Research Article

Copper-catalyzed ascorbate oxidation results in glyoxal/AGE formation and cytotoxicity

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Previously we showed that 10 μM glyoxal compromised hepatocyte resistance to hydrogen peroxide (H_2O_2) by increasing glutathione (GSH) and NADPH oxidation and decreasing mitochondrial membrane potential (MMP) before cytotoxicity ensued. Since transition metal-catalyzed oxidation of ascorbate (Asc) has been shown to result in the generation of both glyoxal and H_2O_2, we hypothesized that glyoxal formation during this process compromises hepatocyte resistance to H_2O_2. We used isolated rat hepatocytes and incubated them with Asc/copper and measured cytotoxicity, glyoxal levels, H_2O_2, GSH levels, and MMP. To investigate the role of Asc/copper on glyoxal-BSA adducts, we measured the appearance of advanced glycation end-products (AGE) in the presence and absence of catalase or aminoguanidine (AG). Asc/copper increased glyoxal and H_2O_2 formation. Hepatocyte GSH levels were decreased and cytotoxicity ensued after a collapse of the hepatocyte MMP. Glyoxal traps protected hepatocytes against Asc/copper-induced cytotoxicity. In cell-free studies with BSA, incubation with Asc and copper resulted in glyoxal-hydroimidazolone formation, which was decreased by both AG and catalase. To the best of our knowledge, this is the first study that illustrates the importance of glyoxal production by transition metal-catalyzed Asc autoxidation. Understanding this mechanism of toxicity could lead to the development of novel copper chelating drug therapies to treat diabetic complications.

Keywords: Advanced glycation end-products / Ascorbate oxidation / Cataract / Glyoxal / Oxidative stress

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1 Introduction

The Maillard reaction or the nonenzymatic glycosylation of proteins has been implicated in age-related changes such as protein crosslinking and yellowing in collagen and crystalline lens [1]. Ascorbate (Asc; vitamin C) levels are high in the human lens and considerable interest has been focused on the potential role of Asc as a mediator of post-translational modifications of crystallins by the Maillard process and its possible role in the pathogenesis of cataract [2, 3]. The recommended daily intake of Asc is 60–95 mg [4]. However, Pauling [5] stated that the amount needed for an adult human to achieve equivalent blood serum levels of Asc in Asc-synthesizing mammals is 6000–18 000 mg. Dietary Asc can also be readily oxidized in foods during preparation, cooking at high temperatures, or storage [5, 6]. The richest natural sources are fruits and vegetables, especially citrus fruits such as oranges, lemon, and grapefruit. It is also present in some cuts of meat (especially liver). Asc is also the most widely consumed nutritional supplement and is available in a variety of formulations.

Asc is an excellent reducing agent and an antioxidant. To retain the highest levels of dietary Asc, foods must be served fresh. Asc oxidation is dependent on pH and requires the presence of catalytic metals such as iron and copper (Cu) [7]. At physiological pH, Asc can undergo transition metal-catalyzed autoxidation to produce H_2O_2 and dehydroAsc [7]. In addition, Asc incubated with a lens culture resulted in the formation of Maillard reaction products involving protein adduct formation and crosslinking [8]. This reaction involved the first oxidation product of Asc, dehydroAsc, which spontaneously hydrolyzed to form reac-

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Abbreviations: AG, aminoguanidine; **AGEs,** advanced glycation end-products; **Asc,** ascorbate; **DCFH-DA,** 2,7-dichlorofluorescin diacetate; **G-H1,** glyoxal-hydroimidazolone; **GSH,** glutathione; **PA,** pyridoxamine; **ROS,** reactive oxygen species

tive aldehyde species such as 2,3-diketogulonate [2, 9] and presumably glyoxal formed by an unknown pathway.

Glyoxal is a reactive dialdehyde that is formed from lipid peroxidation, glucose autoxidation, and DNA oxidation [10]. Glyoxal undergoes the Maillard reaction with amino and thiol groups of biomolecules to form adducts known as advanced glycation end-products (AGEs). These adducts have been implicated in complications associated with Diabetes Mellitus, Alzheimer's disease, and Parkinson's disease [11]. Glyoxal can be detoxified endogenously by the glyoxalase system, which converts glyoxal to glycolate in the presence of glutathione (GSH) [12]. A minor detoxification pathway for glyoxal is catalyzed by reductases such as aldehyde reductase (ALR1), aldose reductase (ALR2), and carbonyl reductase (ALR3). All these enzymes have a broad substrate specificity, are located in the cytosol, and require NADPH or NADH as cofactors [13].

We have shown in isolated rat hepatocytes that the 50% lethal dose of glyoxal is 5 mM and the mechanism of toxicity has been shown to involve oxidative stress [10]. Furthermore, 10 μM glyoxal has been shown to compromise hepatocyte resistance to hydrogen peroxide (H_2O_2) by increasing GSH oxidation, NADPH oxidation, reactive oxygen species (ROS) formation, DNA oxidation, protein carbonylation, and loss of mitochondrial membrane potential before cell death occurred [14]. The cytotoxic mechanism of metal-catalyzed Asc oxidation is currently thought to arise solely from the production of H_2O_2. However, since glyoxal is an oxidation product of Asc [8, 15], we hypothesized that the cytotoxicity of metal-catalyzed Asc oxidation will be augmented by glyoxal formation through its ability to compromise hepatocyte resistance to H_2O_2. In this study, we showed that the production of even minute quantities of glyoxal through the breakdown of Asc may exacerbate oxidative stress in the system.

2 Materials and methods

2.1 Chemicals

1-Bromoheptane, methylglyoxal (40%), glyoxal (40%), 2,4-dinitrofluorobenzene (DNFB), 2,7-dichlorofluorescin diacetate (DCFH-DA), sodium Asc, copper (II) sulfate, aminoguanidine (AG), pyridoxamine (PA), rhodamine 123, and 1,2-diaminobenzene were purchased from Sigma-Aldrich (St. Louis, MO). Type II collagenase was purchased from Worthington (Lakewood, NJ). Monoclonal antibody 1H7G5 against glyoxal-hydroimidazolone (G-H1) was donated generously by Dr. Michael Brownlee (Albert Einstein University, Bronx, New York).

2.2 Glyoxal formation (cell-free system)

Glyoxal levels were measured in a Krebs–Henseleit buffer system as outlined previously [16]. Briefly, 1,2-diaminobenzene was used as a derivatizing agent for the analysis of glyoxal by isocratic HPLC. To a 1 mL sample of cells (1×10^6 cells), 0.2 mL of 5 M $HClO_4$, 0.2 mL of 2,3-dimethylquinoxaline (as an internal standard), 0.2 mL of 10 mM 1,2-diaminobenzene, and water were added to a final volume of 2 mL. After 1 h at 25°C, HPLC analysis was performed. The separation was carried out on a 5 μm, 250 × 4 mm RP-18 (Merck LiChrospher). The mobile phase was 50% v/v 25 mM ammonium formate buffer, pH 3.4, and 50% v/v methanol. A volume of 100 μL was injected. The flow rate was 1.0 mL/min and quinoxalines were detected at 315 nm. Note: In this study, "cell-free" is used to describe the buffer system without hepatocytes.

