Protective Role of Dehydroascorbate in Rat Liver Ischemia-Reperfusion Injury

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Background. **Oxidative stress plays an important role in liver ischemia/reperfusion (I/R) injury. Thus, enhancing the liver antioxidant capacity could be a promising therapeutic strategy. Ascorbate (AA) is considered the perfect antioxidant, but its therapeutic efficacy is greatly limited by its slow achievement of high intracellular levels. This might be circumvented by administering dehydroascorbate (DHA), which presents a several-fold greater uptake than AA, and undergoes rapid intracellular reduction to AA. Thus, our aim was to assess the protective role of DHA in liver I/R injury.**

Materials and methods. **Wistar rats (200–300 g bw) were pretreated iv with different doses of AA or DHA 20 min before liver ischemia, followed by 6 h reperfusion. Liver damage was assessed by biochemical and morphological indices.**

Results. **DHA pretreatment induced a rapid increase in liver ascorbate levels, significantly higher than findings for AA, without any significant reduction in glutathione levels. Liver damage during I/R in controls showed significant increases in serum transaminases and hepatic thiobarbituric acid reactive substances with alterations of liver morphology. DHA administration induced a clear, significant protection against I/R injury, whereas liver damage was only moderately prevented by AA.**

Conclusions. **DHA might represent a simple, effective therapeutic option to prevent liver damage associated with ischemia/reperfusion.** © 2004 Elsevier Inc. All rights reserved.

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INTRODUCTION

Liver ischemia-reperfusion (I/R) injury is a serious, but apparently unavoidable, complication in several circumstances such as hepatic resectional surgery (Pringle maneuver), liver transplantation, and hemorrhagic shock with fluid resuscitation [1, 2]. The consequences of I/R injury include liver and remote organ failure in more severe cases, both of which have high rates of morbidity and mortality. Despite improvements in organ preservation and surgical techniques, I/R injury remains a significant clinical problem, and there is considerable interest in its prevention [3].

Various mechanisms have been proposed for the pathogenesis of postischemic tissue injury, but data obtained by several researchers indicate that the generation of oxygen-derived free-radicals probably represents the most important factor involved [4–6]. According to this hypothesis, "reperfusion injury" is associated with the production of toxic free-radical species after the resupply of blood (or oxygen) to previously ischemic (or hypoxic) tissues [7]. Enhancing the liver antioxidant capacity can thus be viewed as a promising therapeutic strategy [8]. Approaches involving the administration of various antioxidants have been used, but the effectiveness of these interventions has varied [9–12].

Protection against oxidative stress is afforded by several antioxidant systems, including both enzymic and nonenzymic. In view of its biochemical properties (presence in adequate amounts within the cells; capability to react with a variety of free radicals; suitability to be regenerated in the active form),

ascorbic acid is generally considered the perfect antioxidant for the cells of nearly all aerobic organisms (for a recent review, see ref. 13). Ascorbate is thought to act by scavenging aqueous free-radicals [14–16] as well as by reducing lipophilic tocopheroxyl radicals back to α-tocopherol [17]. Cellular accumulation of vitamin C is due to transport of both ascorbate (via Na^+-ascorbate symporters such as SVCT1 and SVCT2) [18] and its oxidized metabolite, dehydroascorbic acid (DHA) [19–21]. Although ascorbate is the predominant, if not the unique, form in blood, DHA may be produced extracellularly in particular pro-oxidant microenvironments [22, 23]. Experiments in neutrophils demonstrated that the rate of cellular DHA uptake is as much as 30-fold greater than that of ascorbate uptake [21, 22]. Once transported, DHA is immediately reduced intracellularly to ascorbate, thus maintaining a favorable gradient for the accumulation of ascorbate via this pathway. DHA uptake followed by intracellular reduction can increase intracellular ascorbate levels 5- to 20-fold within minutes. This process, termed ascorbate recycling, has been demonstrated experimentally in human neutrophils, as well as in other cell types [19–24].

