Research article

Protective effect of L-ascorbic acid against oxidative damage in the liver of rats with water-immersion restraint stress

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We examined whether L-ascorbic acid (AA) (or reduced ascorbic acid) protects against oxidative damage in the liver of rats subjected to water-immersion stress (WIRS). AA (100, 250 or 500 mg/kg) was orally administered at 0.5 h before the onset of WIRS. Rats with 6 h of WIRS had increased serum corticosterone, glucose, total ascorbic acid (T-AA), AA, lipid peroxide (LPO), and NOx concentrations and alanine aminotransferase and aspartate aminotrasferase activities. The stressed rats had increased hepatic LPO, NOx, and dehydroascorbic acid concentrations and myeloperoxidase activity, decreased hepatic T-AA, AA, reduced glutathione concentrations and superoxide dismutase activity, and unchanged hepatic vitamin E concentration. Pre-administered AA attenuated the stress-induced changes in serum LPO and NOx concentrations and alanine aminotransferase and aspartate aminotrasferase activities and hepatic LPO, NOx, and T-AA, AA, dehydroascorbic acid, and reduced glutathione concentrations and myeloperoxidase and superoxide dismutase activities dose-dependently. Pre-administered AA did not affect the stress-induced changes in serum corticosterone and glucose concentrations. These results indicate that pre-administered AA protects against oxidative damage in the liver of rats with WIRS possibly by attenuating disruption of the antioxidant defense system and increases in NO generation and neutrophil infiltration in the tissue.

Keywords: Water-immersion restraint stress, liver, L-ascorbic acid, oxidative damage, nitric oxide, neutrophil

Introduction

The model of water-immersion restraint stress (WIRS) is widely used as an experimental model of stress-induced acute gastric mucosal lesions because this model induces reproducible gastric mucosal lesions without resorting to employing surgical or anesthetic procedures. Ischemia-reperfusion injury is one of the important mechanisms for the gastric mucosal lesions induced by WIRS.[1] Reactive oxygen species (ROS) and nitric oxide (NO) generation, lipid peroxidation, and neutrophil infiltration in the gastric mucosal tissue play a critical role in the pathogenesis of WIRS-induced gastric mucosal lesions.[2–5]

Alpekin *et al.*[6] reported that an increase in the level of lipid peroxide (LPO), a product of lipid peroxidation mediated by ROS, occurred without changes in reduced glutathione (GSH) and vitamin C levels in the liver of rats subjected to WIRS for 2.5 h. Furthermore, the same authors have shown in rats with WIRS that the

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hepatic LPO level increases at 2.5 h, but not 5 h, of WIRS, while the hepatic GSH level decreases at 5 h, but not 2.5 h.[7] Iwai *et al.*[8] reported that, in rats subjected to WIRS for 6 h, hepatic LPO levels increased with concomitant increases in serum LPO levels and alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase activities and that pre-administration of gamazumi fruit juice possessing antioxidant activity for 2 weeks attenuated all these stress-induced changes. Furthermore, the same authors reported that not only an increase in LPO level but also a decrease in the activity of superoxide dismutase (SOD) occurred without changes in the activities of catalase and glutathione peroxidase and GSH level in the liver of rats subjected to WIRS for 6 h.[9] Pre-administration of gamazumi crude extract having antioxidant activity for 2 weeks is known to attenuate the decreased hepatic SOD activity and the increased hepatic LPO level found at 6 h of WIRS.[9] We also reported that, in the liver of rats subjected to WIRS over 6 h, cell damage occurred before the appearance of oxidative stress associated with decreases in reduced ascorbic acid (AA) and GSH concentrations and SOD activity and increases in lipid peroxidation, nitric oxide (NO) generation, and neutrophil infiltration, although there was no change in vitamin E (VE) concentration.[10]

Alpekin *et al.*[6] have shown that administration of vitamin C (10 g/l) in drinking water to rats for 15 days before the onset of WIRS reduces the microscopically observed gastric mucosal lesions and the increase in hepatic LPO level found at 2.5 h of the stress without affecting the hepatic and gastric mucosal vitamin C and GSH levels. We have shown that pre-administration of AA (250 mg/kg) prevents gastric mucosal lesion development by attenuating decreased gastric mucosal AA, non-protein SH, and vitamin E levels and increased gastric mucosal LPO level.[11] AA exerts an antioxidant action not only by scavenging superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and peroxyl radical by itself but also by interacting with GSH or vitamin E.[12–16] AA scavenges ROS generated by activated neutrophils and HOCl derived from myeloperoxidase (MPO) present in neutrophils *in vitro*.[17,18] Furthermore, AA inhibits the adherence of neutrophils to endothelial cells *in vitro*.[19] Thus, AA possesses not only antioxidant properties but also anti-inflammatory properties. However, it is still unclear how pre-administered AA protects against oxidative damage in the liver of rats with WIRS through its antioxidant and/or anti-inflammatory properties.

It is known that rats have increased plasma corticosterone and glucose levels in response to acute stress induced by immobilization and increased plasma corticosterone and adrenocorticotropic hormone levels in response to WIRS.[20,21] These responses to emotional stress occur via the hypothalamic–pituitary–adrenal (HPA) axis.[20,21] However, there is no report on whether pre-administered AA protects against oxidative damage in the liver of rats with WIRS by affecting the stress responses via the HPA axis.