2.3 Hydroimidazolone formation (cell-free system)

BSA was used as the model protein for generating AGE adducts in the presence of Asc/Cu as previously described [17]. Briefly BSA was incubated with 20 mM Asc/Cu 5 μM at 37°C in 0.4 M sodium phosphate buffer (pH 7.5) containing 0.02% sodium azide. BSA was incubated in the presence or absence of Asc/Cu. Catalase and AG were also added where indicated on the figures. Solutions were kept in the dark in sealed polyethylene tubes, continuously rotating for 1 wk. At the end of the week, the samples were dialyzed against PBS overnight at 4°C and subsequently lyophilized and reconstituted in PBS. Samples were then subject to dot blot analysis as mentioned below.

2.4 Hydroimidazolone detection (cell-free system)

Hydroimidazolone adducts of Asc/Cu oxidation were measured by quantitative immunoblotting. Dot blots were performed as *per* the methods outlined by Shinohara *et al.* [18, 19]. Equal amounts of protein were used for quantitative immunoblotting. Glyoxal-derived imidazolone AGE was detected using a 1:10000 dilution of mAb 1H7G5. The antibody used recognizes both methylglyoxal and G-H1 adducts [20]. However, we did not detect any methylglyoxal in this system, therefore, 1H7G5 antibody was used to measure G-H1 adducts. Immunocomplexes were visualized using an enzyme-catalyzed fluorescence kit according to the manufacturer's instructions (Amersham, Piscataway, New Jersey) and were quantitated on a FluorChem™ 8800 (Perkin Elmer, Ontario, Canada).

2.5 Animals

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 and dark cycle (lights on at 0800 h) and an environmental temperature of 21–23°C with a 50–60% relative humidity. The animals were fed with standard chow diet and water *ad libitum*. All the stud-

ies involving animals were carried out in compliance with the guidelines of the Canadian Council on Animal Care, and approved by the University of Toronto Animal Care Committee. Ketamine/zylazine mixture was used as an anesthetic before surgery was performed and animals were sacrificed by exsanguination.

2.6 Primary hepatocytes isolation

Hepatocytes were isolated from rats by collagenase perfusion of the liver as described by Moldeus *et al*. [21]. Isolated hepatocytes (10 mL, 10^6 cells/mL) were suspended in Krebs – Henseleit buffer (pH 7.4) containing 12.5 mM HEPES in continually rotating, 50 mL round-bottom flasks, under an atmosphere of 95% O_2 and 5% CO_2 in a 37°C water bath for 30 min. Stock solutions of catalase, Asc, copper sulfate, and PA were prepared in water, while AG was added as a solid.

2.7 Cell viability

Hepatocyte viability was assessed microscopically by plasma membrane disruption as determined by the trypan blue (0.1% w/v) exclusion test [14]. Hepatocyte viability was determined every 30 min during a 3 h incubation period with an initial viability between 85 and 95%.

2.8 Quantitation of cellular 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 DNFB as *per* the method outlined by Reed *et al.* [22]. 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 × 300 mm column was used. Detection was carried out using UV absorption at 364 nm.

2.9 Determination of ROS

The rate of hepatocyte ROS generation induced by α-oxoaldehydes was determined by adding DCFH-DA to the hepatocyte incubate. DCFH-DA penetrates hepatocytes and becomes hydrolyzed to form nonfluorescent dichlorofluorescein. Dichlorofluorescein then reacts with "ROS" to form the highly fluorescent dichlorofluorescein that effluxes the cell. Aliquots (1 mL) were withdrawn at 15, 45 and 90 min after incubation with glyoxal. These samples were then centrifuged for 1 min at 50 × *g* and the supernatant removed. The cells were resuspended in 1 mL of Krebs – Henseleit media containing 1.6 μM DCFH-DA. The cells were allowed to incubate at 37°C for 10 min. The fluorescence intensity of dichlorofluorescin was measured using a Shimadzu RF5000U fluorescence spectrophotometer. Excitation and emission wavelengths were 500 and 520 nm, respectively [23].

2.10 H_2O_2 measurement

H_2O_2 was measured in hepatocytes by taking samples at 30, 60, and 90 using the FOX 1 reagent (Ferrous Oxidation of Xylenol orange). The FOX 1 reagent consisted of 25 mM sulfuric acid, 250 μM ferrous ammonium sulfate, 100 μM xylenol orange, and 0.1 M sorbitol. At the given time intervals, 50 μL of the hepatocyte (1.0×10^6 cells/mL) suspension in Krebs – Henseleit buffer was added to 950 μL FOX 1 reagent and incubated for 30 min at room temperature. The oxidation of ferrous ammonium sulfate by H_2O_2 in xylenol orange was followed at 560 nm [24].

2.11 Mitochondrial membrane potential assay

The uptake and retention of the cationic fluorescent dye, rhodamine 123, has been used for the estimation of mitochondrial membrane potential. This assay is based on the selective accumulation of rhodamine 123 in active mitochondria by charge-facilitated diffusion. Samples (500 μL) were taken from the cell suspension and incubated 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 separated by centrifugation and the amount of rhodamine 123 appearing 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 in fluorescence intensity between control and treated cells [10].

2.12 Statistical analysis

Statistical analysis was performed by a one-way ANOVA test and its significance was assessed by employing Tukey's post hoc test. Results are represented as the mean ± SEM from three separate animals or cell-free system trials. Refer to Fig. 1 for a compendious flow chart of the experiments.

3 Results

3.1 Cu-induced Asc oxidation leads to glyoxal formation in a cell-free system

Glyoxal formation by Cu-induced Asc oxidation was measured in a cell-free system. Asc (20 mM) formed 19 ± 4 μM of glyoxal after 2 h of incubation (Fig. 2). This was increased four-fold in the presence of Cu. AG (glyoxal trap)

decreased the levels of glyoxal formed by Asc/Cu to below control levels.

3.2 Hydroimidazolone (G-HI) adduct formation on BSA in a cell-free system

HI adduct formation on BSA was measured after 7 days of incubation with Asc/Cu, glyoxal traps, or catalase using a G-H1 antibody (Fig. 3a). Asc (20 mM) increased G-H1 formation by 22-fold as compared to control BSA. However, the levels of G-H1 were increased even further in the presence of Asc/Cu (Fig. 3b). AG was effective in decreasing the level of G-H1 formation by Asc; however, the effect was abolished with the addition of Cu. A combination of AG and catalase significantly decreased G-H1 level formation by Asc/Cu.

