 2009; Sarkar and Sil 2006, 2010). Antioxidant property of the non-essential amino acid, glycine, is also well-established and therefore, could be considered as a powerful candidate against mercury-induced oxidative stress**.** In addition, glycine could prevent nephrotoxicity caused by cyclosporine A (Wheeler et al. 1999) and possess the ability to lower liver injury in a low-flow, reflow perfusion model (Zhong et al. 1996).

In the present study, primary murine hepatocytes were used as this is a useful model for investigating the toxic and protective mechanism of various toxins and protective agents in hepatic pathophysiology. The method of isolation of primary murine hepatocytes is very easy and viable cells can be obtained in a good yield. Moreover, these cells are vulnerable to various toxins and drug-induced oxidative insult so that the oxidative stress condition can be easily imposed in hepatocytes. In our study, the oxidative damage caused by Hg and the protective role of glycine were evaluated by measuring the activities of intracellular antioxidant enzymes, ROS production, levels of cellular metabolites (GSH, GSSG), lipid peroxidation end products and protein carbonylation. The mode of cell death and its protection were investigated by flow cytometric analysis and assessing the effect on the alterations of the Bcl-2 family proteins (Bcl-2, Bad), cytosolic cytochrome C, caspase 3, caspase 9 and PARP protein levels. Molecular mechanism underlying the protective action of glycine was also investigated by evaluating the role of mitogen-activated protein kinase (MAPKs) (p38, ERK and JNK) and NF-κB under this pathophysiological condition. The results of our present study might clear the role of this important amino acid in the prevention of Hg-induced hepatotoxicity, and may shed light on an achievable solution to the serious hepatic problems caused by Hg exposure.

Materials and methods

Materials

Bovine serum albumin (BSA), Bradford reagent, Collagenase type I, Dulbecco's modified Eagle's medium (DMEM), Fetal bovine seram (FBS) were purchased from Sigma-Aldrich Chemical Company, (St. Louis, MO, USA). Kits for measurement of ALT and ALP were purchased from Span diagnostic Ltd., India. Calcium chloride ($CaCl_2$), 1-chloro-2,4-dinitrobenzene (CDNB), dimethyl sulphoxide (DMSO), 5,5′-dithiobis(2-nitrobenzoic acid) [DTNB, (Ellman's reagent)], ethylene diamine tetraacetic acid (EDTA), glacial acetic acid, hydrogen peroxide (H_2O_2), N-ethylmaleimide (NEM), nicotinamide adenine dinucleotide reduced disodium salt (NADH), nitro blue tetrazolium chloride (NBT), oxidized glutathione (GSSG), phenazine methosulphate (PMT), potassium dihydrogen phosphate (KH_2PO_4), reduced glutathione (GSH), sodium azide (NaN_3), thiobarbituric acid (TBA), were bought from Sisco research laboratory, India.

Animals

Healthy adult male albino mice of Swiss strain of 4 weeks, weighing between 20 and 25 g were purchased from Ghosh Enterprises, Kolkata, India. The animals were acclimatized under laboratory condition for a fortnight before starting experiments. The animals were maintained on a standard diet and water ad libitum. They were housed in polypropylene cages and exposed to 10–12 h of daylight under standard conditions of temperature (25°C) and humidity (30%). All the experiments with animals were carried out according to the approval and guidelines of the Bose Institute animal ethical committee (the permit number is: 95/99/CPCSEA).

Hepatocyte isolation

Hepatocytes were isolated from mice liver by the method of (Sarkar and Sil 2006). Briefly, animals were anaesthetized,

sacrificed and livers were collected. After collection, the organs were extensively perfused in situ in phosphate buffer saline to get rid of blood and irrigated in a buffer containing Hepes (10 mM), KCl (3 mM), NaCl (130 mM), NaH_2PO_4–H_2O (1 mM) and glucose (10 mM) pH 7.4 and incubated with a second buffer containing $CaCl_2$ (5 mM), 0.05% collagenase type I mixed with the buffer for about 45 min at 37°C. The liver sample was then passed through wide bore syringe, filtered, centrifuged and the pellet was suspended in DMEM containing 10% FBS and the suspension was adjusted to obtain $\sim 2 \times 10^6$ cells/ml.

Determination of dose-dependent activity of Hg

The cell viability assessment was performed to determine the optimum dose of $HgCl_2$ for cytotoxicity. Six different sets of hepatocytes (1 ml cell suspension $\sim 2 \times 10^6$ in each) were incubated with six different doses of $HgCl_2$ (5, 10, 15, 20, 25 and 30 μM) to determine the maximum damage caused by $HgCl_2$ treatment. MTT assay was performed with these six sets according to the method of (Madesh and Balasubramanian 1997).

Determination of time-dependent activity of Hg

Time-dependent activity of $HgCl_2$ was measured by cell viability assessment. Five different sets of hepatocytes (1 ml cell suspension $\sim 2 \times 10^6$ cells in each) were incubated with $HgCl_2$ at a dose of 20 μM at different times (15, 30 min, 1, 1.5 and 2 h). After the incubation periods, MTT assay was performed with these five sets following the method of (Madesh and Balasubramanian 1997).

Determination of dose-dependent activity of glycine

Cell viability assessment has been carried out to determine the optimum dose of glycine needed for cytoprotection. Six different doses of glycine (20, 30, 45, 60, 75 and 90 mM) were used against mercury-induced cytotoxicity. Six different sets of hepatocytes (1 ml cell suspension $\sim 2 \times 10^6$ cells in each) were incubated with six different doses of glycine and $HgCl_2$ (20 μM) for 1 h. MTT assay was performed with these six sets according to the method of (Madesh and Balasubramanian 1997).

Experimental setup

All experiments were performed using 1 ml of hepatocytes suspension ($\sim 2 \times 10^6$ cells) in each. Hepatocytes kept in culture medium only was served as normal control. A stock solution of 5 mM $HgCl_2$ in water was prepared. From this stock, required amount was added directly for the experiment. In water 500 mM stock solution of glycine was prepared. To investigate the effect of glycine alone, 1 ml of hepatocyte suspension was incubated with glycine (45 mM) for 1 h. The toxin control was prepared by incubating the hepatocytes with $HgCl_2$ (20 μM) for 1 h. The combined effect of glycine and the $HgCl_2$ was studied by incubating hepatocytes with glycine and the $HgCl_2$ together for 1 h. All the incubations were performed at 37°C with gentle shaking. At the end of incubation period, hepatocytes were divided into three parts to carry out each experiment in triplicate.

Determination of protein content

The protein content was measured by the method of (Bradford 1976) using crystalline BSA as standard.

Assessment of markers enzymes

The leakage of marker enzymes, ALT and ALP, an important indicator of cellular membrane damage as well as cell viability, was measured using standard kits as described elsewhere (Sarkar and Sil 2007). Briefly, experimental hepatocytes were centrifuged at approximately 60 g and the content of these enzymes secreted outside the cells because of membrane damage was determined.

Measurement of lipid peroxidation and protein carbonyl content

The lipid peroxidation in normal and experimental hepatocytes in terms of malondialdehyde (MDA) formation was measured following the method of Esterbauer and Cheeseman (1990). The absorbance of thiobarbituric acid reactive substance (TBARS) formed as the end product, was measured at 532 nm and the concentration of the samples was calculated using the extinction coefficient of MDA as 1.56×10^5 $M^{-1}cm^{-1}$. The protein carbonylation of the experimental samples was determined according to the method as described by Uchida and Stadtman (1993). Briefly, the samples were treated with an equal volume of 0.1% (w/v) 2,4-DNPH in 2 N HCl and incubated for 1 h at room temperature. 20% TCA was then added for the precipitate formation which was collected by centrifugation. It was extracted three times with EtOH/EtOAc and dissolved in 8 M guanidine hydrochloride in 133 mM tris solution containing 13 mM EDTA. The absorbance was measured at 365 nm and the results were expressed as nmol of DNPH incorporated/mg protein using the molar extinction coefficient of aliphatic hydrazones as 22,000 $M^{-1}cm^{-1}$.

Assay of cellular metabolites

Reduced glutathione (GSH) level was measured following the method of Ellman (1959) by using DTNB (Ellman's

reagent) as the key reagent. Experimental samples (720 µl) were double diluted and 5% TCA was added. The mixture was centrifuged at 10,000*g* for 5 min and the supernatant was collected. DTNB solution (Ellman's reagent) was then added and the absorbance was measured at 412 nm. A standard curve was drawn using different known concentrations of GSH solution. With the help of this standard curve, GSH contents were calculated. Oxidized glutathione GSSG contents in the experimental samples were determined following the method of Hissin and Hilf (1976). Samples were mixed with 0.04 M NEM and the mixture was incubated at room temperature for 30 min. 0.3 M Na_2HPO_4 solution was then added followed by DTNB reagent. The absorbances of the samples were measured at 412 nm. The results were expressed as nmol per mg protein. The total thiols (total sulfhydryl groups) content was measured according to the method of Sedlak and Lindsay (1968). The absorbance was measured at 412 nm. The content of total thiols was calculated using molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$.