Recently, Huang *et al*. [25] demonstrated that DHA, unlike exogenous ascorbate, confers *in vivo* dose-dependent neuroprotection in a murine model of reperfused and nonreperfused cerebral ischemia. Here, for the first time, we show that DHA administration *in vivo* is able to rapidly raise hepatic ascorbate content well above physiological levels, and that this increase results in a remarkable protection against liver I/R injury, significantly higher than that obtained with ascorbate treatment.

MATERIALS AND METHODS

Hepatic ischemia/reperfusion injury model. Male Wistar rats (200-300 g bw) were purchased from Harlan Italy (Correzzana, MI, Italy). Rats were housed in a controlled environment at 22 ± 1°C under a 12-h/12-h light/dark cycle. Food and water were available *ad libitum*. The experimental protocol followed the *Principles of Laboratory Animal Care* (US NIH Publication No. 83-25, revised 1985) as well as the recommendations of Italian law for the use of experimental animals (DL No. 116/1992), and was approved by the Ethical Committee of the University of Pisa Medical School.

Rats were divided into the three following experimental groups: (1) DHA-pretreated rats, receiving intravenously 125, 250, or 500 mg/kg dehydroascorbic acid dissolved in a sodium acetate/sodium bicarbonate buffer, pH 5.5 [25]; (2) ascorbate (AA)-pretreated rats, receiving intravenously the same amount of ascorbic acid; (3) placebo-pretreated rats receiving intravenously equal volumes of the vehicle only. Twenty minutes after intravenous pretreatment, rats were anesthetized with pentobarbital (50 mg/kg ip); the abdomen was opened through a midline incision, and the hepatoduodenal ligament was exposed. In each rat, the ischemic phase was maintained through complete occlusion of the ligament using an atraumatic microvessel clip for 30 min. At the end of the ischemic phase, the clip was removed and liver reperfusion was allowed for different periods of time (1, 2, 4, or 6 h).

Blood samples were collected from the tail vein just before ischemia and after 0, 1, 2, 4, and 6 h of reperfusion.

Measurement of serum transaminase activities. Serum alanine transaminase (ALT) and aspartate transaminase (AST) activities were measured using commercially available kits (Sigma-Aldrich, Milano, Italy) and the results were obtained as units/liter.

Histological study. Liver samples obtained at various determination points were fixed in 10% buffered formalin and were then embedded in paraffin, in accordance with standard procedures. Sections (5 μm) were stained with hematoxylin and eosin and examined microscopically.

Assay of TBARS in liver tissue. Liver specimens were homogenized with 10 vol of 1.15% KCl, using a Teflon Potter–Elvehjem homogenizer, and were assayed for thiobarbituric acid-reactive substances (TBARS) by the method of Ohkawa et al. [26].

Assay of water-soluble antioxidants in liver tissue. Liver specimens were homogenized with a Teflon pestle in 10 vol of 5% TCA and centrifuged at 12000 g for 20 min. AA, DHA, GSH, and GSSG were quantified on the supernatant by HPLC with electrochemical detection [27].

Liver tissue protein concentration was assayed by the method of Lowry *et al*. [28] using bovine serum albumin as the standard.

Statistical analysis. Data are expressed as means ± SEM. The statistical significance of the experimental treatment was assessed by factorial analysis of variance (ANOVA), followed by Tukey's post-test with multiple comparison. When appropriate, the two-tailed unpaired Student's t test was used as a method of post-hoc analysis to assess two-by-two differences. A P value <0.05 was considered significant.

RESULTS

Ascorbic Acid and Glutathione Levels in Rat Liver after Pretreatment with Ascorbate or Dehydroascorbate

Figure 1 shows the levels of ascorbic acid in the rat liver 20 min after the iv injection of three different amounts (125, 250, or 500 mg/kg bw, respectively) of AA or DHA. Since the amount of oxidized ascorbate detected was always negligible, without any significant difference between groups, only the levels of the reduced form will be shown in this as well in all of the following figures. As expected, both treatments caused a significant, dose-dependent, increase in hepatic ascorbate intracellular levels, but the effect of DHA was significantly greater than that of AA for each dose used. At 250 mg/kg, for example, the percentage increase compared with untreated controls was +59% for AA and +176% in the case of DHA, respectively.

