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The cytotoxic mechanism of glyoxal involves oxidative stress

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Abstract

Glyoxal is a reactive α-oxoaldehyde that is a physiological metabolite formed by lipid peroxidation, ascorbate autoxidation, oxidative degradation of glucose and degradation of glycated proteins. Glyoxal is capable of inducing cellular damage, like methylglyoxal (MG), but may also accelerate the rate of glycation leading to the formation of advanced glycation end-products (AGEs). However, the mechanism of glyoxal cytotoxicity has not been precisely defined. In this study we have focused on the cytotoxic effects of glyoxal and its ability to overcome cellular resistance to oxidative stress. Isolated rat hepatocytes were incubated with different concentrations of glyoxal. Glyoxal by itself was cytotoxic at 5 mM, depleted GSH, formed reactive oxygen species (ROS) and collapsed the mitochondrial membrane potential. Glyoxal also induced lipid peroxidation and formaldehyde formation. Glycolytic substrates, e.g. fructose, sorbitol and xylitol inhibited glyoxal-induced cytotoxicity and prevented the decrease in mitochondrial membrane potential suggesting that mitochondrial toxicity contributed to the cytotoxic mechanism. Glyoxal cytotoxicity was prevented by the glyoxal traps d-penicillamine or aminoguanidine or ROS scavengers were also cytoprotective even when added some time after glyoxal suggesting that oxidative stress contributed to the glyoxal cytotoxic mechanism.

Keywords: α-Oxoaldehydes; Glyoxal; Oxidative stress; Cytotoxicity; Lipid peroxidation; Reactive oxygen species

1. Introduction

Glyoxal is a reactive α-oxoaldehyde and a physiologic metabolite, formed by lipid peroxidation, ascorbate autoxidation, oxidative degradation of glucose, and degradation of glycated proteins [1,2]. Glyoxal like other α-oxoaldehydes, methylglyoxal (MG) and 3-deoxyglucosone, is also capable of inducing cellular damage and protein glycation leading to advanced glycation end-products (AGEs) which, in turn, may also contribute to cytotoxicity. These glycation reactions are also known as Maillard reactions which can impair the function of biological molecules such as proteins, nucleotides, and lipids [3,4]. As carbonyl groups are involved in all of these reactions, the term ''carbonyl stress'' has been suggested for this type of cellular stress [3].

The glycation of nucleotides, lipids, and proteins is suppressed under normal physiological states by the enzymatic detoxification of α-oxoaldehydes [5]. Glyoxal and MG are detoxified by cytosolic glyoxalase system to form glyoxal to glycolate and MG to d-lactate, respectively. The glyoxalase system consists of two enzymes, glyoxalase I and glyoxalase II, and glutathione (GSH) and involves the following three reactions in which the rate of glyoxalase I activity in situ is approximately proportional to the cytosolic concentration of GSH [5].

$$\mathrm{RCOCHO} + \mathrm{GSH} = \underset{\text{hemiacetal}}{\mathrm{RCOCH(OH)\text{-}SG}} \quad (\text{non-enzymatic addition of GSH}) \qquad (1)$$

$$\mathrm{RCOCH(OH)\text{-}SG} \xrightarrow{\text{glyoxalase I}} \underset{S\text{-2-hydroxyacylglutathione}}{\mathrm{RCH(OH)CO\text{-}SG}} \qquad (2)$$

$$\mathrm{RCH(OH)CO\text{-}SG} + \mathrm{H_2O} \xrightarrow{\text{glyoxalase II}} \underset{\text{aldonates}}{\mathrm{RCH(OH)CO_2^-}} + \mathrm{GSH} + \mathrm{H^+} \qquad (3)$$

Another detoxification pathway for glyoxal is through reduction catalyzed by aldehyde reductase (AKR1), aldose reductase (AKR2) and carbonyl reductase (CR) [6,7].

A decrease in cellular GSH concentration during oxidative stress subsequently increases intracellular levels of

Abbreviations: MG, methylglyoxal; AGEs, advanced glycation end-products; GSH, glutathione; GR, glutathione reductase; ROS, reactive oxygen species

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glyoxal/MG and AGE formation [5]. Under these conditions intracellular proteins react with glyoxal and MG and form AGEs [5,8,9]. Protein adduct formation by glyoxal and MG can result in the inactivation of critical cellular proteins that can potentially lead to apoptosis or necrosis or cell growth arrest [5,10–12]. In vivo AGE formation has been linked to the development of disease especially the chronic clinical complications associated with diabetes mellitus such as retinopathy, neuropathy and nephropathy [12–14]. Furthermore, diseases such as cataract, atherosclerosis, kidney failure and Alzheimer's disease have also been associated with the formation of AGE in vivo [12–15].

Glyoxal originates from pathways that have been linked to various oxidative stress pathologies such as glucose autoxidation, DNA oxidation and lipid peroxidation [1–3,15]. However, the mechanism of glyoxal cytotoxicity has not been determined. Previously we showed that glyoxal at low concentrations markedly increases the susceptibility of hepatocytes to hydrogen peroxide (H_2O_2) [16]. In the following study we have investigated the cytotoxic mechanism of glyoxal. Glyoxal cytotoxicity occurred following the loss of hepatocytes mitochondrial membrane potential, GSH oxidation and reactive oxygen species (ROS) formation. Glycolytic substrates, e.g. fructose, sorbitol and xylitol or ROS scavengers inhibited glyoxal-induced cytotoxicity and prevented the decrease in mitochondrial membrane potential suggesting that mitochondrial toxicity and oxidative stress contributed to the cytotoxic mechanism.

2. Materials and methods

1-Bromoheptane, glyoxal (40%), MG, xylitol, 2,4-dinitrofluorobenzene (DNFB), cumene hydrogen peroxide (CHP), dichlorofluorescin diacetate (DCFD), rhodamine 123 and all other chemicals were purchased from Sigma Chemical Co. Type II Collagenase was purchased from Worthington.