Assay of antioxidant enzymes

The activities of antioxidant enzymes, SOD, CAT, GST, GR and GPx have been measured in hepatocytes following the methods as described by Sinha et al. (2007a, b).

Briefly, for the measurement of SOD activity, the hepatocyte suspension containing 5 µg protein was mixed with sodium pyrophosphate buffer, PMT and NBT. The reaction was started by the addition of NADH. Reaction mixture was then incubated at 30°C for 90 s and stopped by the addition of 1 ml of glacial acetic acid. The absorbance of the chromogen formed was measured at 560 nm. One unit of SOD activity is defined as the enzyme concentration required inhibiting chromogen production by 50% in 1 min under the assay conditions.

CAT activity was determined by following the decomposition of H_2O_2 (7.5 mM) at 240 nm for 10 min and it was monitored spectrophotometrically. One unit of CAT activity is defined as the amount of enzyme, which reduces 1 µmol of H_2O_2 per minute.

GST activity was assayed based on the conjugation reaction with glutathione in the first step of mercapturic acid synthesis. The reaction mixture contains 25 µg protein sample, KH_2PO_4 buffer, EDTA, CDNB and GSH. The reaction was carried out at 37°C and monitored spectrophotometrically at 340 nm for 5 min. One unit of GST activity is 1 µmol product formation per minute.

GR activity was determined spectrophotometrically by monitoring the absorbance at 412 nm for 3 min at 24°C. Reaction mixture contained 1 mM EDTA, 0.3 mM DTNB, 2 mM NADPH and 20 mM GSSG. The enzyme activity was calculated using molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$. One unit of enzyme activity is defined as the amount of enzyme, which catalyzes the oxidation of 1 µmol NADPH per minute.

GPx activity was measured by using H_2O_2 and NADPH as substrates. The conversion of NADPH to $NADP^+$ was observed by recording the changes in absorption intensity at 340 nm and one unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol NADPH per minute.

Measurement of intracellular ROS production

All sets of experimental hepatocytes were incubated with DCF-DA (10 mM) for 1 h at 37°C in the dark. DCF-DA diffuses through the cell membrane and enzymatically deacetylated to the more hydrophilic non-fluorescent reduced dye dichlorofluorescin. Non-fluorescent DCFH rapidly oxidized to highly fluorescent product DCF in presence of reactive oxygen metabolites. Earlier report suggests that oxidation of DCFH was unaffected by SOD, catalase or nitric oxide (Zhu et al. 1994). After treatment, the cells were immediately washed and resuspended in PBS. Intracellular ROS production was detected using the fluorescent intensity of the oxidant sensitive probe 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) in a fluorescence microscope.

Detection of cell death pathway by flowcytometry assay

All sets of experimental hepatocytes were incubated with propidium iodide (PI) and FITC-labelled Annexin V for 30 min at 37°C. Excess PI and Annexin V were then washed off; cells were fixed and then stained cells were analyzed by flow cytometry using FACS Calibur (Becton Dickinson, Mountain View, CA, USA) equipped with 488 nm argon laser light source; 515 nm band pass filter for FITC-fluorescence and 623 nm band pass filter for PI-fluorescence using CellQuest software. A scatter plot of PI-fluorescence (*y*-axis) versus FITC-fluorescence (*x*-axis) has been prepared.

Isolation of mitochondria and determination of mitochondrial membrane potential ($\Delta\psi_m$)

For the determination of mitochondrial membrane potential, mitochondria were isolated from all sets of experimental hepatocytes. Cells were taken in 10 ml buffer containing 0.1 M Tris–Mops, pH 7.4, 20 ml 1 M sucrose, and 1 ml 0.1 M EGTA–Tris buffer, pH 7.4. Then the hepatocytes were sonicated and centrifuged at 800*g* for 10 min at 4°C, the supernatant was collected and centrifuged for 30 min at 10,000*g*. The supernatant was then discarded and the mitochondria pellet was re-suspended in

the same buffer and re-centrifuged for 10 min at 10,000*g*. This supernatant was also discarded and the final mitochondrial pellet was re-suspended in PBS. Analytic flow cytometric measurements for the membrane potential ($\Delta\psi_m$) of isolated mitochondria were performed using a FACScan flow cytometer with an argon laser excitation at 488 and 525 nm band pass filter. Mitochondrial membrane potential ($\Delta\psi_m$) was estimated on the basis of cell retention of the fluorescent cationic probe rhodamine 123 (Mingatto et al. 2003).

Immunoblotting

An equal amount of protein (50 μg) from each sample was resolved by 10% SDS-PAGE and transferred to PVDF membrane. Membranes were blocked at room temperature for 2 h in blocking buffer containing 5% non-fat dry milk to prevent non-specific binding and then incubated with anti-p-38 (1:1,000 dilution), anti-ERK1/2 (1:1,000 dilution), anti-p-JNK (1:1,000 dilution), anti-Bad (1:1,000 dilution), anti-Bcl-2 (1:1,000 dilution), anti-caspase3, anti-caspase9 (1:1,000 dilution) and anti-NFκB (1:1,000 dilution) primary antibodies separately at 4°C overnight. The membranes were washed in TBST (50 mmol/L Tris–HCl, pH 7.6, 150 mmol/L NaCl, 0.1% Tween 20) for 30 min and incubated with appropriate HRP conjugated secondary antibody (1:2,000 dilution) for 2 h at room temperature and developed by the HRP substrate 3,3′-diaminobenzidine tetrahydrochloride (DAB) system (Bangalore genei, India).

Statistical analysis

All the values are expressed as mean ± SD ($n = 6$). Significant differences between the groups were determined with SPSS 10.0 software (SPSS Inc., Chicago, IL, USA) for Windows using one-way analysis of variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test (DMRT). A difference was considered significant at the $p < 0.05$ level.

Results

Dose-dependent effect of $HgCl_2$-induced cytotoxicity

Cell viability is an important indicator of finding the degree of cytotoxicity caused by any xenobiotics. Figure 1 shows the dose-dependent effect of $HgCl_2$ in murine hepatocytes. It has been observed that $HgCl_2$ exposure caused decrease in cell viability linearly up to a dose of 20 μM when incubated for 1 h. Effect of $HgCl_2$ remained more or less unaffected beyond this concentration. For all the subsequent experiments in this particular study, this dose (20 μM) of $HgCl_2$ has been taken as optimum.

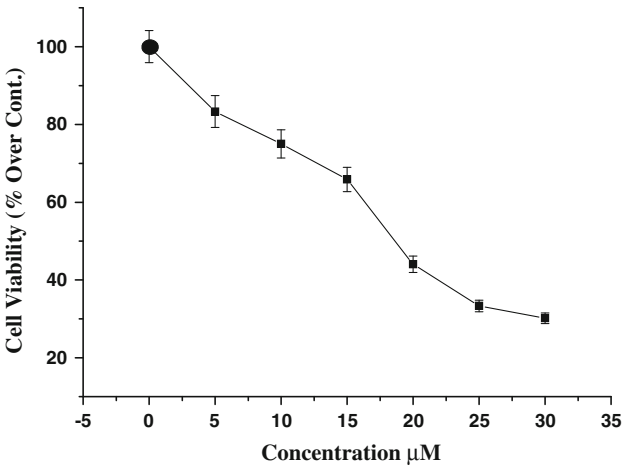

Fig. 1 Dose-dependent effect of $HgCl_2$ on cell viability. MTT assay was carried out for this purpose. Cont: cell viability in normal hepatocytes, Hg-5, Hg-10, Hg-15, Hg-20, Hg-25 and Hg-30: cell viability in Hg-induced hepatocytes for 1 h at a dose of 5, 10, 15, 20, 25 and 30 μM. *a* indicates the significant difference between normal control and toxin-treated cells. Each column represents mean ± SD, $n = 6$; ($p^a < 0.01$)

Time-dependent activity of Hg

Figure 2 demonstrates the result of time-dependent effect of $HgCl_2$ in mouse hepatocytes on MTT assay. From this