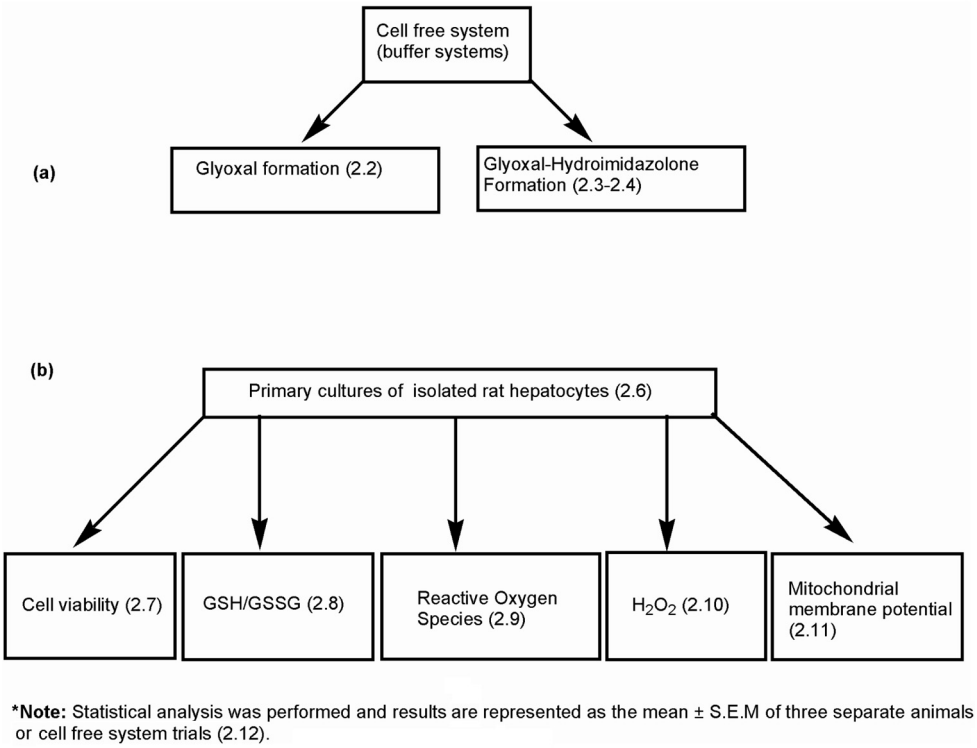

Figure 1. Flow charts of experimental techniques used in this study: (a) experiments with cell-free system and (b) experiments with isolated rat hepatocytes.

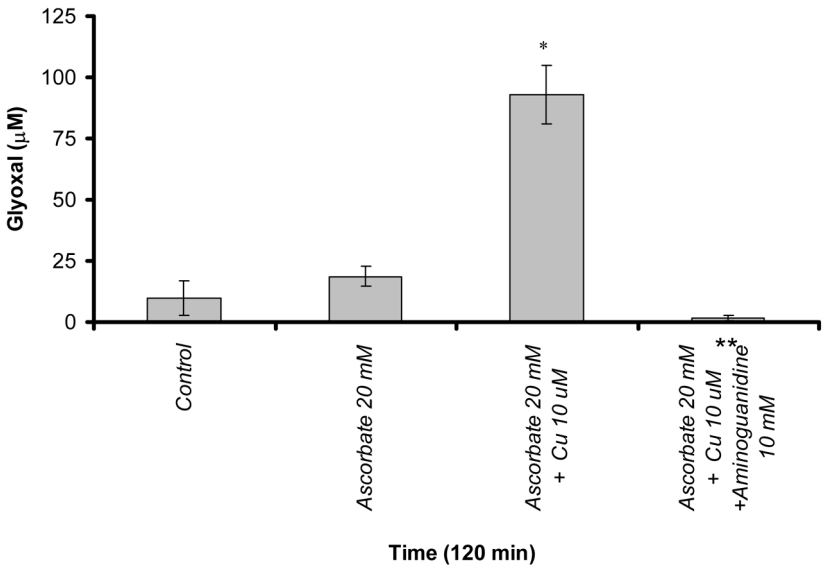

Figure 2. Formation of glyoxal by copper-induced Asc oxidation. A cell-free system was used to measure the amount of glyoxal produced from Asc/Cu. A 10 mL solution of Krebs–Henseleit buffer was incubated at 37°C with 95% O_2/5% CO_2 and various chemicals. 1,2-Diaminobenzene was used as the trapping agent for glyoxal. $n = 3$, *significant as compared to Asc 20 mM ($p < 0.05$); **significant as compared to Asc 20 mM + Cu 10 μM ($p < 0.003$). Results are represented as the mean ± SEM of three separate trials.

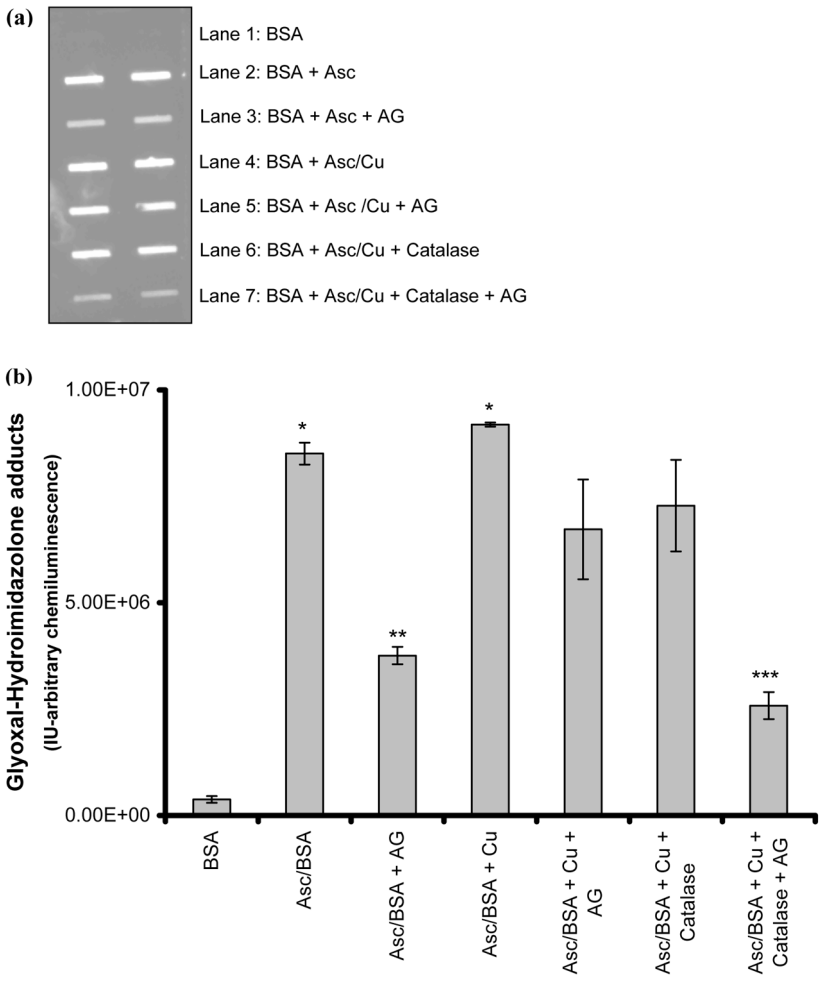

Figure 3. (a) Glyoxal-Hydroimidazolone adduct formation on BSA after incubation with Asc/Cu is prevented by AG and catalase. BSA was incubated at 37°C with Asc/Cu system, glyoxal traps, and catalase for 7 days. At the end of the 7 days, hydroimidazolone adducts of glyoxal were measured *via* dot blot analysis. AG 20 mM, Asc 20 mM, Cu 5 μM, catalase 1000 U/mL, BSA 1 g/mL. (b) Quantitation of hydroimidazolone adduct formation on BSA after incubation with Asc/Cu. $n = 3$, *significant as compared to control ($p < 0.02$); **significant as compared to Asc 20 mM ($p < 0.05$); ***significant as compared to Asc 20/Cu ($p < 0.002$). Results are represented as SEM of three separate trials. AG 20 mM, Asc 20 mM, Cu 5 μM, catalase 1000 U/mL, BSA 1 g/mL.

