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Rapid uptake of oxidized ascorbate induces loss of cellular glutathione and oxidative stress in liver slices

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Abbreviations: GLUT, glucose transporter; TBARS, thiobarbituric acid-reactive substances; WT, wortmannin

Abstract

The observation that ascorbate known to retain pro-oxidant properties induces cell death in a number of immortal cell lines, led us to examine its mechanism and whether it is involved in oxidative stress injury in such asocorbate-enriched tissue cells as hepatocytes. In rat liver homogenates, higher concentrations (1 and 3 mM) of ascorbate suppressed lipid peroxide productions but lower concentrations (0.1 and 0.3 mM) did not. In contrast to the homogenate, ascorbate increased lipid peroxide production in liver slices in a concentration dependant manner. Iso-ascorbate, the epimer of ascorbate did not cause an increase the oxidative stress in liver slices. This differential effect between homogenates and liver slices implies that cellular integrity is required for ascorbate to induce oxidative stress. Wortmannin, an inhibitor of the GLUT (glucose transporter) thought to transport dehydroascorbate into cells, inhibited [^{14}C]-ascorbate uptake and suppressed oxidative stress in liver slices. Wortmannin suppressed that [^{14}C]-ascorbate uptake by GLUT following oxidation to [^{14}C]dehydroascorbate. Taken together, these observations support our hypothesis that ascorbate is oxidized to dehydroascorbate by molecular oxygen in solution (*i.e.*, plasma and culture medium) which is then carried into hepatocytes (*via* a GLUT) where it is reduced back to ascorbate causing oxidative stress.

Keywords: ascorbic acid; dehydroascorbic acid; glucose transporter; glutathione; lipid peroxide

Introduction

Ascorbate acts as a scavenger of reactive oxygen species, including superoxide and hydroxyl free radicals, which are produced by radiation, oxidative reactions and carcinogenic processes (Ozaki *et al.*, 1995). As such the ascorbate anion donate an electron to the free radicals. Ascorbate is also required to maintain the structural integrity of vascular tissues and plays vital roles in a number of other physiological processes, including platelet function, and cholesterol and catecholamine biosynthesis (Agus *et al.*, 1997). With all of these well-established positive attributes it may come as a surprise to some that, in reality, ascorbate is somewhat of a paradoxical molecule. Contrary to the antioxidant properties of ascorbate, it has also been shown to oxidize cellular components under certain experimental conditions (Roginsky and Stegmann, 1994; Pushpendran *et al.*, 1998) and to induce cell death in a number of different cell lines (Sakagami and Satoh, 1997), even though the mechanism is not well understood.

The liver is susceptible to a wide variety of disorders, possibly because it is constantly exposed to so many harmful agents. Liver damage induced by ischemia-reperfusion is generally recognized as being mediated by oxidative stress (Layton *et al.*, 1996; Cerwenka *et al.*, 1999) but considerable uncertainty surrounds the mechanism of oxidative stress-induced damage. The liver is also considered to be a reservoir: maintaining homeostatic level of plasma ascorbate since it stores a large amount of ascorbate (Hornig, 1975; Upston *et al.*, 1999). The physiological significance of high concentration of ascorbate in liver is, however, not well defined.

We hypothesized that the pro-oxidant action of ascorbate may be involved in the liver damage that follows ischemia. This proposition is based on the observation that ascorbate concentrations are increased by 60% in liver extracellular fluid during ischemia (Layton *et al.*, 1996). This indicates that physiological concentrations of ascorbate within hepatocytes (approximately 4.3±0.5 mM) cannot be maintained during an ischemic period (Rose and Bode, 1995). The released ascorbate in plasma is oxidized by molecular oxygen (Pa_{O_2} 95±2 torr) (Walker *et al.*, 1997). During

the reperfusion period, hepatocytes actively take up ascorbate to restore physiological concentrations of the vitamin. A major source of ascorbate reuptake takes the form of dehydroascorbate (DHA) transport *via* a glucose transporter (GLUT) (Banhegyi *et al.*, 1998). The cytosolic dehydroascorbate is rapidly reduced to ascorbate by the action of dehdroascorbate reductase and a non-enzymatic chemical reaction. However, rapid rises in hepatocyte dehydroascorbate concentrations decrease the ability of cellular reducing agents such as glutathione (GSH) to reduce further oxidative stress, and eventually result in liver injury through deregulation of vital cellular components.

In this study, we obtained evidence supporting our putative mechanism that a rapid uptake rate of dehydroascorbate (oxidized ascorbate) is a critical factor in the development of liver injury during the reperfusion period.

Materials and Methods

Male Sprague-Dawley rats were obtained from Charles River Canada (Montreal, Canada). The rats were acclimatized at 25°C for two weeks with 14 h (06:00-20:00) of light daily. Their diets consisted of Purina Lab Chow and tap water supplied *ad libitum*. All of the following experimental protocols were carried out in accordance with guidelines established by the Queen's University Animal Care Committee.