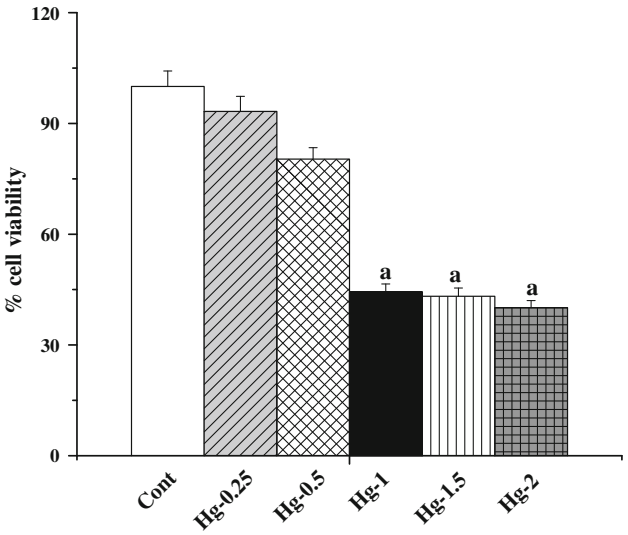

Fig. 2 Time-dependent effect of $HgCl_2$ on cell viability. MTT assay was carried out for this purpose. Cont: cell viability in normal hepatocytes, Hg-15 min (Hg-0.25), Hg-30 min (Hg-0.5), Hg-1 h (Hg-1), Hg-1.5 h (Hg-1.5), Hg-2 h (Hg-2): cell viability in $HgCl_2$ treated hepatocytes for the incubation time of 15, 30 min, 1, 1.5 and 2 h at a dose of 20 μM. *a* the significant difference between normal control and toxin-treated cells. Each column represents mean ± SD, $n = 6$; ($p^a < 0.01$)

study, we found that mercuric chloride caused a significant decrease in cell viability when incubation was carried out for 1 h. Beyond this incubation time (1 h), effect of Hg remained practically unaltered. For all the subsequent experiments in this particular study; this incubation time has been taken as optimum for $HgCl_2$.

Dose-dependent effect of glycine against $HgCl_2$-mediated cellular damage

Results of the dose-dependent effects of glycine against $HgCl_2$-induced oxidative injury in murine hepatocytes have been summarized in the Fig. 3. $HgCl_2$ exposure caused reduction in cell viability at a dose of 20 μM when incubation was accomplished for 1 h. To determine whether this loss could be prevented by glycine treatment, we performed MTT assay. Concurrent incubation of the hepatocytes with glycine and Hg linearly inhibits the diminution in cell viability up to a dose of 45 mM for 1 h and this effect of glycine remained basically unchanged beyond this concentration. In all experiments of this particular study, this dose (45 mM) of glycine has been taken as optimum.

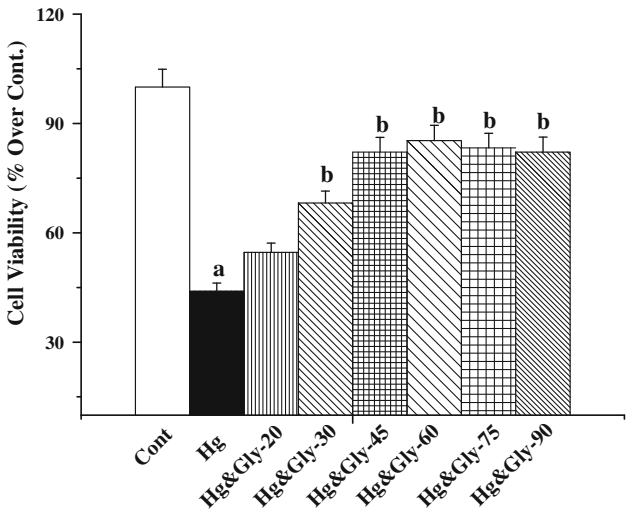

Fig. 3 Dose-dependent effect of glycine against Hg-induced toxic effect on cell viability. MTT assay was carried out for this purpose. Cont: cell viability in normal hepatocytes, Hg-20 (Hg): cell viability in $HgCl_2$ treated hepatocytes for 1 h at a dose 20 μM, Gly-20 (Hg&Gly-20), Gly-30 (Hg&Gly-30), Gly-45 (Hg&Gly-45), Gly-60(Hg&Gly-60), Gly-75 (Hg&Gly-75) and Gly-90 (Hg&Gly-90): cell viability level in hepatocytes treated with glycine and $HgCl_2$ simultaneously for 1 h at a dose of 20, 30, 45, 60, 75 and 90 mM. *a* the significant difference between the normal control and toxin-treated cells, *b* the significant difference between toxin control and glycine-treated cells. Each column represents mean ± SD, $n = 6$; ($p^a < 0.01$, $p^b < 0.01$)

Effect on markers enzymes

The activities of the enzymes ALP and ALT are related to hepatocytes physiology and pathophysiology. Effects of Hg and glycine in normal and experimental sets of isolated hepatocytes are shown in Table 1. The elevation of the activities of these enzymes was observed in toxin exposed cells whereas simultaneous addition of glycine and $HgCl_2$ could prevent the same and maintain the normalcy in hepatocytes.

Effect on the end products of lipid peroxidation and protein carbonylation

Table 2 shows the levels of MDA, as an index of lipid peroxidation and protein carbonylation in normal and experimental sets of isolated hepatocytes. These levels in toxin control were found to be higher than normal hepatocytes. Simultaneous incubation of glycine and $HgCl_2$ reduced these levels compared to the toxin alone treated hepatocytes.

Effect on cellular metabolites

The levels of the cellular GSH and GSSG in the normal and experimental hepatocytes have been depicted in Table 3. Hepatocytes incubated with $HgCl_2$ showed a decrease in the GSH and increase in GSSG levels compared to the normal hepatocytes. Simultaneous incubation of the cells with glycine and $HgCl_2$ modulated these levels compared to the toxin exposed ones.

Effect on antioxidant enzymes

Table 4 shows the effect of glycine on the activities of the antioxidant enzymes CAT, SOD, GST, GR, GPX in $HgCl_2$ exposed hepatocytes. The activities of these antioxidant enzymes in toxin-treated hepatocytes were significantly lower than that of normal cells. Simultaneous incubation of glycine and $HgCl_2$, on the other hand, could prevent that $HgCl_2$-induced decrease in antioxidant enzymes activities.

Effect on intracellular ROS production

Disturbances of the pro-oxidant and anti-oxidant equilibrium in favor of the former play an important role in organs, tissues and cellular pathophysiology. This state of affairs arises as a result of either the increased production of ROS or the decreased level of the antioxidant defense. Hg intoxication creates excess ROS either directly or indirectly in organ pathophysiology. Figure 4a demonstrates the intracellular ROS levels in the normal and experimental hepatocytes. An increase in the intracellular

Table 1 Effect of Hg and glycine on the levels of serum marker enzymes related to hepatocytes dysfunction

Markers enzymes	Normal control	Glycine	Hg	Hg and glycine
ALT (IU/L)	41.23 ± 2.02	40.35 ± 1.98	120.76 ± 5.98[a]	55.45 ± 2.49[b]
ALP (KA units)	33.45 ± 1.51	31.48 ± 1.53	72.23 ± 2.99[a]	41.23 ± 2.12[b]

Values are expressed as mean ± SD, for six animals in each group

[a] Values differs significantly from normal control ($p^a < 0.05$)

[b] Values differs significantly from toxin control ($p^b < 0.05$)

Table 2 Effect of Hg and glycine on the status of lipid peroxidation

Parameters	Normal control	Glycine	Hg	Hg and glycine
MDA (nmol/mg protein)	3.75 ± 0.16	3.45 ± 0.15	7.87 ± 0.36[a]	4.12 ± 0.18[b]
Protein carbonylation (nmol/mg protein)	37.31 ± 1.52	35.76 ± 1.49	63.18 ± 3.11[a]	40.41 ± 1.97[b]

Values are expressed as mean ± SD, for 6 animals in each group

[a] Values differs significantly from normal control ($p^a < 0.05$)

[b] Values differs significantly from toxin control ($p^b < 0.05$)

Table 3 Effect of Hg and glycine on the status of intracellular thiol levels (reduced and oxidised)

Parameters	Normal control	Glycine	Hg	Hg and glycine
GSH (nmol/mg protein)	17.38 ± 0.84	16.12 ± 0.81	8.38 ± 0.41[a]	14.32 ± 0.71[b]
GSSG (nmol/mg protein)	0.46 ± 0.02	0.44 ± 0.021	0.97 ± 0.045[a]	0.51 ± 0.025[b]

Values are expressed as mean ± SD, for 6 animals in each group

[a] Values differs significantly from normal control ($p^a < 0.05$)

[b] Values differs significantly from toxin control ($p^b < 0.05$)

Table 4 Effect of Hg and glycine on the activities of the antioxidant enzymes in hepatocytes

Name of the antioxidant enzymes	Normal control	Glycine	Hg	Hg and glycine
SOD (Unit/mg protein)	129.34 ± 6.32	128.76 ± 5.92	65.69 ± 3.17[a]	93.34 ± 4.22[b]
CAT (μmol/min/mg protein)	287.34 ± 13.45	288.26 ± 13.65	187.56 ± 9.12[a]	252.45 ± 11.45[b]
GST (μmol/min/mgprotein)	3.2 ± 0.14	3.1 ± 0.13	1.13 ± 0.055[a]	2.23 ± 0.11[b]
GR (nmol/min/mg protein)	137.23 ± 5.8	135.43 ± 5.7	64.35 ± 3.13[a]	100.34 ± 5.00[b]
GPx. (nmol/min/mg protein)	149.32 ± 6.67	147.45 ± 5.98	52.56 ± 2.31[a]	115.49 ± 5.31[b]

Values are expressed as mean ± SD, for 6 animals in each group

[a] Values differs significantly from normal control ($p^a < 0.05$)

[b] Values differs significantly from toxin control ($p^b < 0.05$)

ROS level was found in hepatocytes exposed to $HgCl_2$. Simultaneous incubation of the cells with glycine and $HgCl_2$ prevents this increase in the intracellular ROS level compared to the toxin alone exposed groups.