2.1. Animal treatment and hepatocyte preparation

Male Sprague–Dawley rats weighing 275–300 g (Charles River Laboratories) were housed in ventilated plastic cages over PWI 8–16 hardwood bedding. There were 12 air changes per hour, 12 h light photoperiod (lights on at 08:00 h) and an environmental temperature of 21–23 °C with a 50–60% relative humidity. The animals were fed with a normal standard chow diet and water ad libitum. Hepatocytes were isolated from rats by collagenase perfusion of the liver as described by Moldéus and coworkers. Isolated hepatocytes (10^6 cells/ml) (10 ml) were suspended in Krebs–Henseleit buffer (pH 7.4) containing 12.5 mM HEPES in continually rotating 50 ml round-bottomed flasks, under an atmosphere of 95% O_2 and 5% CO_2 in a water bath of 37 °C for 30 min [17]. Stock solutions of chemicals were made in H_2O, dimethylsulfoxide (DMSO), or methanol. GSH depleted hepatocytes were produced by pre-incubating the flasks for 30 min with 1-bromoheptane (200 μM) and sorbinil (200 μM).

2.2. Cell viability

Hepatocyte viability was assessed microscopically by plasma membrane disruption as determined by trypan blue (0.1% w/v) exclusion test [18]. Hepatocyte viability was determined every 30 min during the 3-h incubation, and the cells were at least 80–90% viable before their use.

2.3. Quantitation of cellular glutathione (GSH) and oxidized glutathione (GSSG) content

GSH and GSSG were measured by HPLC analysis of deproteinized samples (25% meta-phosphoric acid) after derivatization with iodoacetic acid and 2,4-dinitrofluorobenzene (DNFB) as per the method outlined by Reed et al. [19]. A Waters HPLC system (Model 150 pumps, WISP 710B auto injector and model 410 UV–vis detector) equipped with waters μBondapak® NH_2 (10 μm) 3.9 mm × 300 mm column was used.

2.4. Determination of reactive oxygen species (ROS)

To determine the rate of hepatocyte ROS generation induced by α-oxoaldehydes dichlorofluorescin diacetate (DCFD) was added to the hepatocyte incubate. DCFD penetrates hepatocytes and becomes hydrolyzed to form non-fluorescent dichlorofluorescin. Dichlorofluorescin then reacts with 'ROS' to form the highly fluorescent dichlorofluorescein that effluxes the cell. One-milliliter samples were withdrawn at 25, 75 and 165 min after incubation with α-oxoaldehydes. These samples were then centrifuged for 1 min at 50 × *g*. The cells were resuspended in H_2O and then 1.6 μM DCFD was added to the cells. The cells were allowed to incubate at 37 °C for 10 min. The fluorescence intensity of ROS product was measured using a Shimadzu RF5000U fluorescence spectrophotometer. Excitation and emission wavelengths were 500 and 520 nm, respectively [20].

2.5. Determination of glyoxal metabolism

Glyoxal content was measured using a variation on the lipid peroxidation assay thiobarbituric acid reactive substances (TBARS) [21]. Samples were taken from hepatocyte incubations at times of analysis. Two hundred and fifty microliters tricholoracetic acid (TCA) was used to stop the reaction and lyse the cells. The samples were then vortexed and centrifuged at high speed for 3 min. An equal volume of 0.8% thiobarbituric acid (TBA) was added to the

samples and then boiled for 20 min. The absorbance was read at 555 nm.

2.6. Determination of glutathione reductase (GR) activity

The GR assay method designed by Vander Jagt et al. was used in this study, with some modifications [22]. The cytosolic fraction of the rat hepatocytes was used to observe the effect of treatment with glyoxal. This was done by monitoring changes in the oxidation of NADPH to $NADP^+$ at 340 nm. Glutathione reductase activity was assayed in a 2-ml reaction mixture consisting of 0.1 M potassium phosphate buffer (pH 7, containing 2 mM Na_2-EDTA), 30 μl microsomes ± various concentrations of glyoxal incubated at 37 °C for up to 6 h. At each time point, a 100-μl aliquot was removed and placed in a cuvette containing 900 μl of buffer to which 0.1 mM NADPH and 50 μM GSSG were added. The rate of NADPH oxidation was then followed at 340 nm using a Shimadzu UV–vis spectrophotometer [13].

2.7. Mitochondrial membrane potential assay

The uptake of cationic fluorescent dye, rhodamine 123, has been used for the estimation of mitochondrial membrane potential. Five hundred microliters samples were taken from the cell suspension incubating at 37 °C and centrifuged at 1000 rpm for 1 min. The cell pellet was then resuspended in 2 ml of fresh incubation medium containing 1.5 μM rhodamine 123 and incubated at 37 °C in a thermostatic bath for 10 min with gentle shaking. Hepatocytes were then separated by centrifugation and the amount of rhodamine 123 remaining in the incubation medium was measured fluorimeterically using Shimadzu RF5000U fluorescence spectrophotometer set at 490 nm excitation and 520 nm emission wavelengths. The capacity of mitochondria to take up the rhodamine 123 was calculated as the difference (between control and treated cells) in rhodamine 123 fluorescence [20].

2.8. Lipid peroxidation assay

Glyoxal reacted with TBA to form pink products and absorbs at 555 nm. Lipid peroxidation was therefore assayed by measuring the formation of formaldehyde, a lipid peroxide decomposition product [23]. Formaldehyde formation was determined colorimetrically using NASH's reagent. This reagent contained a mixture of 4 M ammonium acetate (10 ml), 1 M acetic acid (1 M), 1 M acetyl acetone (400 μl DMSO) and Millipore water (8.6 ml) [24,25]. One-milliliter samples were withdrawn at 15, 60, 180 min from the hepatocyte flasks. Fifty-six microliters of 30% TCA was added to the samples and then vortexed. The mixture was then centrifuged for 1 min at top speed to precipitate the cells. Five hundred microliters of the supernatant was then added to 500 μL of NASH's reagent. The solution was then vortexed and incubated for 60 min at 37 °C with shaking. The formaldehyde levels of the mixture were determined at 412 nm using a Beckman DU-7 spectrophotometer.