Rats (280-300 g) were killed by decapitation under halothane anesthesia and their liver lobes removed. Ten percent liver homogenate was prepared by homogenizing 1 g (wet weight) of liver tissue in 9 ml of PBS (0.15 M NaCl and 10 mM Na_2HPO_4, pH 7.4) using a frosted glass tube-pestle. The homogenate (1 ml/tube) was transferred to glass test tubes (15×75 mm) and the ascorbate (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) added to make 0.1, 0.3, 1, or 3 mM. In some cases as noted in the text, 50 μM ferrous ions [Fe^{2+}, ammonium iron (II) sulfate; Fisher Scientific] were also added to assess the impact of ascorbate plus Fe^{2+} on lipid peroxidation in liver slices and homogenates. Samples were incubated for three hours in a water bath at 37°C.

In order to quantify the concentration of oxidized lipids the amount of thiobarbituric acid-reactive substances (TBARS) was determined. Two ml of 0.67% thiobarbituric acid (Sigma-Aldrich Canada Ltd.) was added to 100 μl of the homogenate in glass test tubes (15×75 mm) and placed into a boiling water bath for 20 min. Tubes were cooled on ice and then centrifuged at 3,000 *g* for 10 min. A spectrophotometer was used to measure the optical density of the solutions at 532 nm. A standard curve was constructed using tetraethoxy propane (Sigma-Aldrich Canada Ltd.) as a reference (r = 0.99), from which the concentrations of TBARS are determined. TBARS concentrations of control group were expressed as 100% to facilitate comparisons among ascorbate concentrations in different samples.

To determine the effect of ascorbate treatment on liver slices, rat livers were placed in RPMI 1640 (Gibco, Grand Island, New York) containing 2.5% fetal bovine serum, 10% horse serum (Gibco) and 50 IU/ml penicillin (Sigma-Aldrich Canada Ltd.) (culture medium). Liver samples were sliced to less than 1 mm using a razor blade. The liver tissue (0.4 g) was placed in Teflon beakers containing 3.6 ml of culture medium. Ascorbate was added to make concentrations of 0.1, 0.3, 1 or 3 mM, respectively. Samples were incubated in a water-jacketed incubator at 37°C under a water-saturated atmosphere of 5% CO_2-95% air for 2 h. Following the incubation period, both the tissue and medium were transferred to a 15 ml screw-cap conical centrifuge tube and centrifuged for 3 min at 1,000 *g*. The media was discarded while the pellet was resuspended in 1 ml of PBS solution. The suspension was recentrifuged at 1,000 *g* for 3 min. A 10% homogenate was prepared and the concentrations of oxidized lipids (TBARS) quantified as described above.

The Lowry protein assay was employed to quantify protein contents in each aliquot sample of homogenate (Lowry *et al.*, 1951). Thus, TBARS levels were expressed as nmol per 100 mg of protein to standardize the amounts of oxidized lipids among tissue samples.

Some of the experiments were aimed at determining the effect of wortmannin (Sigma-Aldrich Canada Ltd.) or GSH on the attenuation of lipid peroxidation induced by ascorbate. In these experiments, wortmannin or glutathione was added for a final concentration of 10 μM or 1 mM respectively. The tissue concentrations of reduced glutathione were measured according to the method of Hissin and Hilf (1976) with minor modifications. Liver homogenates (250 mg) were centrifuged and pellets washed with 0.5 ml of 25% Na_2HPO_3 and 2 ml of 0.1 M sodium phosphate-5 mM EDTA buffer (pH 8.0) by resuspension and recentrifugation. After centrifugation at 10,000 *g* for 30 min, aliquots of the supernatant (0.5 ml) were mixed with 4.5 ml of phosphate-EDTA buffer. Also, 100 μl of O-phthalaldehyde and 1.8 ml of phosphate-EDTA buffer were added to a 100 μl test sample. The solution was mixed, and incubated at room temperature for 15 min. The contents of glutathione were determined fluorometrically with excitation and emission at 350 nm and 420 nm, respectively. Data were calculated on the basis of glutathione calibration curves.

In order to investigate the effect of GLUT inhibitors on dehydroascorbate transportation (Agus *et al.,* 1997;

Song *et al.,* 2002), liver slices were incubated with 1 ml of 10 μM ascorbate containing 0.1 μCi [^{14}C]-ascorbate (8.60 mCi per mmol, Dupont Canada Inc., Mississauga, ON, Canada) in the presence or absence of 1 or 10 μM wortmannin for 4 h in a shaking water bath at 37°C. To collect slices, incubation samples were centrifuged at 1,000 *g* and washed twice with 2 ml PBS (pH 7.4) to remove surface [^{14}C]ascorbate. The liver slices were homogenized in 100 μl PBS and transferred to 4 ml of Scintiverse (Fisher Scientific Canada, Nepean, ON, Canada). Total radioactivity in the samples was determined by liquid scintillation counting (Beckman).