Effect of glycine on $HgCl_2$-induced apoptotic/necrotic death

To investigate whether the toxic effect of $HgCl_2$ on hepatocytes viability and its protection by glycine involves the process of cell apoptosis and/or necrosis, hepatocytes of all groups were assessed by flow cytometric analysis. Flow-cytometric data (Fig. 4b) revealed that, in comparison to control, $HgCl_2$ intoxicated hepatocytes showed maximum Annexin V-FITC-binding (41.23%), but very little PI staining (0.97%) indicating that the nature of the majority of $HgCl_2$ exposed cells was apoptotic. On the other hand, the number of apoptotic cells was significantly low (only 12.26%) in the cell populations exposed to simultaneous incubation of glycine and $HgCl_2$, indicating that glycine could protect hepatocytes from $HgCl_2$ induced apoptotic death.

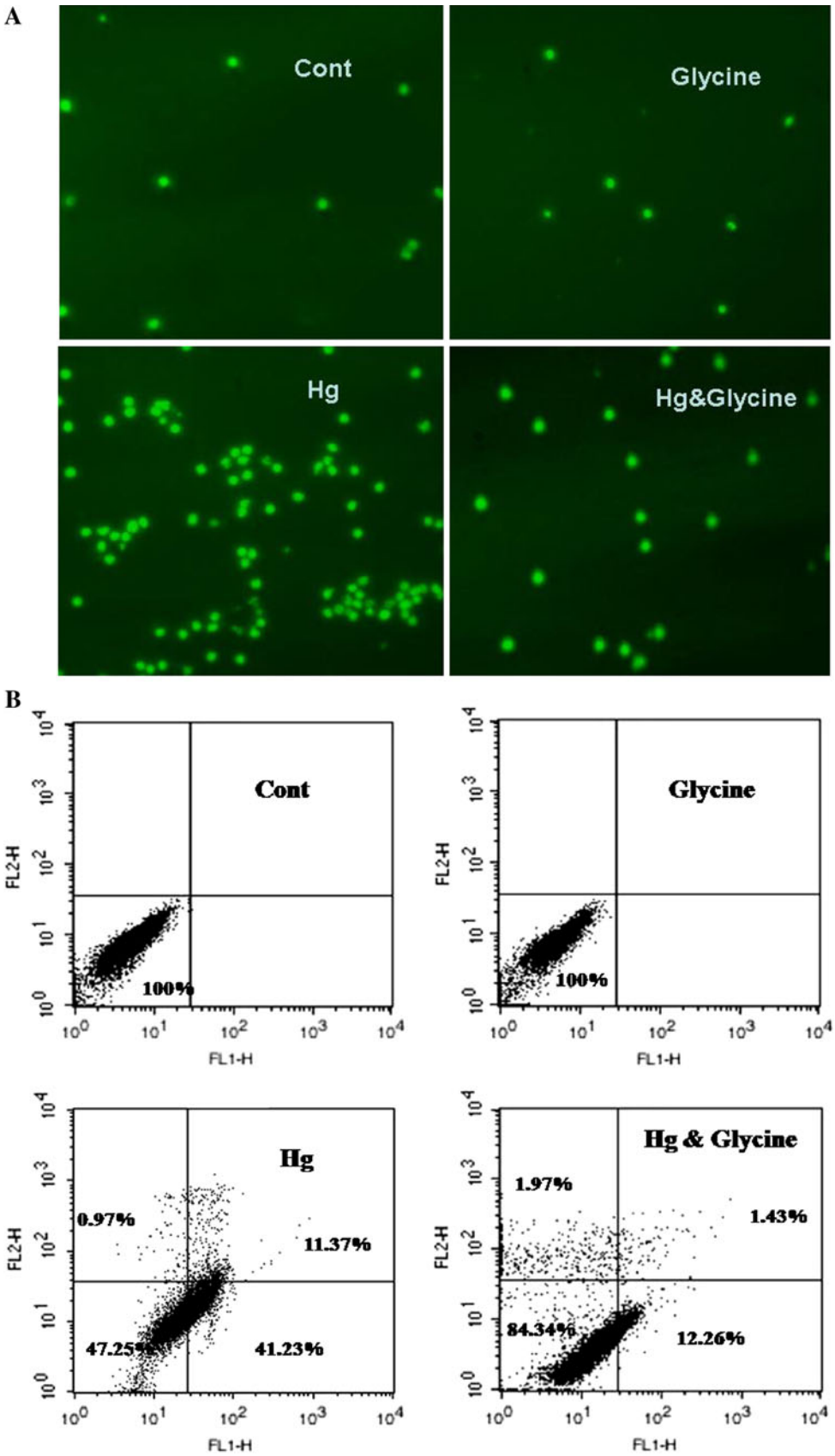

Fig. 4 Impact of Hg and glycine on ROS production and mode of cell death in hepatocytes. **a** The intracellular ROS production was detected by fluorescent microscope using DCF-DA in Hg-exposed hepatocytes in absence (Hg) and presence of glycine (Hg & Glycine). Results represent one of the six independent experiments. **b** Percent distribution of apoptotic and necrotic cells. Cell distribution was analysed using Annexin V binding (taken as *x*-axis) and PI uptake (taken as *y*-axis). The FITC and PI fluorescence were measured using flow cytometer with FL-1 and FL-2 filters, respectively. Results expressed as dot plot representing as one of the six independent experiments

Participations of NFκB

NFκB, a well-known rapidly induced stress-responsive transcription factor, is found to be involved in the intracellular signaling pathways and is exquisitely sensitive to cellular oxidative status, cell transformation, and apoptosis in response to oxidative stress. Therefore, to investigate the role of NF-κB in Hg-induced oxidative stress and cytotoxicity, immunoblotting analysis of NF-κB and IκBα (inhibitor of NF-κB) has been carried out. $HgCl_2$ exposure caused a significant increase in the expression of NF-κB and decrease in the expression of IκBα compared with the relevant control group in association with phosphorylation of IKKα. On the other hand, Hg-induced increase in IKKα phosphorylation, NFκB activation and IκBα degradation were inhibited by the simultaneous treatment of the cells with glycine (Fig. 5).

Effect on MAP kinases

Mitogen-activated protein kinases are the known mediators of cell death due to apoptosis under various pathophysiological conditions (Feuerstein and Young 2000). Intracellular oxidative-stress stimuli can activate both NF-κB and MAP kinase modules (Schulze-Osthoff et al. 1997). So we examined whether the MAPKs are also involved in Hg-induced NF-κB activation in hepatocytes. We determined the protein levels of different MAPKs in Hg intoxicated hepatocytes. Results showed a marked increase in protein content of phosphorylated p38 MAPK, p-JNK and ERK 1/2 in hepatocytes, without any change in total protein content of these kinases. Our results pointed out that Hg effectively phosphorylated p38, JNK and ERK 1/2 MAPKs in addition to NF-κB. From the data shown in Fig. 6, it is evident that the increase in protein contents of phosphorylated MAPKs could be prevented by glycine treatment.

Glycine diminished the pro-apoptotic effects of Hg on Bcl-2 family proteins

Since the process of apoptosis is considered to be regulated by a complex interplay of proapoptotic (Bad) and antiapoptotic (Bcl-2) mitochondrial membrane proteins as well as the activation of caspase-3 (effector caspase) via the activation of caspase-9 (initiator caspase), the status of these signaling molecules was also investigated in Hg intoxicated hepatocytes in the absence and presence of glycine supplementation. Immunoblotting studies demonstrated that Hg downregulated the anti-apoptotic (Bcl-2) and upregulated the proapoptotic (Bad) Bcl-2 family proteins in hepatocytes (Fig. 7). Glycine could, however, protect the cells by preventing the alterations of these proteins.