In the liver of the same animals, we also determined the levels of glutathione; results are shown in Fig. 2. As a consequence of AA pretreatment, no differences were observed in GSH or GSSG hepatic levels with respect to untreated controls at the doses used; on the other hand, in DHA-pretreated rats, probably due to the rapid, significant DHA-induced activation of GSH-dependent ascorbate recycling, a moderate, dose-dependent decrease in GSH levels was observed with a concomitant increase in GSSG levels. These changes were statistically significant only in the case of the highest DHA dose (i.e., 500 mg/kg). For this reason,
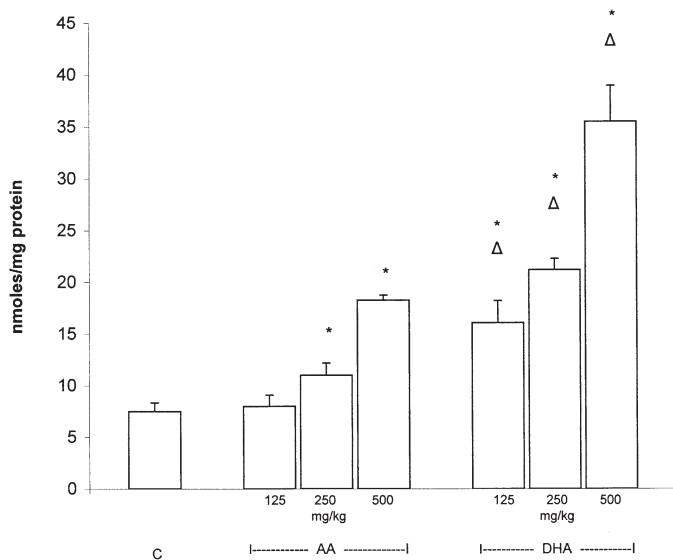
FIG. 1. Hepatic levels of ascorbic acid in untreated control rats (C) and in rats pretreated with different doses of ascorbate (AA) or dehydroascorbate (DHA). Levels were assayed 20 min after the iv injection of AA or DHA. Data are expressed as means of five to six observations for each group ± SEM. Statistical analysis (Student's t-test): $*P < 0.01$ versus untreated controls. $\Delta P < 0.01$ DHA versus the corresponding AA dose.

and also to minimize as far as possible the known adverse effects of DHA when injected intravenously (elevation of blood pressure, salivation and lacrimation) [25, 29], we decided to use in the following experiments (aimed at testing the protective effect of DHA pretreatment against liver I/R injury) only the intermediate DHA dose (i.e., 250 mg/kg), which significantly increased the intracellular hepatic levels of ascorbic acid without inducing a concomitant significant depletion of GSH levels.

Liver Ischemia-Reperfusion Injury: Effects of AA and DHA Pretreatment

In control rats both ALT and AST serum activities increased dramatically after ischemia and during reperfusion (Fig. 3A and B, respectively), reaching their maximum levels after 6 h reperfusion. This increase in serum transaminase activities was only moderately prevented in AA-pretreated rats, whereas the protective role of DHA pretreatment was much more evident (Fig. 3).

As a further biochemical index of liver damage, more directly related to oxidative stress, we assayed also malonyldialdehyde production in the rat liver using the thiobarbituric colorimetric assay. As shown in Fig. 4, during ischemia-reperfusion there was a significant, progressive increase in TBARS levels in the liver of control rats. This increase was only moderately prevented by AA pretreatment, whereas it was almost completely abolished in the liver of DHA-pretreated rats.

Histological changes of the liver after ischemia/reperfusion injury were in keeping with the above biochemical observations. Liver sections of untreated control rats after 6 h reperfusion showed extensive, multifocal, neutrophil infiltrated areas of coagulative necrosis scattered throughout the hepatic parenchyma (Fig. 5A and B). On the contrary, in livers of DHA-pretreated rats, no histological evidence of necrosis was evident and the histological architecture of the liver parenchyma was well preserved, although several areas of neutrophil infiltration were observed (Fig. 5C and D). The histological lesions in the liver of AA-pretreated rats, although generally less severe and widespread than in untreated controls, showed a lesser degree of preservation of the normal hepatic architecture (not shown).