3.3 Effect of Asc/Cu on cytotoxicity

Asc oxidation in the presence of Cu increased the hepatocyte cytotoxicity as compared to Cu or Asc by themselves (Table 1). Furthermore, Asc/Cu-induced cytotoxicity could be prevented by antioxidants and glyoxal traps such as PA and AG, respectively. A combination of PA and AG was the most effective in decreasing the toxicity. Catalase (1000 U/mL) also decreased Asc/Cu-induced toxicity in hepatocytes indicating the involvement of H_2O_2.

3.4 Cellular GSH depletion and GSSG formation

As shown in Figs. 4a and b, Asc/Cu caused hepatocyte GSH depletion and GSSG formation (GSH 42% ± 7 relative to control and GSSG 149% ± 9 relative to control). GSH depletion and GSSG formation were prevented by AG (GSH 79% ± 10 relative to control and GSSG 117% ± 3 relative to control), PA (GSH 83% ± 7 relative to control and GSSG 123% ± 7 relative to control), or catalase (GSH 89% ± 12 relative to control and GSSG 110% ± 13 relative to control). A combination of AG and PA also protected against GSH depletion (94% ± 4 GSH relative to control) and GSSG formation (114% ± 8 GSSG relative to control).

3.5 ROS formation

Asc/Cu-induced ROS formed in a time-dependent manner (Fig. 5). ROS levels were significantly increased before cytotoxicity ensued at 120 min (189 ± 23 FI units). Both PA (82 ± 12 FI units) and AG (97 ± 19 FI units) separately and in combination (75 ± 9 FI units) with each other prevented Asc/Cu-induced cytotoxicity. Treatment with catalase (1000 U/mL) completely prevented Asc/Cu-induced ROS formation (67 ± 7 FI units).

3.6 H_2O_2 formation

Asc/Cu increased H_2O_2 formation (0.405 ± 0.056) in isolated rat hepatocytes. AG (0.178 ± 0.062) was better at decreasing H_2O_2 formation as compared to PA (0.374 ± 0.018). A combination of PA and AG (0.214 ± 0.032) also

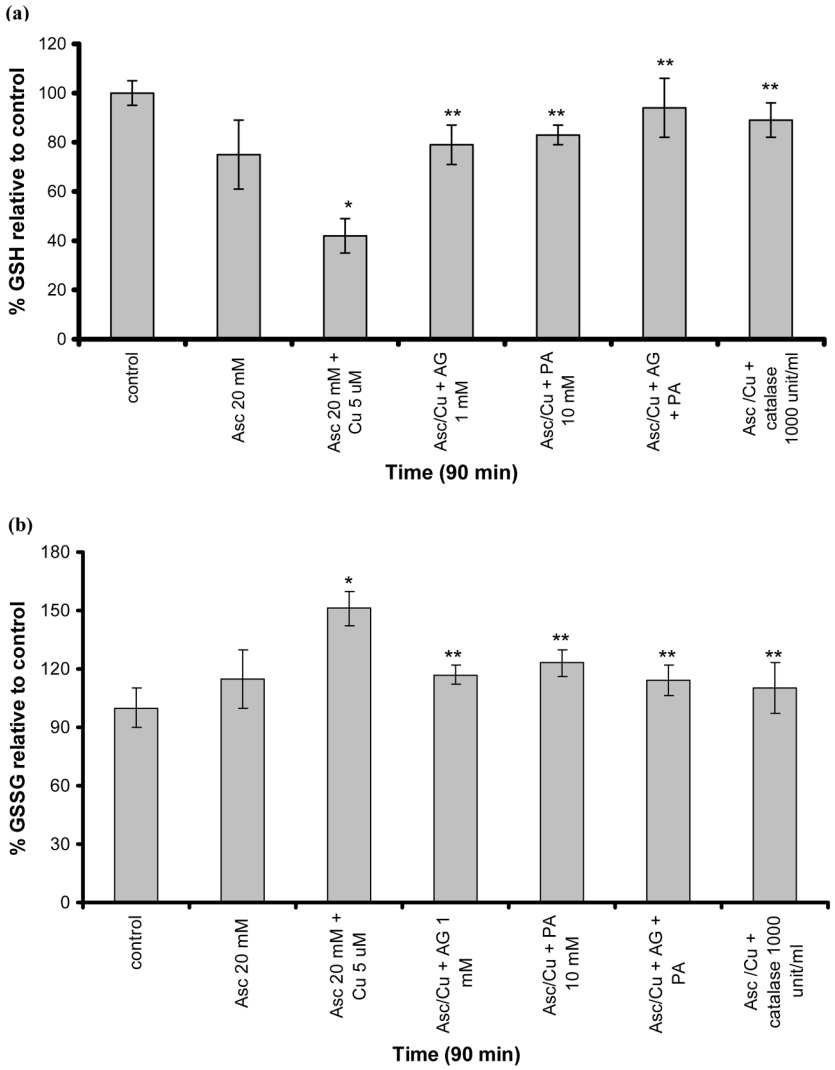

Figure 4. (a) Asc/Cu causes GSH depletion which was prevented by antioxidants and glyoxal traps in isolated rat hepatocytes. The absolute values of GSH in the samples from control to Asc/Cu + catalase are as follows, respectively, in nmol/10^6 cells: 42 ± 4.8, 31.5 ± 3.2, 17.6 ± 2.7, 33.18 ± 4.7, 34.8 ± 2.4, 39.5 ± 4.3, and 37.8 ± 2.6. $n = 3$, *significant as compared to control ($p < 0.05$); **significant as compared to Asc/Cu ($p < 0.05$). Results are represented as the mean ± SEM for three separate animal trials. (b) GSSG formation in isolated rat hepatocytes by Asc/Cu was prevented by antioxidants and glyoxal traps. The absolute values of GSSG in the samples from control to Asc/Cu + catalase are as follows, respectively, in nmol/10^6 cells: 15.3 ± 2.1, 17.3 ± 1.7, 22.4 ± 3.1, 17.6 ± 1.4, 18.5 ± 1.9, 17.1 ± 1.5, and 16.5 ± 1.1. $n = 3$, *significant as compared to control ($p < 0.05$) and **significant as compared to Asc/Cu ($p < 0.05$). Results are represented as the mean ± SEM for three separate animal trials.

decreased H_2O_2 formation significantly. Catalase (1000 U/mL) prevented H_2O_2 formation (0.147 ± 0.025 expressed as absorbance values at 560 nm) by Asc/Cu (Fig. 6).

3.7 Decrease in hepatocyte mitochondrial potential

As shown in Fig. 7, Asc/Cu decreased the hepatocyte mitochondrial membrane potential. This occurred rapidly and before cytotoxicity ensued. The decrease in mitochondrial membrane potential was prevented by PA or AG and by the combination of the two.

4 Discussion

Asc degradation products can undergo Maillard reactions with proteins to form AGE adducts such as pentosidine, N_ε-carboxymethyl-lysine, fluorophore LM-1, pyrraline, glyoxal lysine dimer (GOLD), and oxalic acid monoamide [2, 25, 26]. Asc oxidation has also been shown to be increased by transition metal-catalyzed reactions [9]. In the present study, we have shown for the first time that the metal-catalyzed oxidation of Asc increases H_2O_2 and G-H1 formation (Fig. 8).