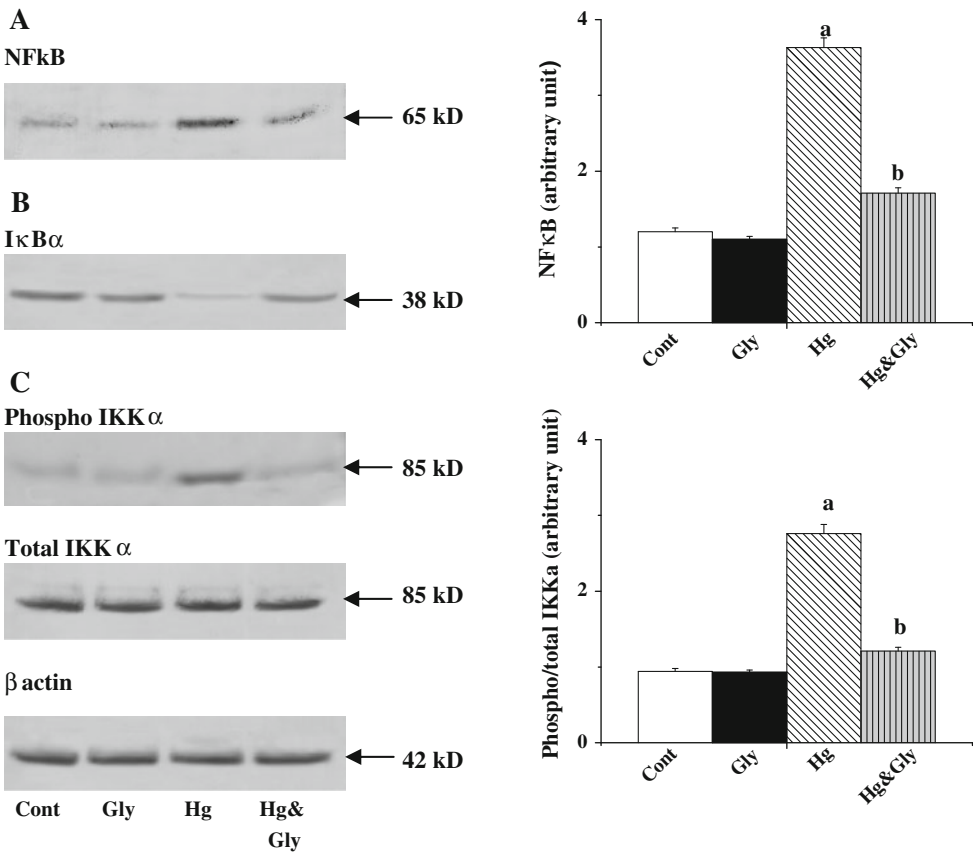

Fig. 5 Immunoblot analysis of NFκB, IκBα andIKKα proteins in response to Hg and glycine (Hg & Glycine). **a** NFκB, *β* actin was used as an internal control. **b** IκBα and **c** IKKα, actin was used as an internal control. Panel Data represent the average ± SD of six separate experiments in each group. *a* The significant difference between the normal control and Hg-exposed hepatocytes, *b* the significant difference between the Hg exposed and glycine treated groups ($P^a < 0.05$, $P^b < 0.05$)

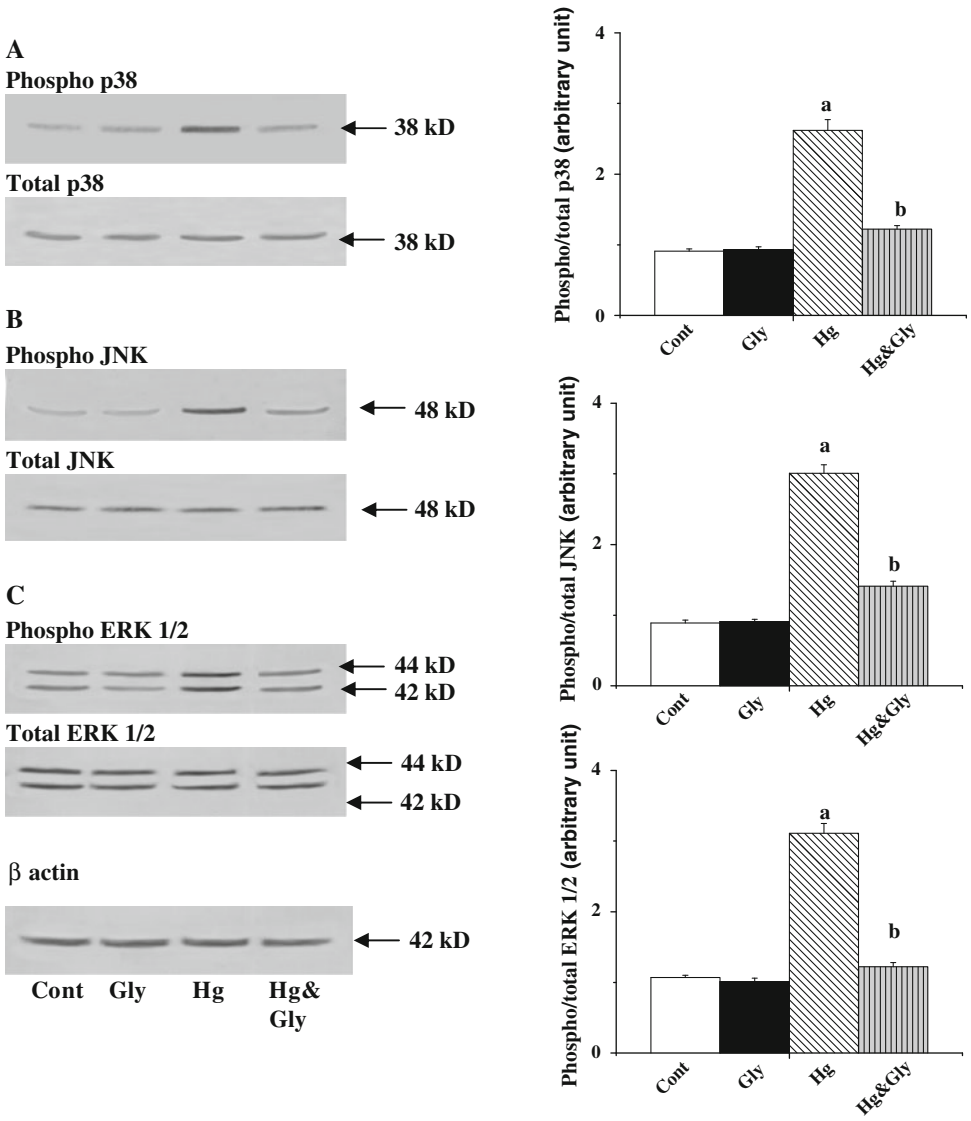

Fig. 6 Immunoblot analysis of mitogen-activated protein kinases (MAPKs) in response to Hg and glycine (Hg & Glycine). **a** Phosphorylated p38 (phospho-p38) and total p38 MAPK, **b** phosphorylated JNK(phospho-p38) and total JNK MAPK, **c** phosphorylated ERK ½ MAPK (phospho-ERK ½ MAPK) and total ERK ½ MAPK. The relative intensities of bands were determined using NIH-image software and the control band was given an arbitrary value of 1. Panel Data represent the average ± SD of six separate experiments in each group. *a* The significant difference between the normal control and Hg-exposed hepatocytes, *b* the significant difference between the Hg exposed and glycine treated groups ($P^a < 0.05$, $P^b < 0.05$)

Effect of glycine against Hg-induced mitochondrion-dependent cell death

A key step in the mitochondrion-dependent apoptotic cell death pathways is evidenced by the loss of mitochondrial membrane potential ($\Delta\psi_m$), release of cytochrome C from mitochondria and subsequent activation of caspase 9 as well as caspase 3. To determine whether glycine exerts its anti-apoptotic action in Hg-induced apoptotic death via above mechanism, mitochondrial membrane potential ($\Delta\psi_m$), cytosolic cytochrome C, caspase 9 as well as caspase 3 levels in hepatocytes were measured. Results showed that Hg administration significantly reduced the mitochondrial membrane potential (Fig. 8a), enhanced the concentration of cytosolic cytochrome C, caspase 9 as well as caspase 3 protein levels (Fig. 8). Hg-induced hepatocytes' apoptosis and its protection by glycine was also studied in terms of PARP cleavage. Cleaved PARP levels were increased dramatically by Hg intoxication. Simultaneous treatment of glycine and $HgCl_2$ could, however, significantly inhibit Hg-induced alterations of the levels of these signalling molecules (Fig. 8).