Changes in the Hepatic Levels of Ascorbic Acid and Glutathione during Ischemia-Reperfusion Injury

We also assayed the variations of the hepatic levels of ascorbic acid during ischemia-reperfusion injury in untreated controls and in rats pretreated with ascorbate or dehydroascorbate (250 mg/kg bw). Quite surprisingly, in all of the experimental groups (untreated controls and AA- or DHA-pretreated rats), no significant differences were detected with respect to the preischemic basal values, either at the end of the ischemic period (30 min of total hepatic ischemia) or after 4 and 6 h of reperfusion (Fig. 6). In other words, at the end of the ischemia-reperfusion time, the livers of the three experimental groups maintained almost unchanged the differences observed 20 min after the intravenous administration
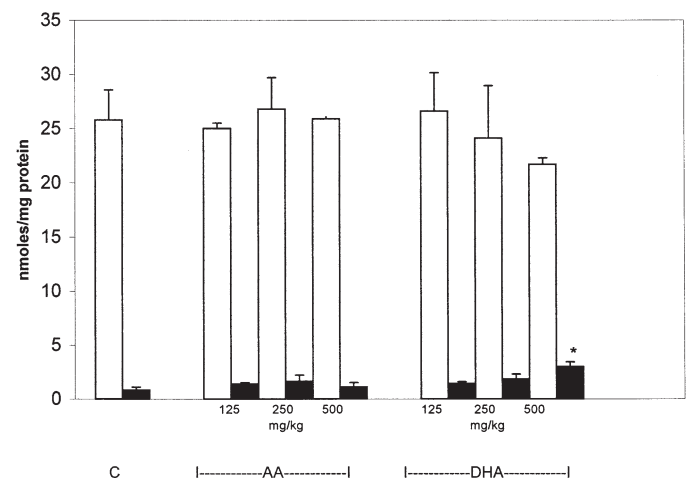