The Graph-Pad Prism (GraphPad, San Diego, CA) was used for all statistical analysis. The one-way ANOVA with Bonferroni *t* post-test and Student's *t*-test were used to assess differences among groups. Data are presented as the mean±SE; *P* values of less than 0.05 were determined to be significant.

Results

In liver slices, ascorbate treatments elevated TBARS concentration in a dose-related manner over a range of concentration between 0 (control) and 3 mM ascorbate (upper panel, Figure 1) ($r = 0.910 \pm 0.048$, $n = 6$). Treatment with 1 and 3 mM ascorbate significantly elevated the TBARS concentrations in liver slices to 152±6% ($P < 0.05$) and 168±14% ($P < 0.01$) but not by treatments of lower concentrations (0.1 and 0.3 mM). Conversely, in liver homogenates TBARS concentrations were elevated by treatment with lower concentrations of ascorbate (0.1 and 0.3 mM) to 135 ±10% and 155±14% ($P < 0.05$), respectively but higher ascorbate concentrations (1 and 3 mM) failed to elevate TBARS concentration forming a bell shaped concentration-response curve (lower panel, Figure 1).

When the liver slices were treated with 50 μM Fe^{2+} in addition to varying concentrations (0, 0.1, 1 and 3 mM) of ascorbate (upper panel, Figure 2), treatment with 50 μM Fe^{2+} alone [control group (0 mM ascorbate) in upper panel, Figure 2] did not increase the TBARS concentration (94±6%) when compared to the control group (100±5%). High concentrations of ascorbate (1 and 3 mM) in the presence of 50 μM Fe^{2+} significantly elevated the TBARS concentrations to 152±16% ($P < 0.05$) and 168±20% ($P < 0.01$, $n = 6$) (upper panel, Figure 2). However, ascorbate-treated groups without (upper panel, Figure 1) and with 50 μM Fe^{2+} (upper panel, Figure 2) did not differ, indicating that 50 μM Fe^{2+} did not contribute to TBARS production in the liver slices.

Liver homogenates were treated with different concentrations (0, 0.1, 0.3, 1 and 3 mM) of ascorbate

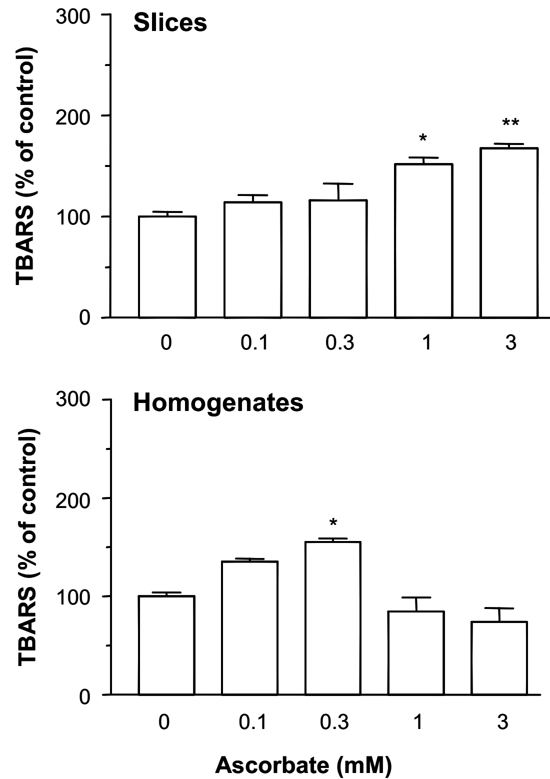

Figure 1. Effects of ascorbate treatment on liver slices (upper panel) and liver tissue homogenates (lower panel). Quantities of TBARS are expressed as percentages of control values. Each bar represents the mean±SEM of six replicates. *$P < 0.05$, **$P < 0.01$.

in the presence of 50 μM Fe^{2+}. While Fe^{2+} alone did not significantly ($P = 0.1041$) increase TBARS mean values above control values (TBARS concentrations with Fe^{2+} were 119±4%, and control values were 100 ±4%), 0.1 and 0.3 mM ascorbate plus 50 μM Fe^{2+} elevated TBARS concentrations to 214±15% ($P < 0.01$, $n = 6$) and 225±28% ($P < 0.01$, $n = 6$), respectively. However, higher concentration of ascorbate (1 and 3 mM plus 50 μM Fe^{2+}) did not increase TBARS concentrations above control values (lower panel, Figure 2). The ascorbate plus 50 μM Fe^{2+} group also showed a bell shaped dose-response curve but the magnitude of elevation of TBARS was higher in 0.1 mM ($P < 0.01$) and 0.3 mM ascorbate-treated groups ($P < 0.001$) (lower panel, Figure 2) than those ascorbate-treated groups without Fe^{2+} (lower panel, Figure 1). Thus, 50 μM Fe^{2+} potentiated ascorbate-induced oxidative stress in the homogenates.