Discussion

Inorganic and organic oxygen free radicals, usually generated from biological reduction of molecular oxygen (O_2), are jointly known as ROS. These highly reactive species display a broad spectrum of pathophysiology (Das et al. 2009a, b, 2010a, b; Ghosh et al. 2008, 2009a, b, 2010a, b). Pourahmad et al. (2003) showed that $HgCl_2$ induced oxidative stress at a dose 20 μM. In the present study, we also performed a dose-dependent experiment on cell viability

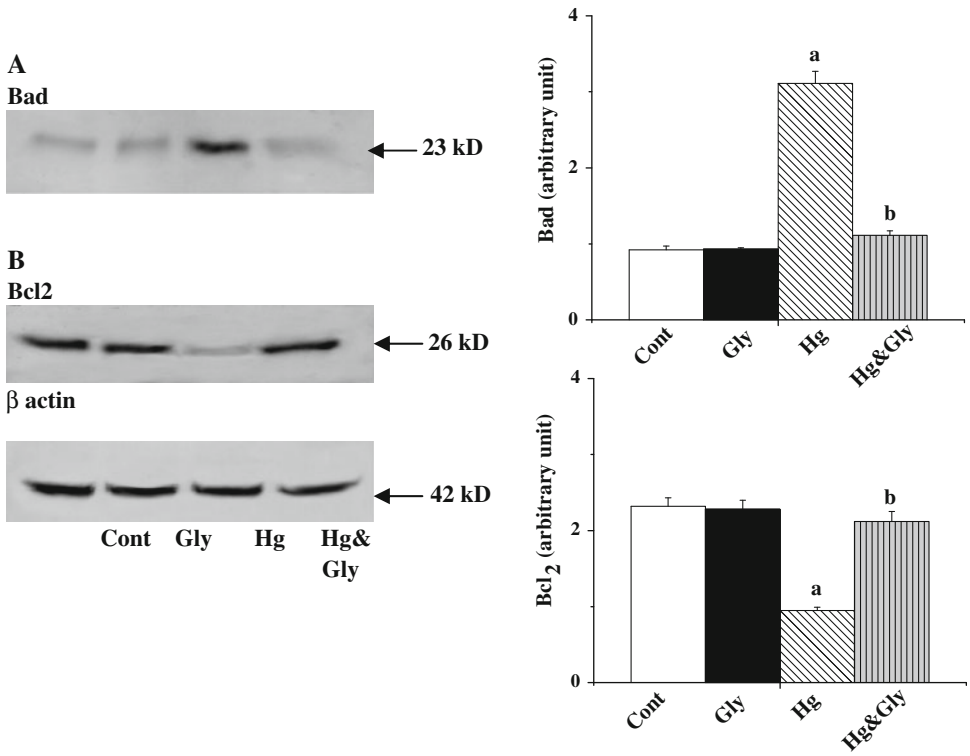

Fig. 7 Investigation of the involvement of Bcl_2 family proteins in absence (Hg) and presence of glycine (Hg & Glycine) in hepatocytes. **a** Bad, **b** Bcl-2, β actin was used as an internal control. Data represent the average $\pm$ SD of six separate experiments in each group. *a* The significant difference between the normal control and Hg-exposed hepatocytes, *b* the significant difference between the Hg-exposed and glycine-treated groups ($P^a < 0.05$; $P^b < 0.05$)

using various concentration of $HgCl_2$ (ranging from 5 to 30 μM). We observed that ~52% cells underwent death at a dose of 20 μM during incubation period of 1 h and beyond this concentration the effect of $HgCl_2$ was not much. Therefore, this dose of $HgCl_2$ was taken as optimum for the subsequent studies. Similarly, we have also performed a dose-dependent experiment on cell viability using various concentration of glycine (ranging from 20 to 90 mM). We observed that ~82% cells remained viable when the hepatocytes were coincubated with glycine (45 Mm) and $HgCl_2$ (20 μM) during incubation period of 1 h and beyond this concentration the protective effect of glycine remained more or less the same. Therefore, this dose of glycine was taken as optimum for the subsequent studies. Our present study also demonstrated that exposure to mercuric chloride ($HgCl_2$) considerably increased ROS creation, enhanced oxidative stresses and induced apoptosis in hepatocytes. These adverse effects in Hg-induced hepatic pathophysiology might possibly, be reduced by glycine supplementation.

Antioxidant defense machineries control ROS production and/or scavenge those species to prevent oxidative stress. Among the different antioxidant molecules, CAT and SOD jointly play a significant role in the exclusion of ROS. With the purpose of removing excess free radicals from the system, GST and GPx use GSH in their course of reactions. Diminish in GSH content, because of oxidative stresses, reduce the actions of GST and GPx with a concomitant decrease in the activity of GSH stimulating enzyme, GR. In the present study, we observed that reduction in antioxidant enzymes activities, GSH depletion and simultaneous GSSG accumulation occur in hepatocytes during Hg-induced oxidative stresses. However, glycine effectively inhibited these oxidative stresses related cellular dysfunctions.

Although recent literatures describe the signaling pathways of the actions of some metals in cellular oxidative stresses and apoptotic or necrotic death (Yamanaka et al. 1991; Chang et al. 2007; Yamauchi et al. 2004; Das et al. 2009a, b, 2010a, b; Ghosh et al. 2010a, b; Ghosh and Sil 2009; Roy et al. 2009), little is known about the mechanism of Hg-induced hepatic pathophysiology. In fact, very little information is available in the literature in this regard. Some reports have been published recently describing only the very basic nature of toxicity in Hg-induced hepatic and other organ pathophysiology (Lee et al. 2009; Ghosh and Sil 2008; Singh et al. 2007). In addition, to the best of our knowledge, there is no report so far describing the mechanism of the protective action of glycine against Hg-induced oxidative stresses followed by apoptosis or necrosis. So, to study the molecular mechanism of the protective action of glycine in Hg-induced oxidative stresses mediated hepatocytes' death, we began to investigate the MAPKs signaling pathways as these signalling molecules are the primary intermediates for the induction of cell death induced by oxidative stresses. Based on the