FIG. 2. Hepatic levels of GSH (open bars) and GSSG (closed bars) in control rats (C) and in rats pretreated with different doses of acsorbate (AA) or dehydroascorbate (DHA). Levels were assayed 20 min after the iv injection of AA or DHA. Data are expressed as means of five to six observations for each group ± SEM. Statistical analysis (Student's t-test): $*P < 0.05$, at least, versus the corresponding level in control rats.
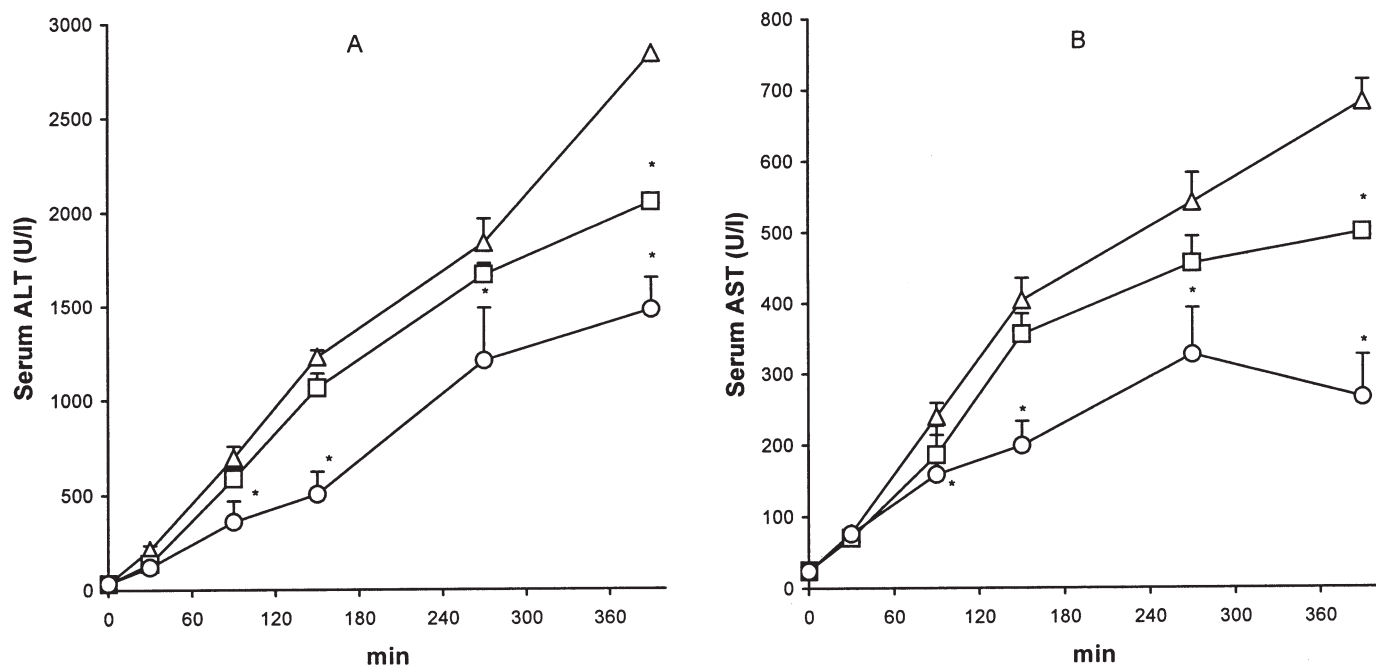
FIG. 3. Variation of serum levels of ALT (A) and AST (B) enzyme activities during liver ischemia (0–30 min) and reperfusion (30–390 min) in control rats (△) and in rats pretreated with 250 mg/kg of ascorbate (□) or dehydroascorbate (○) 20 min before ischemia. Data are expressed as means of five to six observations for each group ± SEM. Statistical analysis (ANOVA and Tukey test) showed that, for both ALT and AST, the protective effect of DHA was significantly greater than that afforded by AA. *$P < 0.01$ *versus* the corresponding level in control rats (Student's t test).

of AA or DHA, illustrated in Fig. 1. On the other hand, 30 min of total liver ischemia caused a significant decrease in hepatic GSH levels with a concomitant increase in GSSG levels (Fig. 7) in all of the experimental groups (with the result that the total glutathione content was not significantly modified at the end of the ischemic time). During reperfusion, GSH levels remained unchanged in the liver of all groups of rats, whereas GSSG levels underwent a significant decline (probably due to the leakage of the oxidized form of glutathione from hepatic cells); consequently, after 6 h reperfusion the total glutathione content was significantly lower in all experimental groups.

DISCUSSION

The objective of this study was to evaluate the possibility of preventing the damage associated with liver I/R by the previous administration of the oxidized metabolite of vitamin C, DHA. Oxygen-free radicals have long been indicated as being responsible for I/R-induced liver damage [4–6]. It was subsequently recognized that this increasing production of oxygen radicals and postischemic damage of the liver have a two-phase time course: an initial phase of injury (roughly within the first 2 h after reperfusion) and a late phase, from approximately 3 to 24 h of blood reflow [5, 30, 31]. In the initial phase of reperfusion, there is an overpro-

duction of oxygen radicals in activated Kupffer cells [30, 31] and in the mitochondria of hepatocytes and endothelial cells, where oxygen reflow encounters highly reduced respiratory chains [5, 31, 32]. Events occurring during this early phase, including activation of Kupffer cells, initiate a complex inflammatory pathway which culminates in the hepatic accumulation of neutrophils [30]. Recruited neutrophils directly damage hepatocytes by releasing oxidants and proteases and are responsible for the later phase of liver injury induced by I/R (for a review see ref. 33).