Trace amounts of metal ions such as ferrous ion are found in chemical reagents as impurities and may influence ascorbate-induced oxidative stress. EDTA (10, 30, 100 and 300 μM) was added to the culture media to eliminate the effects of trace metal ions. When the homogenates were treated with 0.1 mM

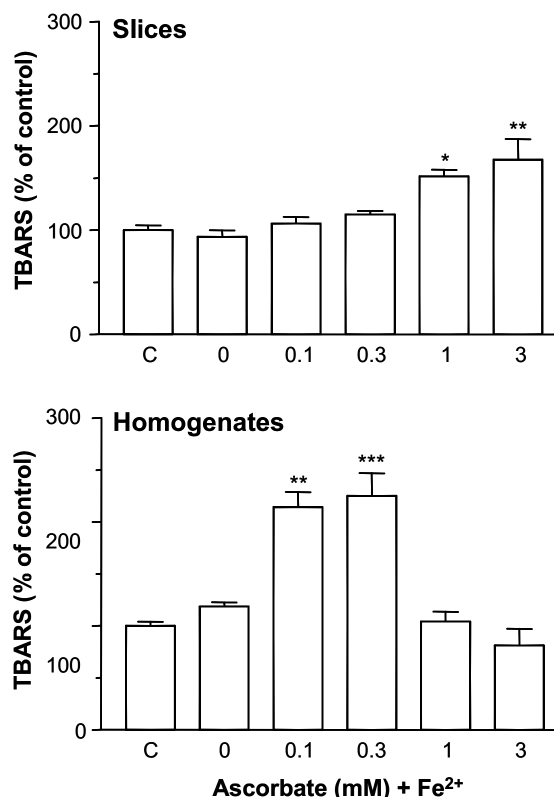

Figure 2. Effect of ascorbate treatment on liver slices (upper panel) and liver tissue homogenates (lower panel) in the presence of 50 μM ferrous ion (Fe^{2+}). C, absolute control without ascorbate and Fe^{2+}. Quantities of TBARS are expressed as percentages of control values. Each bar represents the mean±SEM of six replicates. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

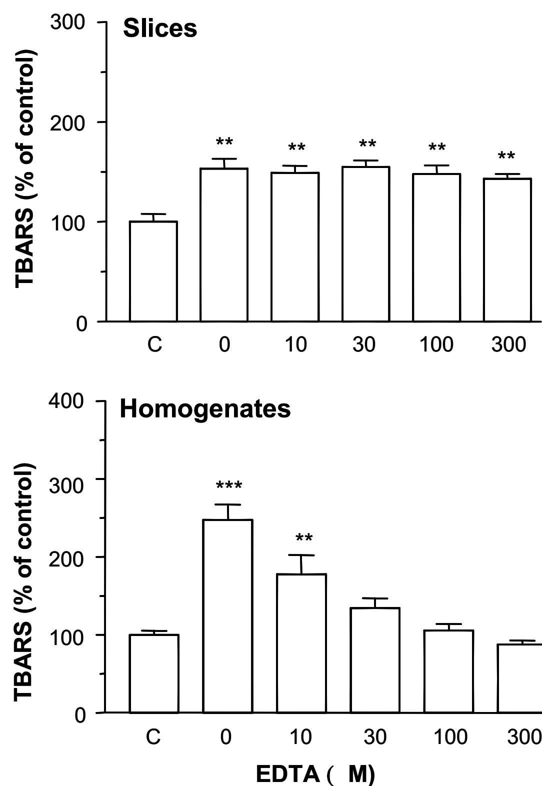

Figure 3. Effects of EDTA treatment on liver slices (upper panel) in the presence of 0.1 mM ascorbate plus 50 μM ferrous ion (Fe^{2+}), and liver tissue homogenates (lower panel) in the presence of 0.l mM ascorbate plus 50 μM Fe^{2+}. C, absolute control without ascorbate, Fe^{2+} and EDTA. Quantities of TBARS were expressed as percentages of control values. Each bar represents the mean±SEM of six replicates. **$P < 0.01$, ***$P < 0.001$.

ascorbate plus 50 μM Fe^{2+}, TBARS concentrations were elevated to 248±20% above control and increasing concentrations of EDTA (0, 30, 100, 300 μM) lowered TBARS concentrations in a concentration dependent manner (lower panel, Figure 3, $r = 0.739$). These observations showed that 300 μM EDTA was sufficient to inactivate 50 μM Fe^{2+}, and thus trace amounts of contaminating transition metal ions should be completely removed by treatment with EDTA.