Asc oxidation in the presence of Cu increased glyoxal formation ~4-fold as compared to Asc by itself (Fig. 2). Furthermore, AG treatment resulted in decreased glyoxal levels in the cell-free system. BSA incubated with Asc/Cu resulted in an increase in the appearance of the glyoxal-specific AGE adduct, hydroimidazolone (Fig. 3a). When dehydroAsc with and without Cu was incubated with BSA, the formation of G-H1 adducts was not observed (data not shown). Therefore, glyoxal is probably not a byproduct of dehydroAsc degradation alone.

AG and catalase decreased AGE formation by Cu-induced Asc oxidation. AG scavenges glyoxal by forming a 1,2,4-triazine derivative and thereby prevents the formation

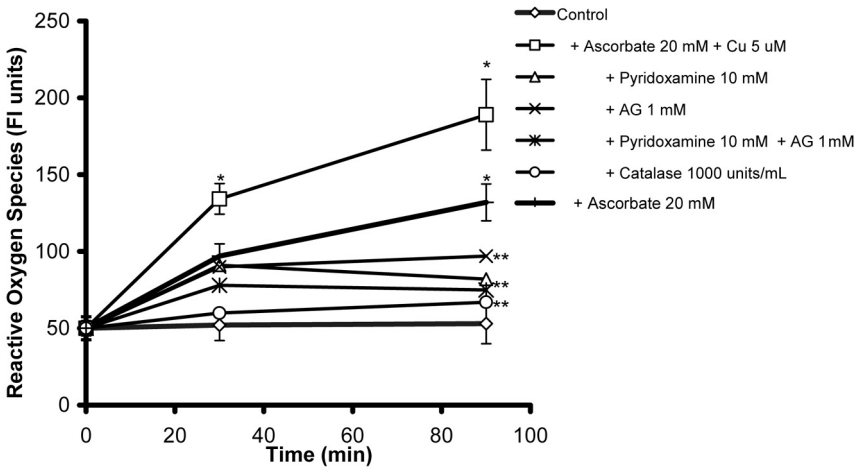

Figure 5. ROS production by Asc/Cu was prevented by antioxidants and glyoxal traps. Isolated rat hepatocytes were incubated at 37°C in rotating round-bottom flasks with 95% O_2 and 5% CO_2 in Krebs – Henseleit buffer (pH 7.4). Fluorimetric measurements for ROS determination were made for 2′,7′ dichlorofluorescin oxidation at λ excitation = 500 nm and λ emission = 520 nm. $n = 3$, *significant as compared to control ($p < 0.02$); **significant as compared to Asc/Cu ($p < 0.002$). Results are represented as the mean ± SEM of three separate animal trials.

Table 1. Cytotoxicity of Asc/Cu was prevented by antioxidants and glyoxal traps in isolated rat hepatocytes

Treatments	Cytotoxicity (% of cells which take up trypan blue)	
	60 min	120 min
Control	23 ± 5	27 ± 4
+Asc 20 mM	26 ± 3	43 ± 5
+PA 10 mM	31 ± 6	32 ± 4[a)]
+AG 1 mM	28 ± 4	26 ± 3[a)]
+PA 10 mM	30 ± 4	29 ± 6[a)]
+Catalase 1000 U	24 ± 5	28 ± 3[a)]
+Asc 20 mM + Cu 5 μM	47 ± 3[b)]	82 ± 8[b)]
+PA 10 mM	22 ± 5[c)]	42 ± 3[c)]
+AG 1 mM	37 ± 8[c)]	40 ± 7[c)]
+PA 10 mM	28 ± 6[c)]	27 ± 3[c)]
+Catalase 1000 U/mL	34 ± 6[c)]	32 ± 4[c)]

Isolated rat hepatocytes were incubated at 37°C in rotating round-bottom flasks with 95% O_2 and 5% CO_2 in Krebs – Henseleit buffer (pH 7.4). Varying treatments were incubated and cytotoxicity was determined using trypan blue up-take assay. $n = 3$. Results are represented as the mean ± SEM of three separate trials.

a) Significant as compared to Asc 20 mM ($p < 0.05$).
b) Significant as compared to control ($p < 0.05$).
c) Significant as compared to Asc 20 mM + Cu 5 μM ($p < 0.005$).

of glyoxal modified proteins [27]. However, in the presence of Cu, AG's inhibitory effect was decreased (Fig. 3b). The formation of glyoxal from the Asc/Cu system in the presence of hepatocytes is likely an amount reflecting both its formation and its metabolism in the hepatocytes. In the albumin system, the Asc/Cu system is expected to generate a significantly higher amount of glyoxal due to the absence of its metabolism. Therefore, the generation of glyoxal from Asc alone would be limited by the rate of Asc autoxidation; but in the presence of a catalytic amount of Cu, Asc oxidation and glyoxal formation would greatly accelerate. Thus in the presence of Cu, the higher levels of glyoxal formation may surmount the ability of AG to effectively neutralize glyoxal resulting in increased adduct formation in the cell-free system and hepatocyte toxicity. A combination of catalase and AG was the best at preventing Asc/Cu-induced G-H1 formation. This could be due to the combined activities of H_2O_2 detoxification by catalase and trapping of glyoxal by AG.

Asc/Cu increased the cytotoxicity more than Asc by itself (Table 1). Asc by itself showed a 16% increase in cytotoxicity as compared to the control; this increase may be due to autoxidation of Asc leading H_2O_2 formation (Fig. 6). However, the Asc/Cu resulted in a significant increase in glyoxal and H_2O_2 formation (Figs. 2 and 6, respectively), which was not observed in cells treated with Asc alone. Normally, hepatocytes are extremely resistant to H_2O_2 as the addition of 20 mM to our isolated hepatocytes was not cytotoxic (data not shown). This high resistance is likely due to the hepatocyte's high catalase activity, GSH levels, GSH peroxidase, and GSH reductase activities. However, we previously showed that glyoxal increased the hepatocyte susceptibility to H_2O_2 at doses as low as 10 μM [14]. This increased vulnerability to oxidative stress caused by glyoxal formation may explain why the antioxidants and glyoxal traps such as PA, catalase, and AG protected against Asc/Cu toxicity (Table 1). PA can react rapidly with glyoxal, forming a Schiff base intermediate that cyclizes to a hemiaminal adduct by intramolecular reaction with the phenolic hydroxyl group of PA. This bicyclic intermediate dimerizes to form a five-ring compound with a central piperazine ring effectively trapping glyoxal [28]. PA can also act as an antioxidant, Cu chelator, and inhibitor of post-Amadori rearrangement product and advanced lipoxidation end-product (ALEs) formation with proteins [29]. The combination of AG and PA resulted in the increased protection of Asc/Cu-induced cytotoxicity. This could be due to an additive effect of increased glyoxal trapping, Cu chelation, and antioxidant activity of these two compounds put together.