AA is one of the water-soluble vitamins. With its strong reducing property and its property of scavenging free radicals, AA is well known as a strong antioxidant agent [13]. However, in a therapeutic perspective, it must be remembered that at least some of harmful consequences of free-radical production can be efficiently counteracted by AA only at supraphysiological concentrations [34]. To circumvent the difficulty to reach and maintain this increase in intracellular levels by the administration of exogenous AA, we used its oxidized metabolite dehydroascorbate. Here we show, for the first time, that the *in vivo* iv administration of DHA rapidly induces a significant dose-dependent increase in hepatic intracellular AA levels, in agreement with previous results obtained *in vitro* [19–24]. Inside the cell, there are several enzymatic systems capable of reducing DHA to AA [35–39], whose main function is believed to be that of restoring the antioxidant poten-
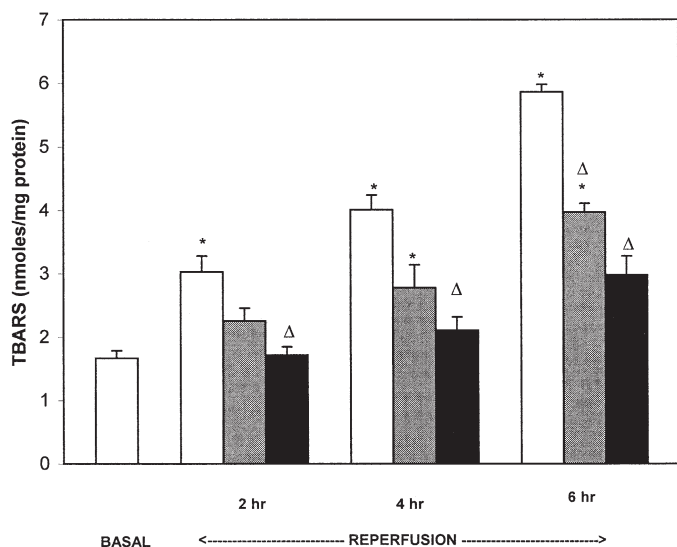
FIG. 4. Hepatic levels of TBARS during ischemia/reperfusion in control rats (white bars) and in rats pretreated with 250 mg/kg of ascorbate (gray bars) or dehydroascorbate (black bars) 20 min before ischemia. Data are expressed as means of five to six observations for each group $\pm$ SEM. Statistical analysis (ANOVA and Tukey test) showed that the protection obtained with DHA pretreatment was significantly higher with respect to that induced by AA. *$P < 0.01$, *versus* the basal (end of ischemia) level; $\Delta P < 0.01$ *versus* the corresponding (same reperfusion time) level in untreated controls (Student's *t*-test).

FIG. 5. Histological lesions in the liver of untreated control rats (A and B) and of rats pretreated 20 min before ischemia with 250 mg/kg of dehydroascorbate (C and D) observed after 6 h reperfusion following 30 min total liver ischemia. (Magnification: A, C, D, $\times 20$; B, $\times 40$).

tial of ascorbate, maintaining an effective steady-state concentration in basal conditions as well as during oxidative stress, independently of *de novo* synthesis and dietary supply.