In liver slices with both 50 μM Fe^{2+} and 1 mM ascorbate, TBARS concentration were increased to 153±10% (upper panel, Figure 3), confirming previous experiments on liver slices (upper panel, Figure 2). However, when different concentrations of EDTA (0, 10, 30, 100, 300 mM) were added to the medium containing 1 mM ascorbate and 50 μM Fe^{2+}, EDTA did not alter TBARS concentrations (upper panel, Figure 3) confirming that ferrous ions did not contribute to generating oxidative stress in liver slices.

We next examined whether iso-ascorbate, an epimer of ascorbate induces oxidative stress. The primary chemical structure of iso-ascorbate is the same as ascorbate. In contrast to the oxidative action of ascorbate in liver slices, iso-ascorbate concentrations up to 3 mM did not elevate TBARS productions above control values (Figure 4).

Ascorbate is easily oxidized to dehydroascorbate, and it is known that cells take up dehydroascorbate from extracellular fluid through a GLUT (Banhegyi *et al.*, 1998), and that GLUT inhibitors such as wortmannin suppress the transport capacity of glucose. We measured the uptake of [^{14}C]ascorbate in liver slices. Treatment groups containing 1 or 10 μM wortmannin showed significantly lower quantities of radioactivity (76±8%, $P < 0.05$ or 62±5%, $P < 0.01$) than the control group (100±5%) in hepatocytes after 4 h incubations. These observations support the concept that [^{14}C]ascorbate is taken up through a GLUT after oxidation to [^{14}C]dehydroascorbate as wortmannin suppressed uptake of the labeled compound (Figure 5).

In order to further elucidate the mechanism of oxidative stress induced by ascorbate, the effects of glutathione and wortmannin on TBARS production

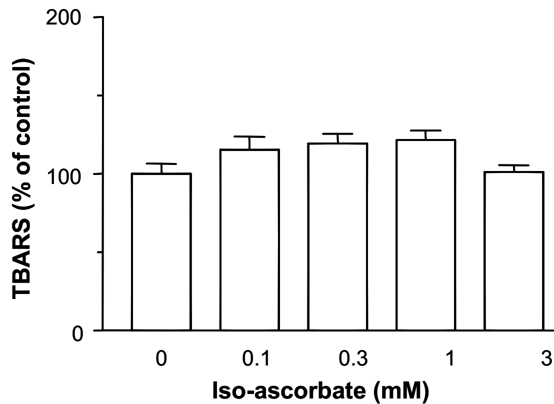

Figure 4. Effects of iso-ascorbate treatment on liver slices. Quantities of TBARS were expressed as percentages of control values. Each bar represents the mean±SEM of six replicates. No significant differences were found. Each bar represents the mean±SEM of six replicates.

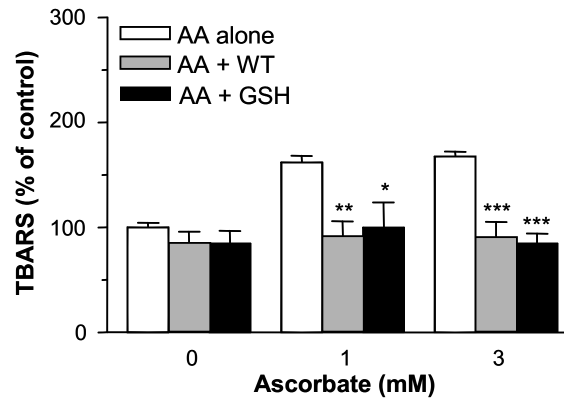

Figure 6. Effects of wortmannin and glutathione on TBARS production induced by ascorbate. Liver slices were treated with different concentrations of ascorbate (0, 1, and 3 mM) in presence of 10 μM wortmannin (AA+WT) or 1 mM glutathione (AA+GSH). Each bar represents the mean±SEM of six replicates. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* AA alone.

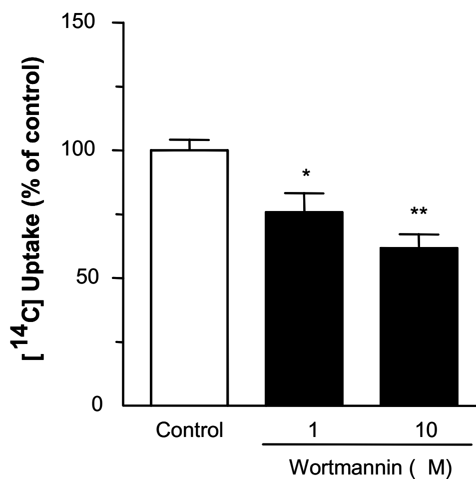

Figure 5. Effects of wortmannin on uptake of ^{14}C-labeled compound. Liver slices were treated with either 1 μM or 10 μM wortmannin in culture medium containing 0.1 μCi [^{14}C]ascorbate plus 10 μM ascorbate. DPM of ^{14}C-labeled compound were counted, converted to DPM/mg protein and expressed as percentages of control values. Each bar represents the mean±SEM of six replicates. $^{*}P < 0.05$, $^{**}P < 0.01$.