Table 1
Glyoxal increases hepatocyte cytotoxicty, ROS and formaldehyde production while decreasing its metabolism

Compounds added	Percent cytotoxicity (trypan blue uptake)			Lipid peroxidation (absorbance at 412 nm)	Percent glyoxal remaining
	60 min	120 min	180 min	180 min	60 min
Control-hepatocytes	26 ± 3	25 ± 1	32 ± 4	0.103 ± 0.020	0
+Glyoxal 2 mM	26 ± 3	28 ± 3	28 ± 5	0.246 ± 0.012	4 ± 2
+Sorbinil 200 μM	33 ± 4	43 ± 7	98 ± 2	0.489 ± 0.023	54 ± 3
+Glyoxal 3 mM	28 ± 3	31 ± 3	38 ± 5	0.256 ± 0.022	16 ± 5
+Glyoxal 4 mM	29 ± 3	37 ± 3	49 ± 5	0.250 ± 0.018	22 ± 3
+Glyoxal 5 mM	42 ± 2[a]	51 ± 6[a]	98 ± 2[a]	0.526 ± 0.023[a]	92 ± 1[a]
+BHA 50 μM	27 ± 3[b]	31 ± 2[b]	33 ± 4[b]	0.153 ± 0.008[b]	15 ± 5[b]
+Quercetin 100 μM	29 ± 5[b]	27 ± 4[b]	35 ± 2[b]	0.172 ± 0.012[b]	20 ± 6[b]
+Desferoxamine 300 μM	24 ± 4[b]	33 ± 3[b]	38 ± 6[b]	0.169 ± 0.012[b]	18 ± 4[b]
+Xylitol 10 mM	33 ± 4[b]	34 ± 6[b]	37 ± 3[b]	0.183 ± 0.014[b]	47 ± 3[b]
+Methylglyoxal 10 mM	27 ± 4	34 ± 3	37 ± 5	0.208 ± 0.031	–
+Sorbinil 200 μM	41 ± 5[c]	48 ± 3[c]	59 ± 6[c]	0.349 ± 0.029[c]	–
GSH depleted cells	25 ± 3	26 ± 3	29 ± 3	0.178 ± 0.017	4 ± 2
+Glyoxal 2 mM	28 ± 4[d,e]	32 ± 4[d,e]	100[d,e]	0.899 ± 0.042[d,e]	91 ± 3[d,e]

$n = 3$.
[a] Significant difference as compared to control ($P < 0.005$).
[b] Significant difference as compared to glyoxal 5 mM ($P < 0.05$).
[c] Significant difference as compared to methylglyoxal 10 mM ($P < 0.005$).
[d] Significant difference as compared to GSH depleted cells ($P < 0.05$).
[e] Significant difference as compared to glyoxal 2 mM ($P < 0.02$).

2.9. Statistical analysis

Statistical analysis was performed by the use of one-way ANOVA. A probability of less than 0.05 was considered significant.

3. Results

3.1. Glyoxal cytotoxicity

As shown in Table 1 the addition of glyoxal to hepatocytes caused lipid peroxidation and cytotoxicity as the glyoxal concentration increased. Lipid peroxidation and cytotoxicity was markedly increased by sorbinil, an aldehyde reductase inhibitor, or by depleting GSH beforehand. Lipid peroxidation and cytotoxicity were prevented by butylated hydroxyanisole (BHA), an antioxidant, quercetin (ROS scavenger), desferoxamine (iron chelator) and xylitol (a glycolytic substrate that generates NADH). MG was not cytotoxic but became cytotoxic in the presence of sorbinil. Hepatocyte glyoxal metabolism was inhibited as the glyoxal concentration increased and was markedly inhibited by sorbinil or GSH depletion. Glyoxal metabolism was increased by BHA, desferoxamine or xylitol.

3.2. Measurement of ROS

Glyoxal-induced hepatocytes ROS production, which increased with time and increasing doses of glyoxal (Fig. 1). ROS production was inhibited by antioxidants such as BHA and quercetin.

3.3. Cellular GSH depletion and GSSG formation

As shown in Fig. 2 glyoxal-induced hepatocyte GSH depletion and GSSG formation, which was dependent on the glyoxal concentration. With 0.5 mM glyoxal hepatocytes GSH levels started to recover and GSSG levels decreased at later time points whereas GSH recovery was not seen at higher concentrations of glyoxal. The control sample was not treated with any chemicals and little GSH depletion occurred over the incubation period of 135 min.

3.4. Inhibition of cytosolic GR by glyoxal

Cytosolic GR was initially inhibited rapidly by glyoxal followed by a much slower phase. This increased as the concentration of glyoxal increased (Fig. 3). Glyoxal at 0.5 mM inhibited GR slightly (20%) while 3 mM was required to inhibit 50% of GR activity.