Our results show that the administration of DHA can cause in rat liver a decrease in GSH with a concomitant increase in GSSG levels, which is particularly evident at the highest DHA dose (500 mg/kg). Recently, May *et al.* [40] reported that cultured bovine aortic endothelial cells (BACEs) have a high capacity of recycling ascorbate, and that GSH levels can be affected in a similar way by this reduction. Incubation of BACEs with relatively high concentrations of DHA caused a small but progressive depletion in intracellular GSH, which was significant at DHA concentrations of 1 mM and higher [40]. For the above reasons, we decided to perform our protection experiments *in vivo* using the intermediate DHA dose (250 mg/kg), which still significantly increases intracellular AA levels, but is devoid of this GSH-depleting capability. In our model, 30 min total liver ischemia, obtained by clamping the hepatoduodenal ligament, was followed by different times of reperfusion (up to 6 h). Liver damage induced by I/R was clearly demonstrated in control rats by either biochemical or histological parameters. As a result of the increased hepatic levels of ascorbate obtained with the intravenous injection of AA or DHA 20 min before ischemia, both pretreatments afforded significant protection against liver damage. However, whereas pro-
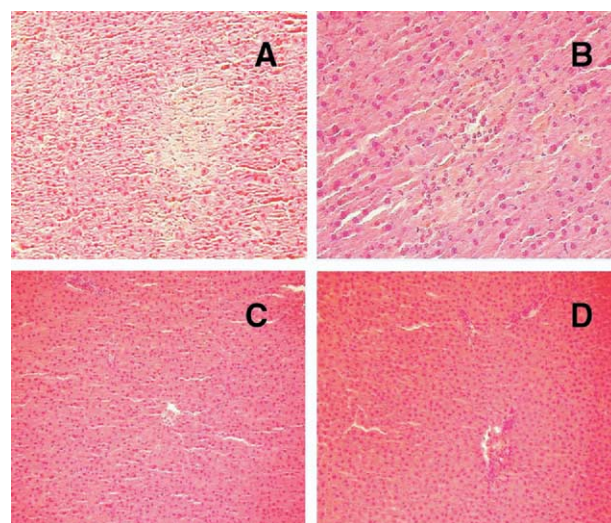
tection induced by AA pretreatment was only moderate, the injection of DHA, which induced the largest increase in liver ascorbate levels, produced a much greater protection, judging by the various damage parameters. In this regard, we may mention here that as unavoidable consequence of the used surgical procedure (total occlusion of the hepatoduodenal ligament) a certain degree of mesenteric congestion was always present in the animals subjected to liver ischemia. Therefore, we must consider that DHA pretreatment was effective in decreasing oxygen-free radical damage not only from the liver but also from gut. The partial protective effect of AA pretreatment was in agreement
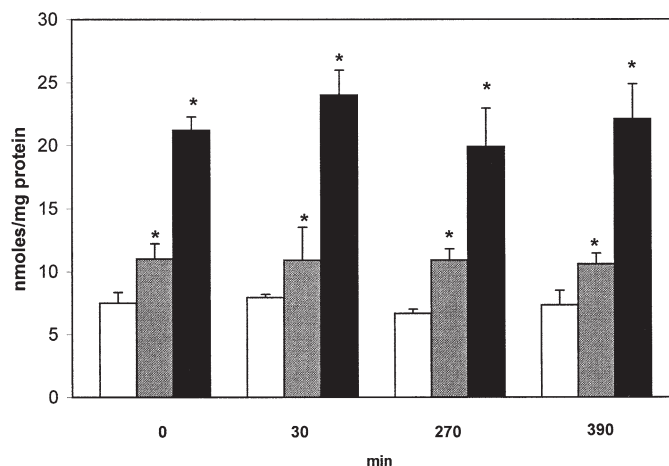