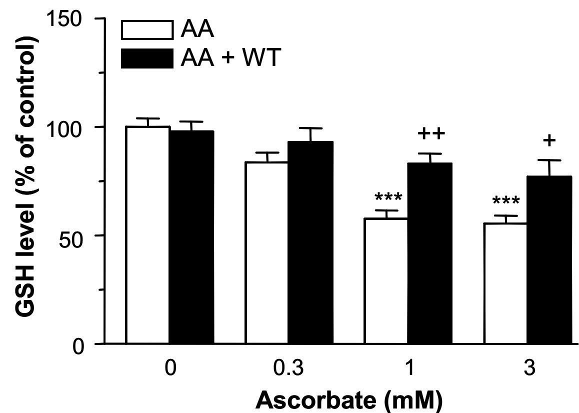

Figure 7. Effects of wortmannin on ascorbate-induced changes of endogenous glutathione (GSH) levels in liver slices. Liver slices were treated with different concentrations of ascorbate (0, 1, and 3 mM) without (AA) or with 10 μM wortmannin (AA+WT). Each bar represents the mean±SEM of six replicates. $^{***}P < 0.001$; difference between control and treatment group. $^{+}P < 0.05$, $^{++}P < 0.01$; difference between treatment groups of ascorbate and ascorbate plus wortmannin.

were tested. While 1 mM glutathione or 10 μM wortmannin alone did not change TBARS concentration (control group, Figure 6), we again confirmed that 1 and 3 mM ascorbate stimulated TBARS productions to 162±6% and 167±14%, respectively (Figure 6). The 1 or 3 mM ascorbate-induced TBARS production in the presence of 1 mM glutathione were suppressed from 162±6% to 109±24% ($P < 0.05$) or from 168 ±14% to 104±10% ($P < 0.001$), respectively (Figure 6, $n = 6$). Thus, 1 mM glutathione prevents oxidation of ascorbate to dehydroascorbate. The TBARS concentrations in the presence of 10 μM wortmannin and 1 mM (109±17, $P < 0.01$) or 3 mM ascorbate (101 ±14%, $P < 0.01$) were significantly lower than those of 1 mM (162±6%, $P < 0.01$) or 3 mM (167±14%, $P < 0.001$) ascorbate alone (Figure 6), supporting our hypothesis that wortmannin inhibits the "dehydroascorbate transporter" (GLUT).

In order to further support the notion that ascorbate generates oxidative stress, endogenous glutathione concentrations in the liver slices were determined. Endogenous glutathione concentrations in liver slices were significantly reduced in a concentration-dependent manner after ascorbate treatments (Figure 7,

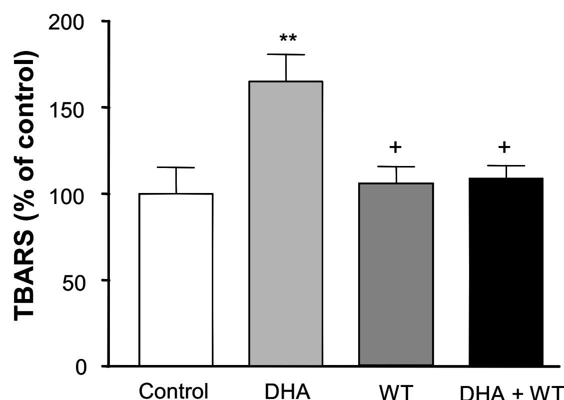

Figure 8. Effect of dehydroascorbate in liver slices. Liver slices were treated with 1 mM dehydroascorbate without (DHA) or with 10 μM wortmannin (DHA+Wortmannin). Each bar represents the mean±SEM of six replicates. $^{**}P < 0.01$, difference between control and treatment group. $^{+}P < 0.05$, difference between treatment groups of DHA and DHA plus wortmannin.

r = 0.806). Higher concentrations of ascorbate (1 and 3 mM) significantly decreased glutathione levels to 58 ±4% ($P < 0.001$) and 56±4% ($P < 0.001$), respectively. The glutathione concentrations in treatment groups with ascorbate (1 and 3 mM) and 10 μM wortmannin were higher than those induced by ascorbate alone (Figure 7), as confirmed by TBARS levels in liver slices. Interestingly, treatment of liver slices with 1 mM dehydroascorbate resulted in a TBARS level that was 165±16% ($P < 0.01$, $n = 6$) greater than control values and TBARS levels in liver slices incubated with 1 mM dehydoascorbate in the presence of 10 μM wortmannin was significantly lower than that of dehydroascorbate alone (109±7%, $P < 0.05$) (Figure 8), consistent with other observations that high levels of dehydroascorbate have been proven to be toxic to various cells and tissues (Paolicchi *et al*., 1996; Song *et al*., 1999 and 2001).