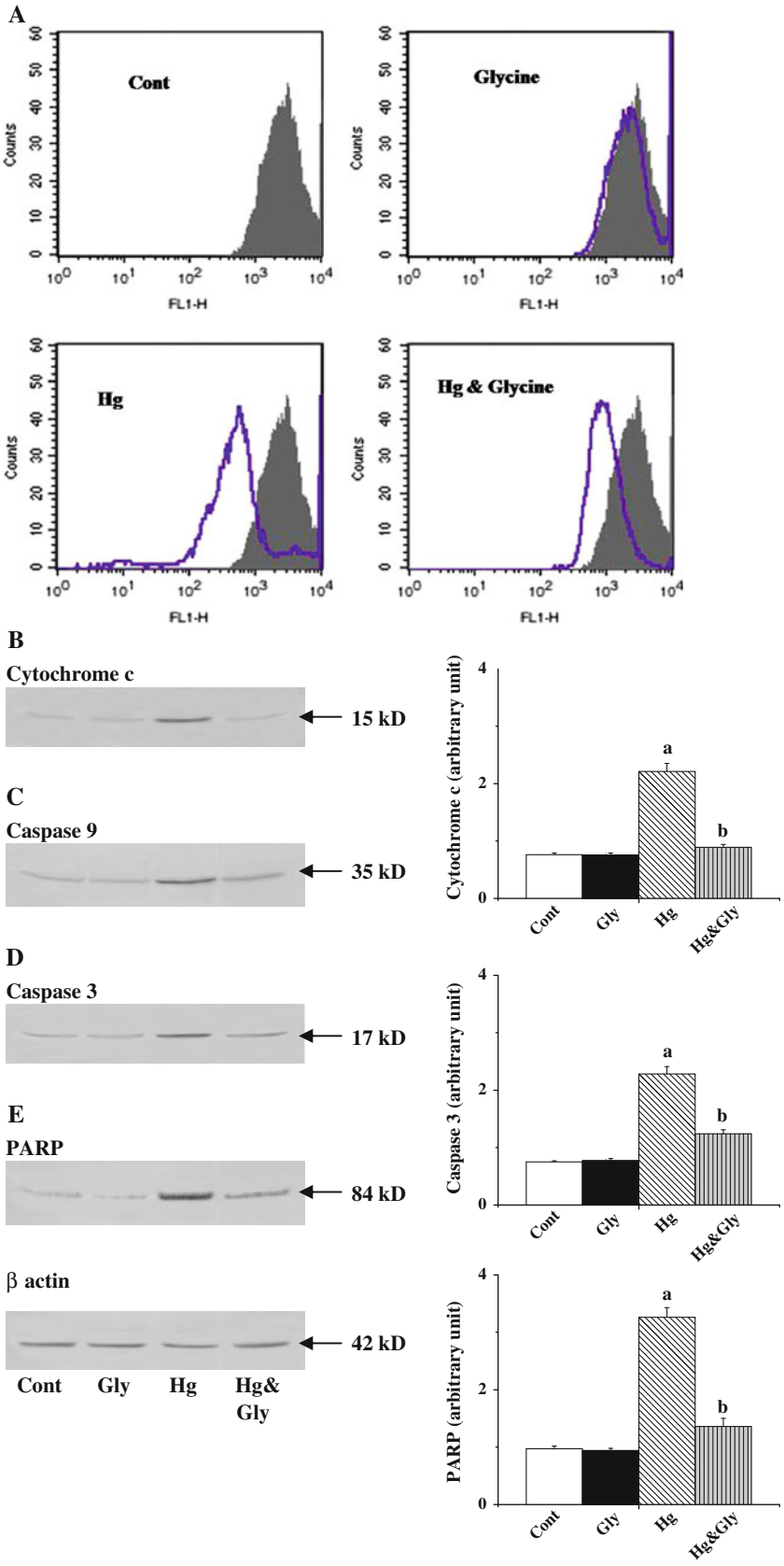

Fig. 8 Investigation of Mitochondrion-dependent pathway in absence (Hg) and presence of glycine (Hg & Glycine) in hepatocytes. **a** Mitochondrial membrane potential ($\Delta\psi_m$) was measured using a fluorescent cationic probe rhodamine-123 by flow cytometer with FL-1 filter. Results represent one of the six independent experiments. **b** Cytosolic cytochrome c, **c** caspase 9, **d** caspase 3 and **e** PARP. β actin was used as an internal control. Data represent the average $\pm$ SD of six separate experiments in each group. *a* The significant difference between the normal control and Hg-exposed hepatocytes, *b* the significant difference between the Hg-exposed and glycine-treated groups ($P^a < 0.05$; $P^b < 0.05$)

nature of the stress-inducers and cell types, different members of the MAPKinase family (ERK1/2, JNKs and p38-MAPKs) are activated by cellular stresses, including oxidative stresses and are thought to correlate with cell death. In the present study, we observed that Hg exposure upregulated the expression of phospho-ERK1/2 phospho-JNK and phospho-p38 whereas no significant changes were detected in the expression of total ERK1/2, JNK and p38. Several other investigators have also shown that MAP-kinase proteins are expressed due to Hg exposure and treatment with their inhibitors significantly resists the cells against Hg-induced cytotoxicity (Korashy and El-Kadi 2008; Choi et al. 2006). Therefore**,** these observations are also supporting our present experimental findings and help us to conclude that MAPkinase proteins play a significant role in inducing Hg-induced cytotoxicity in hepatocytes. Another very important transcription factor (a major regulator of stress responses as well) playing significant role in cellular dysfunction is NF-κB. A number of recent studies revealed either pro-apoptotic or anti-apoptotic function of NF-κB in response to toxicant injury (Ghosh et al. 2009a, b). In our study, we detected the up-regulation of NF-κB (p65); suggesting its pro-apoptotic role in response to Hg exposure to hepatocytes. It has been established that the release of transcriptionally capable NF-κB dimers in oxidative stresses induced pathophysiology is accelerated after phosphorylation-induced degradation or dissociation of IκBα molecules. In our study, we observed that Hg exposure down-regulated the expression of IκBα with a concomitant upregulation of NF-κB and IKKα phosphorylation. Glycine might possibly, on the other hand, successfully suppress the Hg induced up-regulation of phospho-ERK 1/2, phosphor-JNK phospho- p38, NF-κB and phospho-IKKα.

Apoptosis, a phenomenon of programmed cell death, is a dynamic cellular process of gene-directed self-destruction in which cells ultimately die in a controlled way either spontaneously or in response to a variety of environmental stimuli or chemicals and mitochondria play an important role in regulating cell death pathways (Green and Reed 1998; Green and Kroemer 2004). Oxidative stresses and ROS are predominantly produced in mitochondria and play a significant role in apoptosis. The balance between the up and down regulations of the members of proapoptotic (like Bad) and antiapoptotic (like Bcl-2) Bcl-2 family proteins controls the destiny of the cells either to undergo apoptosis or to survive in extreme stressed conditions. Moreover, Bcl-2 family proteins are the upstream controllers of mitochondrial events like membrane potential ($\Delta\psi_m$); its damage can lead cell death through the release of cytochrome C into the cytosol. In the present study, we found that Hg down-regulated the expression of Bcl-2 and up-regulated the expression of Bad in hepatocytes (Fig. 7) in association with the reduction of mitochondrial membrane potential, enhancement of the release of cytochrome C, activation of caspases (caspase 3 and caspase 9) and a consequent modulation of PARP cleavage from its full-length form (116 kDa) to the cleaved form (84 kDa). Simultaneous treatment of glycine and Hg, on the other hand, effectively suppressed the activation of caspases, release of cytochrome C and improved mitochondrial membrane potential ($\Delta\psi_m$) through the mutual regulation of Bcl-2/Bad in Hg-induced hepatic pathophysiology.

In conclusion, the results of the present study, for the first time, demonstrate that glycine exerts its protective effect in Hg-induced hepatic oxidative stresses via its strong ROS scavenging and anti-oxidative properties**.** Moreover, glycine also acts as an antiapoptotic molecule by down-regulating the activation of phospho-ERK1/2, phospho-JNK, phospho-p38, NF-κB and other mitochondrial-dependent signalling molecules in addition to up-regulating the expression of Bcl-2 and down-regulating the expression of Bad.

Acknowledgments The authors are grateful to Mr. Prasanta Pal for excellent technical assistance for the study.

Conflict of interest The authors have declared that no conflict of interest exists.

References

Addaya S, Chakravarti K, Basu A, Santra M, Haldar S, Chatterjee GC (1984) Effects of mercuric chloride on several scavenging enzymes in rat kidney and influence of vitamin E supplementation. Acta Vitaminol Enzymol 6:103–107

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chang SI, Jin B, Youn P, Park C, Park JD, Ryu DY (2007) Arsenic-induced toxicity and the protective role of ascorbic acid in mouse testis. Toxicol Appl Pharmacol 218:196–203

Choi CH, Bark H, Chung JM, Park EK, Kim SH (2006) Elevated reactive oxygen species but not glutathione regulate mercury resistance to AML-2/DX100 cells. Immunopharmacol Immunotoxicol 28:545–555

Coccine Y, Randin G, Candura S, Nappi R, Prockop L, Luigi M (2000) Low level exposure of methyl mercury modifies muscarinic cholinergic receptor binding characteristic in rat brain and lymphocyte: physiologic implication and new opportunities in biological monitoring. Environ Health Perspect 108:29

Das J, Ghosh J, Manna P, Sil PC (2010) Protective role of taurine against arsenic-induced mitochondria-dependent hepatic apoptosis via the inhibition of PKCδ-JNK pathway. PLoS One 5:e12602; 1–19

Das J, Ghosh J, Manna P, Sinha M, Sil PC (2009a) Taurine protects rat testes against NaAsO(2)-induced oxidative stress and apoptosis via mitochondrial dependent and independent pathways. Toxicol Lett 187:201–210

Das J, Ghosh J, Manna P, Sinha M, Sil PC (2009b) Arsenic-induced oxidative cerebral disorders: Protection by taurine. Drug Chem Toxicol 32:93–102

Das J, Ghosh J, Manna P, Sil PC (2010b) Acetaminophen induced acute liver failure via oxidative stress and JNK activation: protective role of taurine by the suppression of cytochrome P450 2E1. Free Radic Res 44:340–355

Ellman GL (1959) Tissue sulphydryl group. Arch Biochem Biophy 82:70–77

Esterbauer H, Cheeseman KH (1990) Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. Method Enzymol 186:407–421

Feuerstein GZ, Young PR (2000) Apoptosis in cardiac diseases: stress- and mitogen activated signaling pathways. Cardiovasc Res 45:560–569

Ghosh J, Das J, Manna P, Sil PC (2010a) Protective effect of the fruits of Terminalia arjuna against cadmium-induced oxidant stress and hepatic cell injury via MAPK activation and mitochondria dependent pathway. Food Chem 123:1062–1075