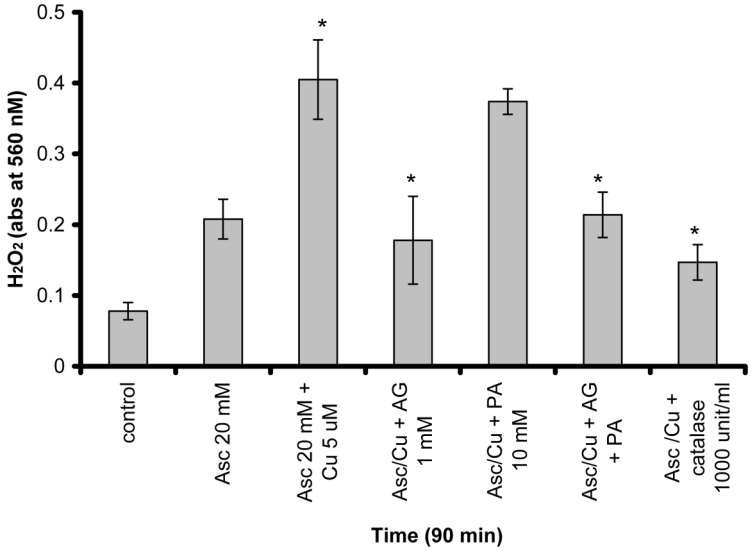

Figure 6. H_2O_2 formation by Asc/Cu was prevented by antioxidants and glyoxal traps at 90 min. Isolated rat hepatocytes were incubated at 37°C in rotating round-bottom flasks with 95% O_2 and 5% CO_2 in Krebs – Henseleit buffer (pH 7.4). H_2O_2 was measured by the FOX1 reagent. $n = 3$, *significant as compared to control ($p < 0.03$); **significant as compared to Asc/Cu ($p < 0.05$). Results are represented as the mean ± SEM of three separate animal trials.

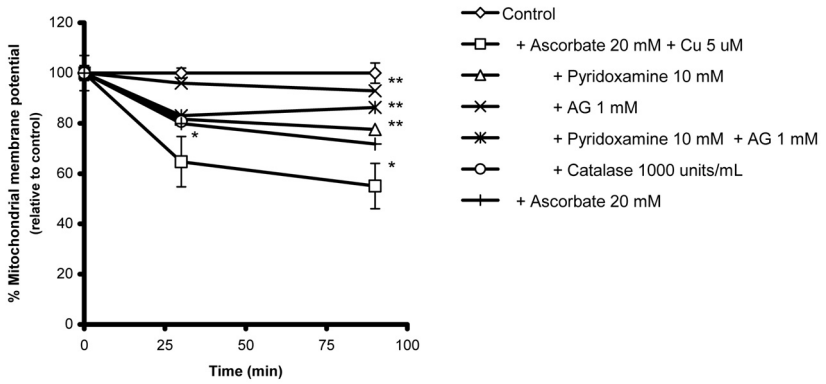

Figure 7. Mitochondrial membrane potential collapse induced by Asc/Cu was prevented by glyoxal traps and antioxidants. Isolated rat hepatocytes were incubated at 37°C in rotating round-bottom flasks with 95% O_2 and 5% CO_2 in Krebs – Henseleit buffer (pH 7.4). Rhodamine 123 was used to assess the mitochondrial toxicity of glyoxal in isolated hepatocytes. Fluorimetric measurements were made at $\lambda_{excitation} = 490$ nm and $\lambda_{emission} = 520$ nm. $n = 3$, *significant as compared to control ($p < 0.05$); **significant as compared to Asc/Cu ($p < 0.05$). Results are represented as the mean ± SEM of three separate animal trials.

Catalase is an important cellular antioxidant defense enzyme that defends against oxidative stress. It serves to protect the cell from the toxic effects of higher concentrations of H_2O_2 by catalyzing its decomposition into molecular oxygen and water without the production of free radicals. In the present study, catalase showed protective effects against Asc/Cu-induced G-H1 formation and toxicity. This result indicates that oxidative stress and H_2O_2 formation are involved in the cytotoxic mechanism since catalase can detoxify the H_2O_2 produced as a byproduct of Asc-enhanced transition metal redox cycling. A decrease in H_2O_2 levels, however, prevented GSH oxidation, subsequently resulting in increased GSH-mediated glyoxal detoxification.

Since GSH detoxifies both glyoxal and H_2O_2, cellular GSH levels were measured. Asc/Cu resulted in GSH oxidation (Figs. 4a and b). This GSH oxidation likely occurred for three reasons: (i) as a result of ROS formation caused by Asc/Cu-mediated H_2O_2 formation, (ii) glyoxal detoxification, and (iii) Asc recycling from dehydroAsc [30]. As a result of GSH depletion, the hepatocytes became susceptible to the cytotoxic effects of glyoxal. Mitochondrial toxicity (Fig. 7) was demonstrated previously in hepatocytes exposed to formaldehyde, another reactive aldehyde [31]. Similarly, Asc/Cu treatment decreased the mitochondrial membrane potential which preceded cytotoxicity. Furthermore, the combined cytotoxic effect of reactive aldehyde formation and oxidative stress was implicated as cytotoxicity was prevented by AG, PA, and catalase. It has been shown by Reber *et al*. [32] in E1A-NR3 cell line that glyoxal caused mitochondrial toxicity which resulted in apoptosis.

Increased cytotoxicity in the Asc/Cu system can also be due to direct effects of glyoxal on antioxidant enzymes. Previously, we showed that glyoxal inactivated GSH reductase [10]. Furthermore, others have shown that the NADPH generating enzymes such as cytosolic glucose-6-phosphate dehydrogenase (G6PDH) and mitochondrial isocitrate dehydrogenase (ICDH) were also inhibited by glyoxal [33]. Consequently, GSH depletion would be exacerbated by the inhibition of GSH recycling from GSSG. The inhibition of G6PDH and ICDH by glyoxal and H_2O_2 may contribute significantly to a lowering of the cellular NADPH supply [14].

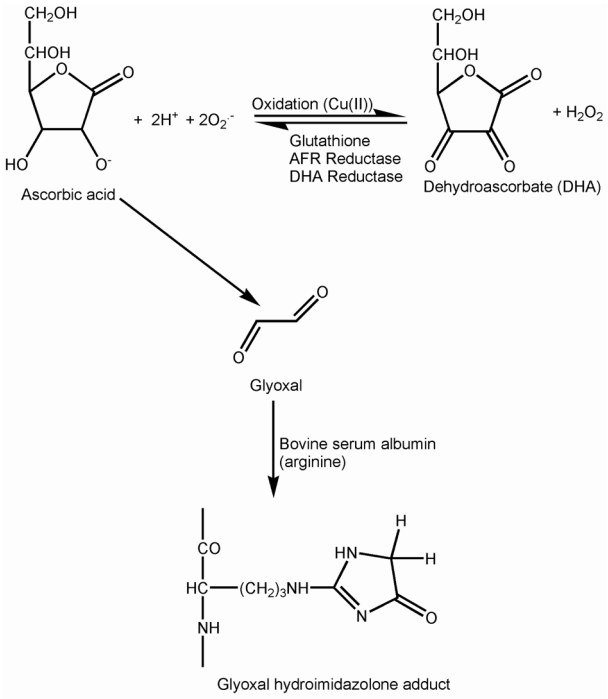

Figure 8. Possible mechanism of glyoxal formation from metal-catalyzed Asc oxidation (modified from [8]).

Consistent with these findings are our results showing that the addition of AG, PA, and catalase can help maintain the cellular redox state by reducing the effects of glyoxal on antioxidant enzymes and detoxifying ROS derived from H_2O_2.