Discussion

Few mechanisms have been proposed to explain the pro-oxidant action of ascorbate. One of them is the Fenton reaction in which ascorbate reacts with ferrous ion (Fe^{2+}) to generate free hydroxyl radicals that have a powerful oxidative potential (Cardoso *et al*., 1998; Miller and Aust, 1989). This is a logical explanation since a substantial amount of iron is found in hemoglobin and its breakdown products and also since ascorbate is highly concentrated in several tissues such as adrenal glands and liver (Hornig, 1975). We have confirmed that ferrous ions potentiate ascorbate-induced oxidative stress in liver homogenates (lower panels, Figure 1 and 2). In liver slices, however, the level of ascorbate-induced oxidative stress did not further increase when the slices were also treated with ferrous ions (upper panels, Figure 1 and 2). Oxidative stress indicated by TBARS levels in liver slices treated with ascorbate plus Fe^{2+} contradict other studies that suggest an ascorbate-Fe^{2+} interaction is responsible for inducing lipid peroxidation (Ramassamy *et al*., 1994; Cardoso *et al*., 1998). However, none of the encountered studies examined the impact of ascorbate treatment in culture medium. It is believed that in serum containing medium, iron binds to the proteins and is thus unable to interact with the ascorbate in order to induce oxidative stress. To further confirm that oxidative stress is not due to an interaction between ascorbate and ferrous ions, we examined the extent of lipid peroxidation in culture medium containing ascorbate, Fe^{2+} and EDTA. EDTA is a powerful metal chelator (Song *et al*., 1999). Thus, if no significant difference is detected among groups containing EDTA or not, such would further support the proposition that no heavy metal ion significantly influences the extent of lipid peroxidation. In present study, EDTA eliminated ferrous ion-induced oxidative stress in liver homogenates (lower panel, Figure 3), but was ineffective in liver slices in reducing TBARS concentrations generated by both 0.1 mM ascorbate and 50 μM ferrous ion (upper panel, Figure 3). This result was consistent with our previous observation that the combination of ascorbate (10-100 μM) and ferrous ion increased concentrations of TBARS in PC12 cells and cerebrocortical homogenates, but did not increase TBARS when the medium was supplemented with 10% fetal bovine serum or albumin (Song *et al.,* 2001). Thus, it is unlikely that the Fenton reaction is a mechanism of ascorbate-induced oxidative stress in culture medium containing serum or in *in situ* tissues where plasma is freely available.

We proposed an alternative mechanism to explain ascorbate-induced oxidative stress. Ascorbate is a powerful reducing agent and thus is easily oxidized to ascorbyl radical and then to dehydroascorbate. Ascorbate is known to be transported into cells *via* three different mechanisms: (i) Na^{+} dependent uptake (Spector and Lorenzo, 1973), (ii) glutamate-ascorbate heteroexchange (Miele *et al*., 1994) and (iii) *via* a GLUT (Vera *et al*., 1993). The first two mechanisms of ascorbate uptake do not generate any oxidative stress since ascorbate cannot accept additional electrons under normal conditions. GLUT is an efficient transporter for ascorbate uptake in the form of dehydroascorbate (Welch *et al*., 1995), and the only carrier system which can generate oxidative stress (Song *et al.,* 2001 and Brown *et al.,* 2002). After being taken up into the cytosol, dehydroascorbate is rapidly reduced to ascorbate by enzymatic (Bode *et al*., 1993; May *et al*., 1998; Savini *et al*.,1998) and

chemical reactions. Such reduction of dehydroascorbate to ascorbate means that dehydroascorbate snatches electrons from cytosolic components such as glutathione and other vital cellular compounds to form ascorbate, thus generating oxidative stress. The hepatocyte would not undergo oxidative stress if production rates of reducing agents such as glutathione were higher than uptake rates of oxidative agents. Therefore, high uptake rates of dehydroascorbate would be more critical than the total amount of dehydroascorbate uptake in generating oxidative stress because ingestion of large amounts of ascorbate is unrelated to oxidative stress. Ascorbate treatment decreased glutathione concentrations in liver slices and increased quantities of TBARS (lipid peroxides). These observations strongly support our hypothesis that dehydroascorbate (oxidized ascorbate) uptake generates oxidative stress.

Glutathione suppressed ascorbate-induced oxidative stress (Figure 6). The suppression by exogenous glutathione may not likely be caused by scavenging the oxidative potential in hepatocytes where glutathione does not easily diffuse into. Glutathione prevents the oxidation of ascorbate in medium, thus inhibiting production of dehydroascorbate. The action of glutathione is consistent with the effects of wortmannin described below. In order to explain the role of the GLUT on dehydroascorbate transport into the hepatocytes, the effects of wortmannin were tested (Figure 6, 7 and 8). Wortamannin inhibits phosphatidylinositol 3-kinase (PI3-kinase) which plays many different roles in transduction systems (Arcaro and Wymann, 1993; Clarke *et al.*, 1994). PI3-kinase activates GLUT by translocation from its cytosolic sites to the plasma membrane (active sites). Insulin enhances glucose uptake by mobilizing GLUT from intra-cellular compartments to the plasma membrane by activating PI3-kinase (Cushman and Wardzala, 1980; Okada *et al.*, 1994; Evans *et al.*, 1995). Inhibition of PI3-kinase suppressed the transport capacity of GLUT (Figure 5 and 6). We, therefore, hypothesized that rates of dehydroascorbate uptake will be decreased and thus intensity of oxidative stress will be decreased in the presence of wortmannin. Our observations with wortmannin (Figure 5 and 6) support the hypothesis.