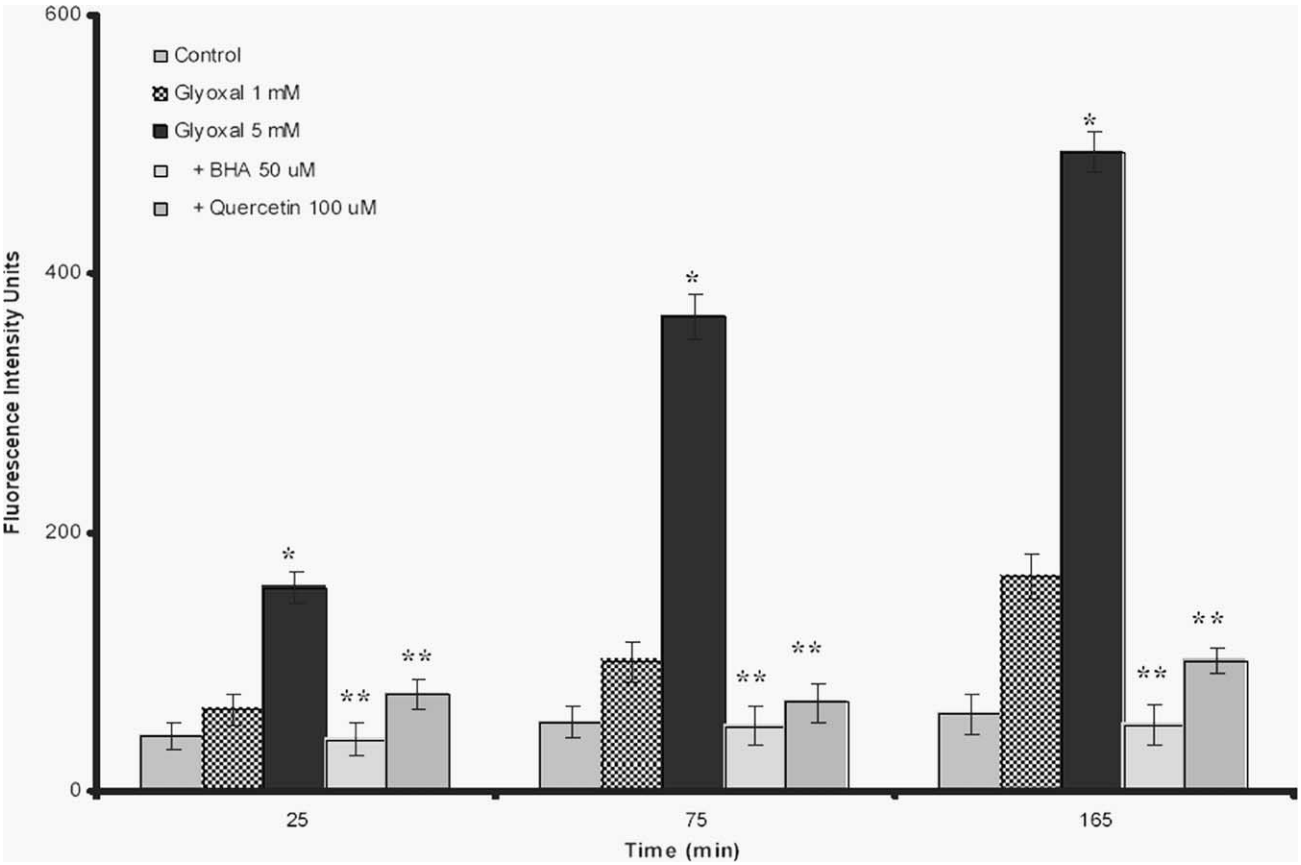


Fig. 1. Glyoxal-induced hepatocyte reactive oxygen species (ROS) production is inhibited by antioxidants. $n = 3$, significant as compared to the control ($^{*}P <$ 0.002) and significant as compared to glyoxal 5 mM ($^{**}P < 0.05$).