FIG. 6. Hepatic levels of ascorbic acid in control rats (white bars) and in rats pretreated with 250 mg/kg ascorbate (gray bars) or dehydroascorbate (black bars) during ischemia (0–30 min) and reperfusion (30–390 min). Data are expressed as means of five to six observations for each group $\pm$ SEM.
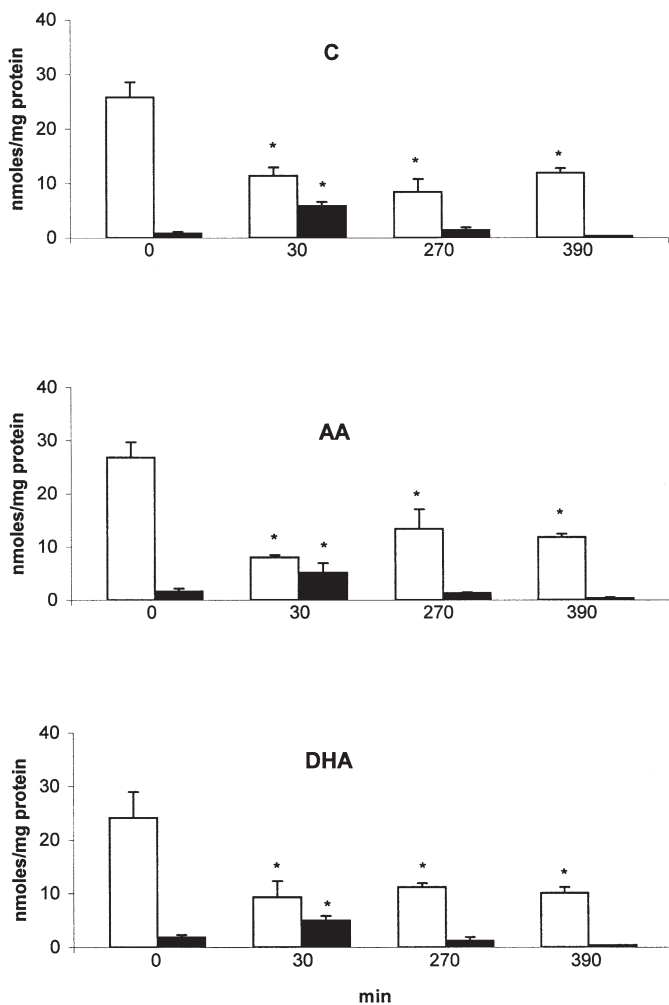
FIG. 7. Variations of the hepatic levels of GSH (open bars) and GSSG (closed bars) during liver ischemia (0–30 min) and reperfusion (30–390 min) in control rats (C) and in rats pretreated with 250 mg/kg of ascorbate (AA) or dehydroascorbate (DHA) 20 min before ischemia. Data are expressed as means of five to six observation for each groups ± SEM. Statistical analysis (Student's t-test): *$P < 0.05$, at least, *versus* the corresponding levels at time 0′.

which has been described in connection with the administration of high doses of AA [42]. In this study we have investigated the possibility of preventing liver damage occurring especially during the early phase of I/R, i.e., the phase most directly related to the overproduction of oxygen-free radicals. The capability of DHA pretreatment to influence also the subsequent phase of liver I/R injury (secondary to the intervention of activated neutrophils) is still an open question and deserves further investigation.

Our results indicated that the degree of protection against liver I/R injury was proportional to the hepatic levels of the antioxidant ascorbate obtained before ischemia. On the basis of our results, the principal effect of both AA and DHA pretreatment seems to be the increase of the total antioxidant capacity of the liver, principally related to the significant rise in intracellular ascorbate to supraphysiological levels. These increased levels of vitamin C could make the liver more effective in scavenging radicals and/or in reducing peroxidative reactions, affording protection from what is generally considered the main physiopathological mechanism involved in liver I/R injury. From this point of view, it is important to note that our results show also a significant decrease in liver GSH levels during I/R. This decrease was almost equivalent in all of the experimental groups and was associated with the preservation of liver ascorbate levels during ischemia and until 6 h of reperfusion. These results could be interpreted considering the fall of GSH as a result of the oxidative stress to which the hepatocytes were subjected, and directly responsible for the maintenance of ascorbate levels by recycling. Thus, cells maintained a steady state of the principal antioxidant at the expense of GSH consumption, and the resulting degree of damage was inversely related to the previous levels of ascorbate, which were higher in DHA-pretreated rats.

with previous descriptions by Ozaki *et al*. [41], but our results were apparently discordant as regards to the protective effect of DHA. Indeed, these authors reported that DHA was less effective than AA in the suppression of lipid peroxidation of the reoxygenated liver tissue [41]. This discrepancy could be, at least in part, attributable to the different experimental protocol (60 min partial *versus* 30 min total liver ischemia; pretreatment 60 min *versus* 20 min before ischemia; intraperitoneal *versus* intravenous injection). On the other hand, Ozaki *et al*. [41] in their article did not report the hepatic ascorbate levels after DHA pretreatment or the effect of DHA injection on the increase in serum transaminase activities induced by liver damage. As a further comment, it may be observed here that another potential advantage of DHA pretreatment is that it can avoid the paradoxical pro-oxidant effect

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