Also our hypothesis is supported by the observation that ascorbate consistently elevated lipid peroxide production in liver slices but that iso-ascorbate did not (Figure 4). The primary chemical structure of iso-ascorbate is the same as ascorbate and thus their red-ox potentials are equal. Therefore, the red-ox potential (reducing ability) of ascorbate is unrelated to the generation of oxidative stress. The differences in ios-ascorbate and ascorbate tertiary configurations do not confirm tertiary structure of GLUT (after oxidation of dehydroascorbate), and thus dehydro-iso-ascorbate transport into the hepatocytes is hindered. We understand that this inability of transportation by GLUT is the major reason that iso-ascorbate (*via* dehydroisoascorbate) cannot generate oxidative stress.

In liver homogenate dose-response curves between ascorbate concentrations and TBARS productions were a bell shape (lower panel, Figire 1 and 2). At lower concentrations (0.1 and 0.3 mM), ascorbate enhanced TBARS production, but was ineffective at higher concentrations (1 and 3 mM). We postulated that subcellular organelles and resealed broken down cells may pick up dehydroascorbate *via* a GLUT and generate oxidative stress, while the reducing property of the higher (1 and 3 mM) concentrations of ascorbate in medium overrides the oxidative stress (lower panels, Figure 1 and 2). The physical structure of subcellular organelles may be weaker than that of intact hepatocytes and cannot protect against the reducing environment in the medium.

While the results obtained in liver slices are similar to those observed during similar experiments carried out in brain cortical slices, the increases in lipid peroxidation induced by incubation with ascorbate in this experiment were less marked. For example, in the present experiment, treatment with 1 mM ascorbate resulted in a 162±6.4% increase in TBARS levels, but exposure of brain slices to 1 mM ascorbate resulted in a 236±20% increase in TBARS levels as compared to control (Song *et al.*, 1999, 2001). Results may be less amplified than in those obtained in brain cortical slice studies for two reasons. While ascorbate levels increase between five and six-fold in the brain extracellular fluid during ischemia, it increases by 60% in the liver extracellular fluid during ischemia (Layton *et al.*, 1996). The extent of dehydroascorbate transport into liver cells in order to restore the physiological concentrations of ascorbate would not significantly differ between liver and brain. Therefore comparatively smaller amounts of dehydroascorbate would be reduced back to ascorbate in liver cells, necessitating the removal of fewer electrons from the lipids that comprise the cellular membranes. In addition, the extent of peroxidation induced by incubation with ascorbate in the hepatocytes may have been lower than in cortical brain slices because the liver possesses relatively high levels of glutathione as compared to other organs. Levels of glutathione in the liver have been estimated to be 5.3±0.3 mM, while in the kidney levels are 3.00±0.18 mM and in the colon only 0.21±0.01 mM (Rose and Bode, 1995).

Ascorbic acid concentrations in extracellular fluid were 60% higher than controls after hypoxia (Layton *et al.*, 1996), showing that high concentrations (4.3±0.5 mM) of ascorbate in the liver (Oriot *et al.*, 1995; Rose and Bode, 1995) cannot be sustained without

a sufficient energy supply. The released ascorbate is oxidized to dehydroascorbate, likely by molecular oxygen in plasma. This dehydroascorbate is then efficiently carried into the hepatocytes *via* GLUT and reduced to ascorbate. That dehydroascorbate is then recycled to ascorbate within hepatocytes, and cellular components are then oxidized during the reduction of dehydroascorbate. We suggested that dehydroascorbate could mimic the effects of ascorbate in hepatocytes, which can be considered to be a cause of liver oxidative injury during hepatic ischemia. Oxidative stress generated in liver during the post-surgical reperfusion can be attenuated by inhibiting the uptake of dehydroascorbate. Our observations hold potentially important physiological implications and the possibility that ascorbate (*via* dehydroascorbate) plays a pro-oxidant role in liver damage during hepatic surgery which has not previously been addressed.

In conclusion, present results support the hypothesis that the prooxidant effects of ascorbate are in fact mediated by dehydroascorbate transport. Ascorbate is easily oxidized to dehydroascorbate within the extracellular environment and then carried into cells *via* a GLUT. Once inside the cell, reduction back to ascorbate necessitates the removal of electrons from intracellular components. However, if these electrons are removed from vital cell components, for example the lipids that comprise cell membranes, oxidative stress may result.

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