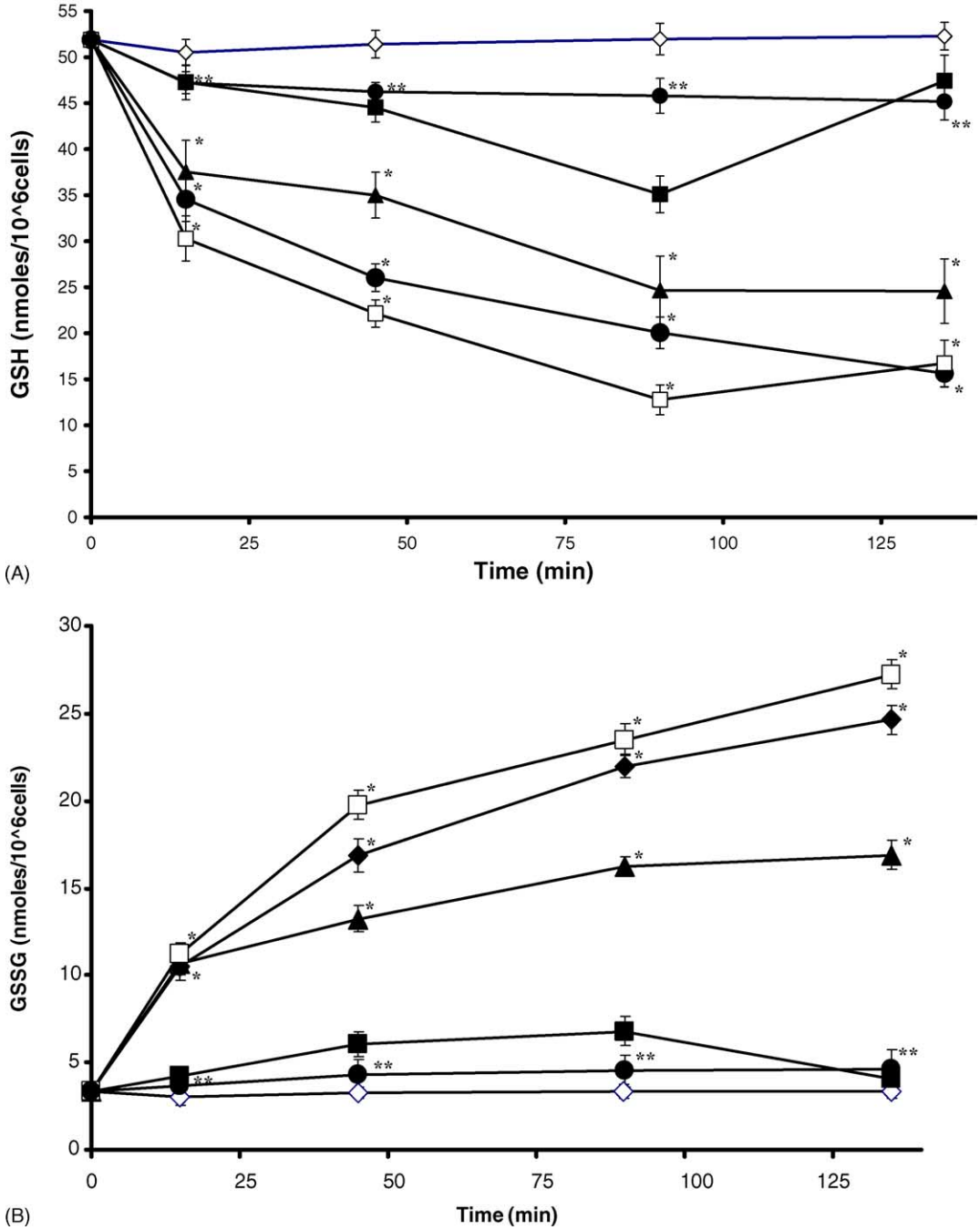


Fig. 2. Glyoxal-induced hepatocyte GSH oxidation is prevented by an antioxidant. (A) Intracellular GSH levels under different glyoxal concentrations: (■) 0.5 mM, (▲) 1 mM, (◆) 5 mM, (□) 10 mM, (◇) control-intracellular GSH and treatment of (●) 10 mM glyoxal with BHA 50 μM. (B) Extracellular GSSG levels under different glyoxal concentrations: (■) 0.5 mM, (▲) 1 mM, (◆) 5 mM, (□) 10 mM, (◇) control-extracellular GSSG and after treatment of (●) 10 mM glyoxal with BHA 50 μM. $n = 3$, significant as compared to control ($^{*}P < 0.05$) and significant as compared to glyoxal 10 mM ($^{**}P < 0.05$).

3.5. Decrease in hepatocyte mitochondrial potential

As shown in Fig. 4 glyoxal above a 2 mM concentration decreased the hepatocytes mitochondrial membrane potential. This was dose dependent and occurred rapidly before cytotoxicity ensued. The decrease in mitochondrial membrane potential was prevented by BHA and quercetin.

3.6. Inhibition of glyoxal cytotoxicity

As shown in Table 2 cytotoxicity was prevented by glyoxal traps. The most cytoprotective agent was d-penicillamine 10 mM. Aminoguanidine 20 mM, arginine 20 mM, cysteine 10 mM and pyridoxamine 10 mM were also effective. These trapping agents also prevented GSH

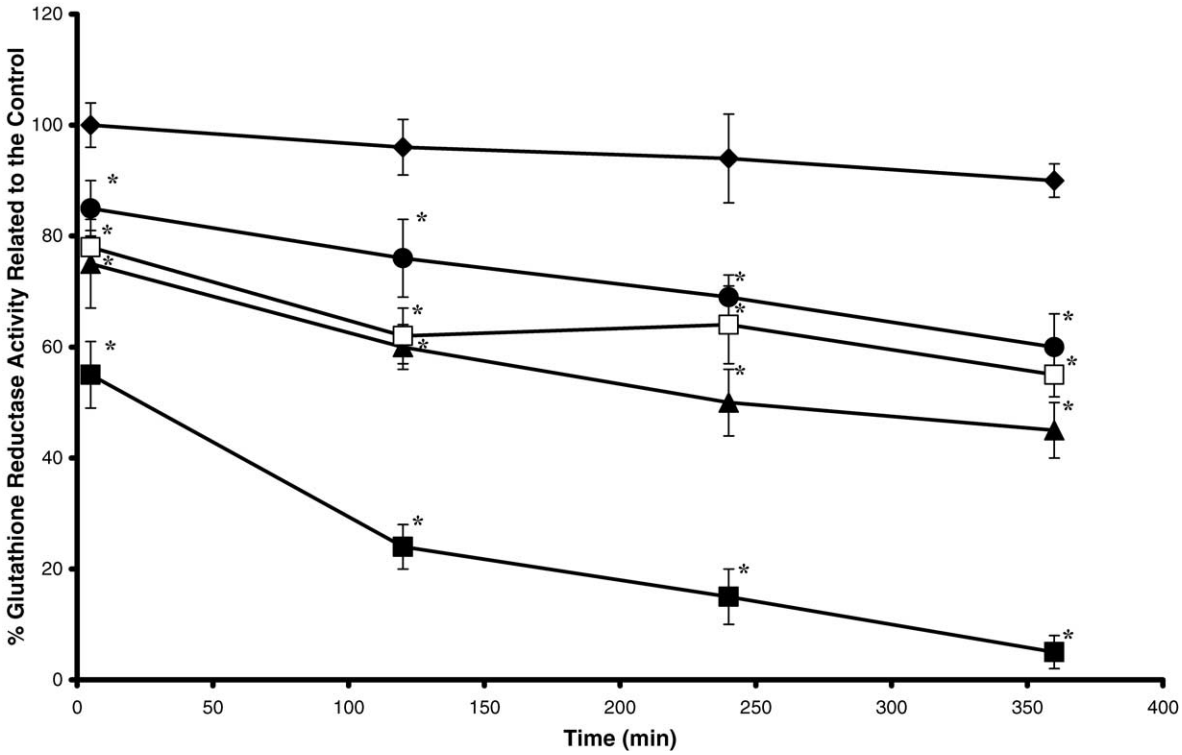


Fig. 3. The dose-dependent inhibition of cytosolic glutathione reductase (GR) by glyoxal. Doses of glyoxal: (•) 0.1 mM, (□) 1 mM, (▲) 3 mM, (■) 5 mM, (◆) control. $n = 3$, significant compared to control ($^{*}P < 0.05$).

depletion and lipid peroxidation. Aminoguanidine was the best glyoxal trap in terms of increasing glyoxal metabolism and preventing cytotoxicity and GSH depletion. Glyoxal cytotoxicity was also prevented by the glycolytic ATP generators, xylitol or fructose. Ethanol, an NADH generator, also protected the hepatocytes. Xylitol also prevented glyoxal from causing hepatocytes GSH oxidation.