Although the Asc concentrations used in this study were much higher than those found in the plasma, levels of Asc are in the millimolar range intracellularly, in the adrenal medulla and in the ocular lens, particularly in some diabetics [1, 34]. The role of oxidative stress in the diabetic liver is at present controversial, but secondary diseases which often occur combined with diabetes such as nonalcoholic or alcohol-associated steatohepatitis can cause oxidative stress in the liver. Furthermore, the already lower Asc concentrations observed in the lens of rat diabetic models may indirectly indicate that elevated glyoxal formation due to Asc oxidation may contribute to diabetes-associated ocular complications [35, 36]. Because it appears that diabetes results in oxidative stress, treatment with a high dose of Asc has been proposed as an adjuvant to current therapies (for instance, as a prophylactic agent against diabetic hypertension [37]). We propose that the possible formation of glyoxal from such treatments should be considered before widespread use. Clinically, the accumulation of α-oxoaldehyes has been implicated in chronic complications associated with Diabetes Mellitus *via* AGE formation and activation of proinflammatory response by monocytes/macrophages [27, 38].

The role of Cu in diabetes has been controversial and complicated. Cu deficiency, like diabetes, is characterized by increased glycation, peroxidation, and nitration, due to a decrease in Cu containing antioxidant defense enzymes such as CuZn-superoxide dismutase [39]. Copper can increase the rate of AGE formation, which has been implicated in secondary complications associated with diabetes [40]. Plasma concentrations of Cu have been reported to be higher in diabetic patients with complications such as retinopathy, hypertension, and microvascular disease as compared to nondiabetics [41, 42]. There have been several studies that indicate that glycation of Cu containing proteins (*i.e.*, CuZn-superoxide dismutase, ceruloplasmin, *etc*.) can result in the release of Cu which can participate in Fenton-type reactions to produce hydroxyl radicals that can further fragment biomolecules [43 – 45]. Excessively high concentration of glycated CuZn-superoxide dismutase has been found in diabetic rat lenses and is postulated to be involved in lens pathology [46]. Furthermore, glycated proteins have high affinity for transition metals like Cu [47] providing active sites for producing free radicals contributing to the increased oxidative stress observed in diabetes. AGE inhibitors have been shown to have potent Cu chelating or free-radical scavenging activities [48, 49].

This study shows that the Asc/Cu cytotoxic mechanism in isolated rat hepatocytes involves oxidative stress, which leads to a decrease in cellular GSH levels, AGE formation, and mitochondrial membrane potential and ultimately cell death. Our results demonstrate for the first time that glyoxal formation may both exacerbate oxidative stress derived from the transition metal-catalyzed oxidation of Asc as well as lead to G-H1 formation. Understanding this mechanism of toxicity could lead to the development of novel Cu chelating drug therapies to treat complications associated with oxidative stress in diabetes (*e.g.*, cataract). This research also highlights the contributory role of reactive carbonyls in Asc-mediated cell death and may help explain the purported anticancer activity of Asc [50 – 52].

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5 References

[1] Grandhee, S. K., Monnier, V. M., Mechanism of formation of the Maillard protein cross-link pentosidine. Glucose, fructose, and ascorbate as pentosidine precursors, *J. Biol. Chem.* 1991, *266*, 11649 – 11653.

[2] Saxena, P., Saxena, A. K., Monnier, V. M., High galactose levels *in vitro* and *in vivo* impair ascorbate regeneration and increase ascorbate-mediated glycation in cultured rat lens, *Exp. Eye Res.* 1996, *63*, 535 – 545.

[3] Bensch, K. G., Fleming, J. E., Lohmann, W., The role of ascorbic acid in senile cataract, *Proc. Natl. Acad. Sci. USA* 1985, *82*, 7193 – 7196.

[4] Levine, M., Conry-Cantilena, C., Wang, Y., Welch, R. W., *et al.*, Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance, *Proc. Natl. Acad. Sci. USA* 1996, *93*, 3704 – 3709.

[5] Pauling, L., *How to Live Longer and Feel Better*, W. H. Freeman and Company, New York 1986.

[6] Hickey, S. Roberts, H., *Ascorbate: The Science of Vitamin C*, Lulu Press, Napa, CA 2004.

[7] Buettner, G. R., Jurkiewicz, B. A., Catalytic metals, ascorbate and free radicals: combinations to avoid, *Radiat. Res.* 1996, *145*, 532 – 541.

[8] Nagaraj, R. H., *et al.*, High correlation between pentosidine protein crosslinks and pigmentation implicates ascorbate oxidation in human lens senescence and cataractogenesis, *Proc. Natl. Acad. Sci. USA* 1991, *88*, 10257 – 10261.

[9] Saxena, A. K., *et al.*, Protein aging by carboxymethylation of lysines generates sites for divalent metal and redox active copper binding: relevance to diseases of glycoxidative stress, *Biochem. Biophys. Res. Commun.* 1999, *260*, 332 – 338.

[10] Shangari, N., O'Brien, P. J., The cytotoxic mechanism of glyoxal involves oxidative stress, *Biochem. Pharmacol.* 2004, *68*, 1433 – 1442.

[11] Thornalley, P. J., Langborg, A., Minhas, H. S., Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose, *Biochem. J.* 1999, *344*, 109 – 116.

[12] Abordo, E. A., Minhas, H. S., Thornalley, P. J., Accumulation of alpha-oxoaldehydes during oxidative stress: a role in cytotoxicity, *Biochem. Pharmacol.* 1999, *58*, 641 – 648.

[13] Vander Jagt, D. L., Robinson, B., Taylor, K. K., Hunsaker, L. A., Reduction of trioses by NADPH-dependent aldo-keto reductases. Aldose reductase, methylglyoxal, and diabetic complications, *J. Biol. Chem.* 1992, *267*, 4364 – 4369.

[14] Shangari, N., Chan, T. S., Popovic, M., O'Brien, P. J., Glyoxal markedly compromises hepatocyte resistance to hydrogen peroxide, *Biochem. Pharmacol.* 2006, *71*, 1610 – 1618.

[15] Mistry, N., *et al.*, Immunochemical detection of glyoxal DNA damage, *Free Radic. Biol. Med.* 1999, *26*, 1267 – 1273.

[16] Okado-Matsumoto, A., Fridovich, I., The role of alpha, beta-dicarbonyl compounds in the toxicity of short chain sugars, *J. Biol. Chem.* 2000, *275*, 34853 – 34857.

[17] Booth, A. A., Khalifah, R. G., Todd, P., Hudson, B. G., *In vitro* kinetic studies of formation of antigenic advanced glycation end products (AGEs). Novel inhibition of post-Amadori glycation pathways, *J. Biol. Chem.* 1997, *272*, 5430 – 5437.

[18] Hammes, H. P., *et al.*, Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy, *Nat. Med.* 2003, *9*, 294 – 299.

[19] Shinohara, M., *et al.*, Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endocytosis, *J. Clin. Invest.* 1998, *101*, 1142 – 1147.

[20] Kilhovd, B. K., *et al.*, Increased serum levels of the specific AGE-compound methylglyoxal-derived hydroimidazolone in patients with type 2 diabetes, *Metabolism* 2003, *52*, 163 – 167.

[21] Moldeus, P., Hogberg, J., Orrenius, S., Isolation and use of liver cells, *Methods Enzymol.* 1978, *52*, 60 – 71.