Ghosh J, Das J, Manna P, Sil PC (2008) Cytoprotective effect of arjunalic acid in response to sodium fluoride mediated oxidative stress and cell death via necrotic pathway. Toxicol in Vitro 22:1918–1926

Ghosh J, Das J, Manna P, Sil PC (2009a) Taurine prevents arsenic-induced cardiac oxidative stress and apoptotic damage: Role of NF-κB, p38 and JNK MAPK pathway. Toxicol Appl Pharm 240:73–87

Ghosh J, Das J, Manna P, Sil PC (2009b) Arjunolic acid, a triterpenoid saponin, prevents acetaminophen (APAP)-induced liver and hepatocyte injury via the inhibition of APAP bioactivation and JNK-mediated mitochondrial protection. Free Radical Biol Med 48:535–553

Ghosh J, Das J, Manna P, Sil PC (2010b) Acetaminophen induced renal injury via oxidative stress and TNF-alpha production: Therapeutic potential of arjunolic acid. Toxicology 268:8–18

Ghosh A, Sil PC (2008) A Protein from *Cajanus indicus* Spreng Protects Liver and Kidney against Mercuric Chloride-Induced Oxidative Stress. Biol Pharm Bull 31:1651–1658

Ghosh A, Sil PC (2009) Protection of acetaminophen induced mitochondrial dysfunctions and hepatic necrosis via Akt-NF-kappaB pathway: role of a novel plant protein. Chem Biol Interact 177:96–106

Green DR, Kroemer G (2004) The pathophysiology of mitochondrial cell death. Science 305:626–629

Green DR, Reed JC (1998) Mitochondria and apoptosis. Science 281:1309–1312

Hansen JM, Zhang H, Hones DP (2006) Differential oxidation of thioredoxin-1, thioredoxin-2, and glutathione by metal ions. Free Radic Biol Med 40:138–145

Hissin PJ, Hilf RA (1976) A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 74:214–226

Jezek P, Hlavata L (2005) Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. Int J Biochem Cell Biol 37:2478–2503

Kaur P, Aschner M, Syversen T (2006) Glutathione modulation influences methyl mercury induced neurotoxicity in primary cell cultures of neurons and astrocytes. Neurotoxicology 27:492–500

Korashy MH, El-Kadi AOS (2008) The role of redox-sensitive transcription factors NF-κB and AP-1 in the modulation of the Cyp1a1 gene by mercury, lead, and copper. Free Radic Biol Med 44:795–806

Lee S, Cha M, Kang C, Sohn ET, Lee H, Munawir A, Kim JS, Kim E (2009) Mutual synergistic toxicity between environmental toxicants: a study of mercury chloride and 4-nonylphenol. Environ Toxicol pharmacol 27:90–95

Madesh M, Balasubramanian KA (1997) A microlitre plate assay for superoxide using MTT reduction method. Indian J Biochem Biophys 34:535–539

Manna P, Sinha M, Sil PC (2007a) Arjunolic acid, a triterpenoid saponin, ameliorates arsenic-induced cyto-toxicity in hepatocytes. Chem Biol Interact 170:187–200

Manna P, Sinha M, Sil PC (2007b) Protection of arsenic-induced hepatic disorder by arjunolic acid. Basic Clin Pharmacol Toxicol 101:333–338

Manna P, Sinha M, Sil PC (2008a) Protection of arsenic-induced testicular oxidative stress by arjunolic acid. Redox Report 13:67–77

Manna P, Sinha M, Sil PC (2008b) Arsenic induced oxidative myocardial injury: protective role of arjunolic acid. Arch Toxicol 82:137–149

Manna P, Sinha M, Sil PC (2009a) Prophylactic role of arjunolic acid in response to streptozotocin mediated diabetic renal injury: activation of polyol pathway and oxidative stress responsive signaling cascades. Chem Biol Interact 181:297–308

Manna P, Sinha M, Sil PC (2009b) Protective role of arjunolic acid in response to streptozotocin-induced type-I diabetes via the mitochondrial dependent and independent pathways. Toxicology 257:53–63

Manna P, Ghosh J, Das J, Sil PC (2010a) Contribution of type 1 diabetes to rat liver dysfunction and cellular damage via activation of NOS, PARP, IκBα/NF-κB, MAPKs, and mitochondria-dependent prophylactic role of arjunolic acid. Free Radic Biol Med 48:1465–1484

Manna P, Ghosh J, Das J, Sil PC (2010b) Streptozotocin induced activation of oxidative stress responsive splenic cell signaling pathways: protective role of arjunolic acid. Toxicol Appl Pharmacol 244:114–129

Mingatto FE, Rodrigues T, Pigoso AA, Uyemura SA, Curti C, Santos AC (2003) The critical role of mitochondrial energetic impairment in the toxicity of nimesulide to hepatocytes. J Pharm Exp Therap 303:601–607

Pourahmad J, O'Brien PJ, Jokar F, Daraei B (2003) Carcinogenic metal induced sites of reactive oxygen species formation in hepatocytes. Toxicol Vitro 17:808–810

Roy A, Manna P, Sil PC (2009) Prophylactic role of taurine on arsenic mediated oxidative renal dysfunction via MAPKs/ NF-kappaB and mitochondria dependent pathways. Free Radic Res 43:995–1007

Sarkar K, Sil PC (2006) A 43 kDa protein from the herb *Cajanus indicus* L. protects thioacetamide induced cytotoxicity in hepatocytes. Toxicol In Vitro 20:634–640

Sarkar K, Ghosh A, Kinter M, Mazumder B, Sil PC (2006) Purification and characterization of a 43kD hepatoprotective protein from the herb *Cajanus indicus* L. Protein J 25:411–421

Sarkar K, Sil PC (2007) Attenuation of acetaminophen-induced hepatotoxicity *in vivo* and *in vitro* by a 43 kD protein from the herb *Cajanus indicus* L. Toxicol Mech Methods 17:305–315

Sarkar MK, Kinter M, Mazumder B, Sil PC (2009) Purification and characterization of a novel antioxidant protein molecule from *Phyllanthus niruri*. Food Chem 114:1405–1412

Sarkar MK, Sil PC (2010) Prevention of tertiary butyl hydroperoxide induced oxidative impairment and cell death by a novel antioxidant protein molecule isolated from the herb, *Phyllanthus niruri*. Toxicol In Vitro 24:1711–1719

Schulze-Osthoff K, Ferrari D, Riehemann K, Wesselborg S (1997) Regulation of NF-κB activation by MAP kinase cascades. Immunobiology 198:35–49

Sedlak J, Lindsay RH (1968) Estimation of total, protein-bound, and non protein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 24(25):192–205

Singh V, Joshi D, Shrivastava S, Shukla S (2007) Effect of monothiol along with antioxidant against mercury-induced oxidative stress in rat. Indian J Expt Biol 45:1037–1044

Sinha M, Manna P, Sil PC (2007a) Taurine, a conditionally essential amino acid, ameliorates arsenic-induced cytotoxicity in murine hepatocytes. Toxicol In Vitro 21:1419–1428

Sinha M, Manna P, Sil PC (2007b) Attenuation of cadmium chloride induced cytotoxicity in murine hepatocytes by a protein isolated from the leaves of the herb *Cajanus indicus* L. Arch Toxicol 81:397–406

Sinha M, Manna P, Sil PC (2008) Protective effect of arjunolic acid against arsenic-induced oxidative stress in mouse brain. J Biochem Mol Toxicol 22:15–26

Uchida K, Stadtman ER (1993) Covalent attachment of 4-hydroxynonenal to glyceraldehydes-3-phosphate dehydrogenase. J Biol Chem 268:6388–6393

Wheeler MD, Ikejema K, Enomoto N, Stacklewitz RF, Seabra V, Zhong Z (1999) Glycine: a new anti-inflammatory immunonutrient. Cell Mol Life Sci 56(9–10):843–856

Yamauchi H, Aminaka Y, Yoshida K, Sun G, Pi J, Waalkes MP (2004) Evaluation of DNA damage in patients with arsenic poisoning: urinary 8-hydroxydeoxyguanine. Toxicol Appl Pharmacol 198:291–296

Yamanaka K, Hesegwa A, Sawamuna R, Okada S (1991) Cellular response to oxidative damage in lung induced by the administration of dimethylarsinic acid, a major metabolite of inorganic arsenics, in mice. Toxicol Appl Pharmacol 108:205–213

Zhong Z, Jones S, Thurman RG (1996) Glycine minimizes reperfusion injury in a low-flow, reflow liver perfusion model in the rat. Am J Physiol 270:G332–G338

Zhu H, Bannenberg GL, Moldéus P, Shertzer HG (1994) Oxidation pathways for the intracellular probe 2', 7'-dichlorofluorescein. Arch Toxicol 68:582–587