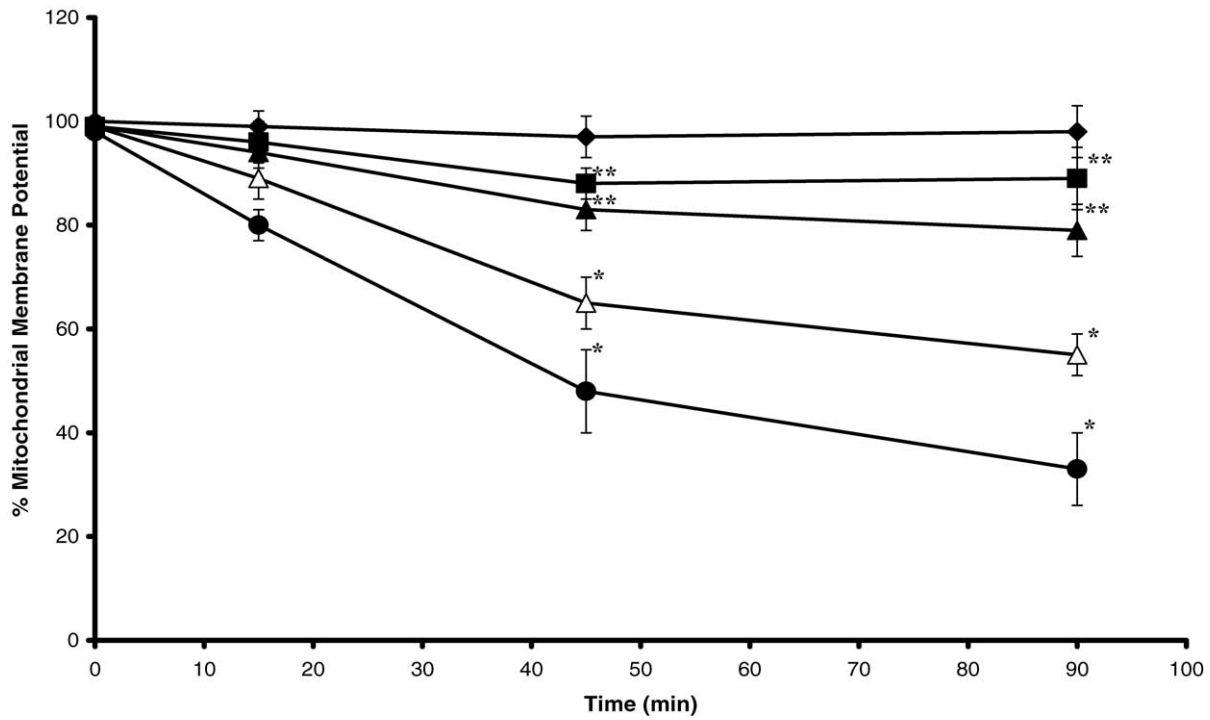


Fig. 4. Glyoxal-induced hepatocyte mitochondrial toxicity and its dependence on glyoxal concentration. Doses of glyoxal: (□) 5 mM, (•) 8 mM, (◆) control. Eight millimolar glyoxal was treated with (■) BHA 50 μM and (▲) quercetin 100 μM. $n = 3$, significant difference as compared to control ($^{*}P < 0.05$) and significant difference as compared to glyoxal 8 mM ($^{**}P < 0.02$).

Table 2
Glyoxal cytotoxicity is prevented by ATP/NAD(P)H generators, thiamin and glyoxal traps (aminoguanidine, arginine, pyridoxamine, penicillamine)

Compounds added	Percent cytotoxicity (trypan blue uptake)		Percent hepatocyte GSH	Percent glyoxal remaining	Lipid peroxidation (absorbance at 412 nm)
	120 min	180 min	120 min	60 min	180 min
Control	25 ± 1	32 ± 4	98 ± 2	0	0.103 ± 0.020
+Glyoxal 5 mM	51 ± 6[a]	98 ± 2[a]	13 ± 4[a]	92 ± 1[a]	0.526 ± 0.023[a]
+Ethanol 10 mM	28 ± 5[b]	46 ± 4[b]	64 ± 3[b]	77 ± 3	0.210 ± 0.021[b]
+Fructose 10 mM	33 ± 4[b]	48 ± 5[b]	73 ± 5[b]	76 ± 4	0.243 ± 0.017[b]
+Aminoguanidine 20 mM	32 ± 2[b]	35 ± 5[b]	67 ± 3[b]	6 ± 4[b]	0.152 ± 0.009[b]
+Arginine 20 mM	35 ± 4[b]	39 ± 2[b]	70 ± 3[b]	43 ± 3[b]	0.189 ± 0.016[b]
+Pyridoxamine 10 mM	31 ± 4[b]	36 ± 4[b]	75 ± 4[b]	24 ± 5[b]	0.289 ± 0.018[b]
+Penicillamine 10 mM	28 ± 3[b]	26 ± 4[b]	64 ± 5[b]	61 ± 3	0.212 ± 0.022[b]
+Cysteine 10 mM	38 ± 4[b]	42 ± 5[b]	39 ± 4	72 ± 6	0.321 ± 0.023

$n = 3$.
[a] Significant difference as compared to control ($P < 0.05$).
[b] Significant difference as compared to glyoxal 5 mM ($P < 0.05$).

4. Discussion

While isolated rat hepatocytes were resistant to MG (10 mM), hepatocyte cytotoxicity was induced by glyoxal (5 mM). Glyoxal induced much more cytototxicity in GSH depleted hepatocytes than control. Glyoxal metabolism was also slower in GSH depleted hepatocytes than control hepatocytes. Glyoxal cytotoxicity was preceded by lipid peroxidation and ROS which was prevented by the antioxidant BHA. This suggests ROS formation was due to lipid peroxidation induced by glyoxal. Furthermore, BHA prevented glyoxal-induced cytotoxicity which suggests that lipid peroxidation contributed to the cytotoxicity. A collapse of the hepatocyte mitochondrial membrane potential also preceded the cytotoxicity and both were prevented by antioxidants.