[22] Reed, D. J., *et al.*, High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides, *Anal. Biochem.* 1980, *106*, 55 – 62.

[23] Pourahmad, J., O'Brien, P. J., A comparison of hepatocyte cytotoxic mechanisms for Cu2+ and Cd2+, *Toxicology* 2000, *143*, 263 – 273.

[24] Ou, P., Wolff, S. P., A discontinuous method for catalase determination at ‚near physiological' concentrations of H2O2 and its application to the study of H2O2 fluxes within cells, *J. Biochem. Biophys. Methods* 1996, *31*, 59 – 67.

[25] Monnier, V. M., *et al.*, Structure of advanced Maillard reaction products and their pathological role, *Nephrol. Dial. Transplant.* 1996, *11*, 20 – 26.

[26] Tessier, F., Obrenovich, M., Monnier, V. M., Structure and mechanism of formation of human lens fluorophore LM-1. Relationship to vesperlysine A and the advanced Maillard reaction in aging, diabetes, and cataractogenesis, *J. Biol. Chem.* 1999, *274*, 20796 – 20804.

[27] Thornalley, P. J., Glutathione-dependent detoxification of alpha-oxoaldehydes by the glyoxalase system: involvement in disease mechanisms and antiproliferative activity of glyoxalase I inhibitors, *Chem. Biol. Interact.* 1998, *111 – 112*, 137 – 151.

[28] Voziyan, P. A., Metz, T. O., Baynes, J. W., Hudson, B. G., A post-Amadori inhibitor pyridoxamine also inhibits chemical modification of proteins by scavenging carbonyl intermediates of carbohydrate and lipid degradation, *J. Biol. Chem.* 2002, *277*, 3397 – 3403.

[29] Metz, T. O., Alderson, N. L., Thorpe, S. R., Baynes, J. W., Pyridoxamine, an inhibitor of advanced glycation and lipoxidation reactions: a novel therapy for treatment of diabetic complications, *Arch. Biochem. Biophys.* 2003, *419*, 41 – 49.

[30] Winkler, B. S., Orselli, S. M., Rex, T. S., The redox couple between glutathione and ascorbic acid: a chemical and physiological perspective, *Free Radic. Biol. Med.* 1994, *17*, 333 – 349.

[31] Teng, S., *et al.*, The formaldehyde metabolic detoxification enzyme systems and molecular cytotoxic mechanism in isolated rat hepatocytes, *Chem. Biol. Interact.* 2001, *130 – 132*, 285 – 296.

[32] Reber, F., *et al.*, Alteration of the intracellular pH and apoptosis induction in a retinal cell line by the AGE-inducing agent glyoxal, *Graefes Arch. Clin. Exp. Ophthalmol.* 2002, *240*, 1022 – 1032.

[33] Morgan, P. E., Dean, R. T., Davies, M. J., Inactivation of cellular enzymes by carbonyls and protein-bound glycation/glycoxidation products, *Arch. Biochem. Biophys.* 2002, *403*, 259 – 269.

[34] Chaplen, F. W., Fahl, W. E., Cameron, D. C., Evidence of high levels of methylglyoxal in cultured Chinese hamster ovary cells, *Proc. Natl. Acad. Sci. USA* 1998, *95*, 5533 – 5538.

[35] Mitton, K. P., Trevithick, J. R., High-performance liquid chromatography-electrochemical detection of antioxidants in vertebrate lens: glutathione, tocopherol, and ascorbate, *Methods Enzymol.* 1994, *233*, 523 – 539.

[36] Mitton, K. P., Dzialoszynski, T., Sanford, S. E., Trevithick, J. R., Cysteine and ascorbate loss in the diabetic rat lens prior to hydration changes, *Curr. Eye Res.* 1997, *16*, 564 – 571.

[37] Goldenberg, H., Vitamin C: from popular food supplement to specific drug, *Forum Nutr.* 2003, *56*, 42 – 45.

[38] Lapolla, A., *et al.*, Glyoxal and methylglyoxal levels in diabetic patients: quantitative determination by a new GC/MS method, *Clin. Chem. Lab Med.* 2003, *41*, 1166 – 1173.

[39] Saari, J. T., Copper deficiency and cardiovascular disease: role of peroxidation, glycation, and nitration, *Can. J. Physiol. Pharmacol.* 2000, *78*, 848 – 855.

[40] Jakus, V., Bauerova, K., Rietbrock, N., Effect of AG and copper(II) ions on the formation of advanced glycosylation end products. *In vitro* study on human serum albumin, *Arzneimittelforschung* 2001, *51*, 280 – 283.

[41] Isbir, T., Tamer, L., Taylor, A., Isbir, M., Zinc, copper and magnesium status in insulin-dependent diabetes, *Diabetes Res.* 1994, *26*, 41 – 45.

[42] Ruiz, C., *et al.*, Selenium, zinc and copper in plasma of patients with type 1 diabetes mellitus in different metabolic control states, *J. Trace Elem. Med. Biol.* 1998, *12*, 91 – 95.

[43] Islam, K. N., *et al.*, Fragmentation of ceruloplasmin following non-enzymatic glycation reaction, *J. Biochem. (Tokyo)* 1995, *118*, 1054 – 1060.

[44] Kaneto, H., *et al.*, DNA cleavage induced by glycation of Cu, Zn-superoxide dismutase, *Biochem. J.* 1994, *304*, 219 – 225.

[45] Kawamura, N., *et al.*, Increased glycated Cu, Zn-superoxide dismutase levels in erythrocytes of patients with insulin-dependent diabetis mellitus, *J. Clin. Endocrinol. Metab.* 1992, *74*, 1352 – 1354.

[46] Takata, I., *et al.*, Glycated Cu, Zn-superoxide dismutase in rat lenses: evidence for the presence of fragmentation in vivo, *Biochem. Biophys. Res. Commun.* 1996, *219*, 243 – 248.

[47] Eaton, J. W., Qian, M., Interactions of copper with glycated proteins: possible involvement in the etiology of diabetic neuropathy, *Mol. Cell Biochem.* 2002, *234 – 235*, 135 – 142.

[48] Price, D. L., Rhett, P. M., Thorpe, S. R., Baynes, J. W., Chelating activity of advanced glycation end-product inhibitors, *J. Biol. Chem.* 2001, *276*, 48967 – 48972.

[49] Sajithlal, G. B., Chithra, P., Chandrakasan, G., An *in vitro* study on the role of metal catalyzed oxidation in glycation and crosslinking of collagen, *Mol. Cell Biochem.* 1999, *194*, 257 – 263.

[50] Chen, Q., *et al.*, Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a prodrug to deliver hydrogen peroxide to tissues, *Proc. Natl. Acad. Sci. USA* 2005, *102*, 13604 – 13609.

[51] Prasad, K. N., Sinha, P. K., Ramanujam, M., Sakamoto, A., Sodium ascorbate potentiates the growth inhibitory effect of certain agents on neuroblastoma cells in culture, *Proc. Natl. Acad. Sci. USA* 1979, *76*, 829 – 832.

[52] Roginsky, V. A., Bruchelt, G., Bartuli, O., Ubiquinone-0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone) as effective catalyzer of ascorbate and epinephrine oxidation and damager of neuroblastoma cells, *Biochem. Pharmacol.* 1998, *55*, 85 – 91.