Glyoxal cytotoxicity in isolated rat hepatocytes was dose and time dependent. As compared to other cell types hepatocytes were relatively resistant to glyoxal cytotoxicity. In lung epithelial cell line L132, 400 μM of glyoxal caused cytotoxicity and in E1A-NR3 cells 800 μM of glyoxal-induced cytotoxicity [3,15]. Glyoxal decreased mitochondrial membrane potential in a dose- and time-dependent fashion (Fig. 4). This decrease preceded cytotoxicity. It has been shown by Reber et al. in E1A-NR3 cell line that 800 μM glyoxal caused the mitochondria to become rounded, have blebs and move to the perinuclear region of the cell [15]. Thus, the cytotoxic mechanism of glyoxal involves a decrease in the mitochondrial membrane potential which can result in the opening of the mitochondrial permeability transition pore. The opening of the mitochondrial permeability transition pore results in the non-selective increase in the permeability of the inner membrane. This results in the depolarization of the mitochondria and the loss of the H^+ gradient that is normally present across the inner membrane. The non-selective entry of water and other ions into the matrix causes an increase in the volume of the mitochondrium [26]. Cytotchrome *c*, which is normally stored between the inner and outer membranes of the mitochondria, gets released into the cytosol due to a change in mitochondrial potential. The release of cytochrome *c* into the cytosol causes activation of caspases as cytochrome *c* binds to Apaf-1 that results in the formation of an oligomeric complex that activates procaspase-9, an activated caspase-9 that then cleaves and activates other caspases downstream and ultimately results in cytotoxicity [27].

Furthermore, the remarkable resistance of hepatocytes to MG as compared to glyoxal can be explained as Speer et al. have recently shown that MG at 2 mM but not glyoxal is highly protective to isolated rat mitochondria by binding to an arginine of the permeability transition pore and keeping it closed so that it prevents calcium efflux-induced swelling. Additionally, it also prevents cytochrome *c* release [28]. Furthermore, other cells such as cortical neurons in culture required 130 μM MG for 24 h and leukemia HL60 cells required 238 μM for 24 h to cause cytotoxicity [29,30].

GSH depleted hepatocytes were more sensitive to glyoxal indicating that GSH is required for the detoxification of glyoxal by the glyoxalase system. Although the glyoxalase system results in the recovery of GSH because of the slower catalytic rate of glyoxalase II as compared to glyoxalase I, at high concentrations of glyoxal or MG GSH maybe trapped as *S*-2-hydroxyacylglutathione, resulting in GSH depletion in the liver [31,32]. GSH is also important in metabolism as it plays a protective role against ROS. It has also been shown that depletion in GSH facilitates lipid peroxidation which can initiate oxygen derivatives and induce cellular stress [32,33].

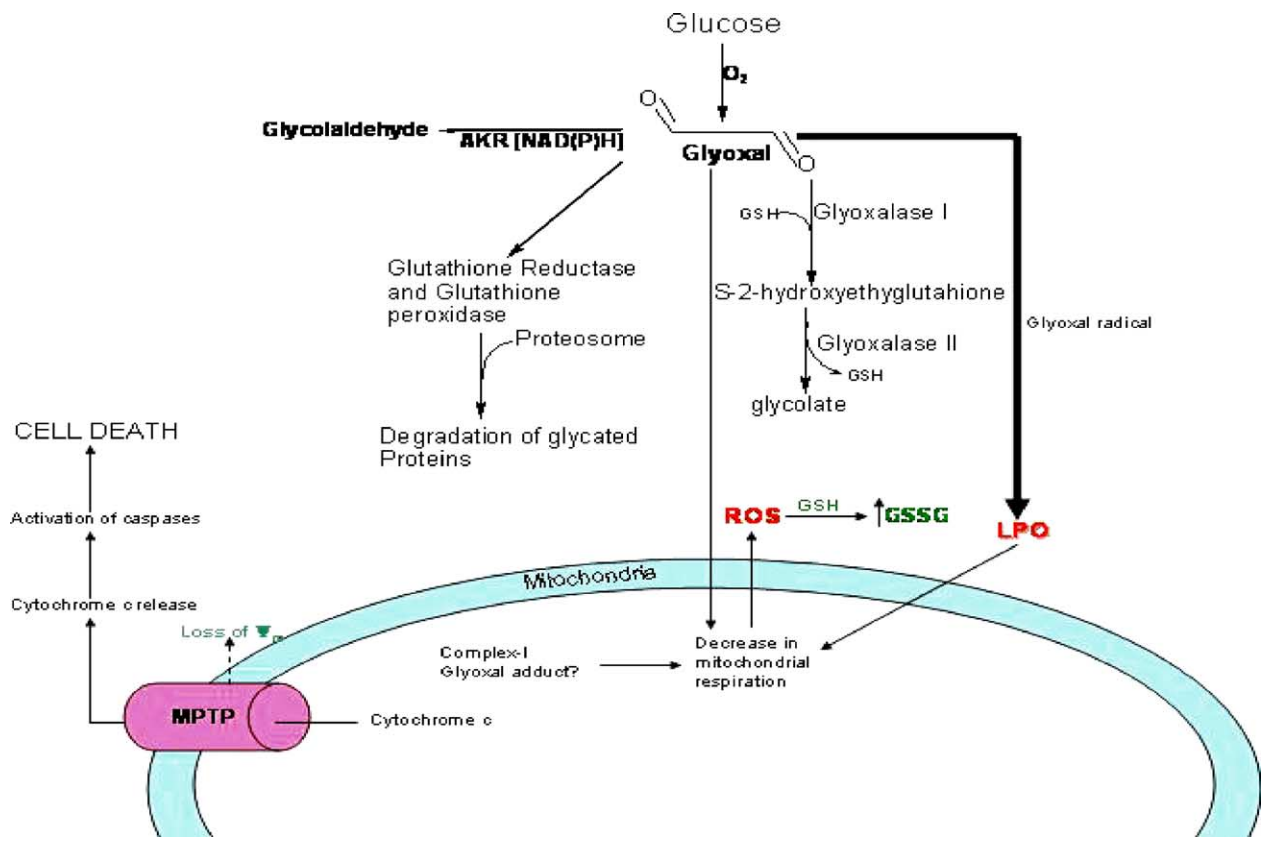
Glyoxal induced a dose- and time-dependent depletion of GSH and increased GSH oxidation formation of GSSG which was re-reduced to GSH at low glyoxal concentrations (Fig. 2). This GSH oxidation probably occurred as a result of the ROS formation caused by mitochondrial toxicity as previously demonstrated for formaldehyde [34]. As well as inhibition of enzymes involved in GSSG reduction such as GR and NADPH generating enzymes such as glucose-6-phosphate dehydrogenase (G6PDH) or

isocitrate dehydrogenase. In turn the GSH depletion would inhibit glyoxal detoxification by the glyoxalase system and by AKR. Therefore, to account for this GSSG increase glyoxal was incubated with a cytosolic fraction of hepatocytes and it was found that glyoxal inhibited GR thus making the cells more susceptible to oxidative stress. GR has two lysine (K48 and K56) residues and one arginine (R413) residue in its cavity at the dimer interface and its active site contains the redox active pair cysteine 39 and cysteine 44. Also present at the active site are lysine 32 which is one of the residues involved in binding FAD. The lysine, arginine and cysteine residues that are essential for enzyme function are probably targeted by glyoxal thus inhibiting the enzyme [35]. At high concentrations of glyoxal (90% of the enzyme activity was inhibited as compared to the control (Fig. 3). However, enzymes other than GR must also be inactivated by glyoxal.

At high glyoxal concentrations the metabolism of glyoxal was inhibited. This could be due to the inhibition of aldehyde reductases and NAD(P)H producing enzymes by glyoxal. A depletion of GSH by glyoxal causes a decrease in the activity of the glyoxalase system, thus inhibiting its major detoxification pathway. AKR inhibitors such as sorbinil increased glyoxal and MG cytotoxicity indicating the important role that these reductases play in metabolism. AKRs are NAD(P)H dependent; therefore, a decrease in NAD(P)H supply renders these enzymes inactive. Therefore, when xylitol (10 mM), an NADH generator, was added to the hepatocytes it had a protective effect. At high concentrations glyoxal could be effectively inhibiting its own metabolism by decreasing the cellular levels of GSH and inhibiting NADPH generating enzymes.

As mentioned earlier, GSH depletion can result in ROS and lipid peroxidation. We measured ROS formation and looked at the aldehydic decomposition products of lipid peroxidation, i.e. formaldehyde [34]. Glyoxal increased ROS in hepatocyte which was prevented by BHA. This suggests ROS formation was due to lipid peroxidation induced by glyoxal. Furthermore, BHA prevented glyoxal-induced cytotoxicity which suggests that lipid peroxidation contributed to the cytotoxicity.

Formaldehyde has also been shown to cause cytotoxicity by decreasing mitochondrial membrane potential, inhibiting respiratory complex I, interacting with the mitochondrial permeability transition pore to release cytochrome *c* from the mitochondria to the cytosol leading to apoptosis [34,36,37]. Xylitol prevented lipid peroxidation likely by the reductive metabolism of glyoxal or formaldehyde by AKR or aldehyde dehydrogenase (ADH), respectively (Table 1). We have also shown that trapping agents and ATP/NADH generators prevented glyoxal cytotoxicity (Table 2).



Scheme 1. Proposed mechanism of glyoxal cytotoxicity. ROS: reactive oxygen species; LPO: lipid peroxidation; GSH: glutathione; GSSG: oxidized glutathione; MPTP: mitochondrial permeability transition pore; AKR: aldose/ketose reductase.

Trapping agents like d-penicillamine and aminoguanidine reacted with glyoxal and prevented it from causing cellular damage (Table 2). Reaction of d-penicillamine and glyoxal under conditions of physiological temperature and pH converts glyoxal into a thiazolidine derivative [38]. Aminoguanidine scavenges glyoxal by forming a 1,2,4-triazine derivative and thereby prevents the formation of glyoxal-modified proteins [39]. While, the ATP generators provide further evidence of mitochondrial toxicity as they act as an alternate energy source and allow the cell to sustain its defense systems. Furthermore, these traps and energy generators prevented GSH depletion thus keeping the antioxidant function of cellular GSH intact and assuring that glyoxal was metabolised.

Scheme 1 shows our proposed mechanism of glyoxal cytotoxicity. The sequences of events that cause glyoxal to be cytotoxic are as follows: glyoxal causes lipid peroxidation that generates ROS, which leads to decrease in cellular GSH levels that in turn decreases glyoxalase I activity. Glyoxal likely inactivates GR, glutathione peroxidase, and enzymes that supply NAD(P)H. Glyoxal is likely also an inhibitor of the mitochondrial electron transport chain. Decrease in the respiratory chain activity leads to more ROS formation that leads to more lipid peroxidation. This causes GSH levels to decrease even further hindering the ability of the cell to combat oxidative stress. We hypothesize that glyoxal and/or lipid peroxidation aldehydic decomposition products could interact with the mitochondrial permeability transition pore to release cytochrome *c* and activate caspases that lead to programmed cell death (apoptosis).

The range of glyoxal concentrations used in this study were much higher than those found under physiological (∼12.5 μg/ml) and diabetic (∼27.2 μg/ml) conditions [40]. However, this is the first study that illustrates the role of oxidative stress in the mechanism of glyoxal toxicity. Knowing the mechanism of endogenous glyoxal formation and toxicity will enable us to develop novel drug therapies for complications associated with diabetes mellitus, cataract, Parkinson's disease and Alzeihmer's disease to name a few.

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