In the present study, therefore, we attempted to clarify the effect of pre-administered AA on oxidative damage in the liver of rats subjected to WIRS.

Materials and methods

Materials

Xanthine, 3,3′,5,5′-tetramethylbenzidine (TMB), and SOD (purified from bovine erythrocytes) were purchased from Sigma Chemical Co. (St Louis, MO, USA); XO (purified from beef milk), and dithiothreitol (DTT) from Roche-Diagnostic Co. (Tokyo, Japan); AA (L-form), bovine serum albumin, *N*,*N*-dimethylformamide, α,α′-dipyridyl, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), ethylenediaminetetraacetic acid (EDTA), *N*-ethylmaleimide (NEM), GSH, 2-thiobarbituric acid, α-tocopherol (α-Toc), δ-tocopherol, and other chemicals were from Wako Pure Chemical Ind., Ltd (Osaka, Japan). All chemicals used were of reagent grade and were not further purified.

Animals

Male Wistar rats aged 6 weeks were purchased from Nippon SLC Co. (Hamamatsu, Japan). The animals were housed in cages in a ventilated animal room with controlled temperature (23 ± 2ºC) and relative humidity (55 ± 5%) with 12 h of light (7:00 to 19:00). The animals were maintained with free access to rat chow, Oriental MF (Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum* for one week. All animals received humane care in compliance with the Guidelines of the Management of Laboratory Animals in Fujita Health University.

Induction of WIRS and AA administration

Seven-week-old rats were starved for 24 h prior to experiments, but were allowed free access to water. The animals were randomly divided into 8 groups: the unstressed control group without AA administration (n = 5); the unstressed group with administration of AA (100 mg/kg) (n = 5); the unstressed group with administration of AA (250 mg/kg) (n = 5); the unstressed group with administration of AA (500 mg/kg) (n = 5); the group subjected to WIRS for 6 h without AA administration (n = 8); the group with 6 h

of WIRS and administration of AA (100 mg/kg; *n* = 8); the group with 6 h of WIRS and administration of AA (250 mg/kg; *n* = 8); and the group with 6 h of WIRS and administration of AA (500 mg/kg; *n* = 8). Rats were restrained in wire cages and immersed up to the depth of the xiphoid process in at 23ºC water bath to induce WIRS, as described by Takagi and Okabe.[1] AA dissolved in distilled water was orally administered to rats at a volume of 1 ml/100 g body weight with a stomach tube at 0.5 h before the onset of WIRS. Rats not administered with AA received the same volume of distilled water in the same manner at the same time point.

Determinations of serum and hepatic components and enzymes

All rats used for the determinations of serum and hepatic components and enzymes were sacrificed under ether anesthesia. The blood was collected from the inferior vena cava, and serum was obtained from the collected blood by centrifugation. Immediately after sacrifice, livers were well perfused with ice-cold 0.9% NaCl to remove blood remaining in the tissue. The collected serum and livers were stored at –80ºC until use. Serum ALT and AST were assayed using a commercial test kit of Iatrozyme TA-L_Q (Dai-Iatron Co., Tokyo, Japan). These enzyme activities are expressed as an international unit (IU/l). Serum T-AA, AA, and DHA were determined by the methods of Zannoni *et al.*[22] and Okamura[23] as follows: for the determination of T-AA, 0.3 ml of serum was incubated with 0.1 ml of 10 mM DTT at 37ºC for 30 min to convert all DHA in each sample to its reduced form and then the excess DTT was removed with 0.1 ml of 0.5% NEM. An aliquot of the supernatant obtained after deproteinization with 0.5 ml of ice-cold 10% TCA was used for the assay of the resultant AA plus the original AA. For the determination of AA, 0.3 ml of serum was mixed with 0.2 ml of a solution of 10 mM DTT-0.5% NEM. An aliquot of the supernatant obtained after deproteinization with 0.5 ml of ice-cold 10% TCA was used for the assay of AA. AA in each sample was measured by the α,α′-dipyridyl method. The concentration of AA was determined using the standard curve of authentic AA. The concentration of DHA in serum was estimated from the difference between the concentrations of T-AA and AA determined. Serum LPO was assayed by the thiobarbituric acid method[24] using tetramethoxypropane as a standard. The concentration of serum LPO is expressed as that of malondialdehyde (MDA) equivalents. Serum NOx was quantified using a commercial NO colorimetric assay kit (Roche-Diagnostic Co., Tokyo, Japan) based on the Griess reaction-dependent method.[25]

The isolated liver was homogenized in 9 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA to prepare 10% homogenate. The liver homogenate was used for the determinations of T-AA, AA, DHA, GSH, vitamin E, and LPO. T-AA, AA, and DHA in the liver homogenate was determined by the same α,α′-dipyridyl method as used for the assays of serum T-AA, AA, and DHA. GSH in the liver homogenate was assayed by the DTNB method[26] using GSH as a standard. VE in the liver homogenate was assayed by the high-performance liquid chromatographic method with electrochemical detection using α-tocopherol as an internal standard as described in our previous report.[27] The amount of hepatic VE is expressed as that of α-Toc. LPO in the liver tissue was assayed by the thiobarbituric acid method[28] using tetramethoxypropane as a standard except that 1 mM EDTA was added to the reaction mixture. The amount of hepatic LPO is expressed as that of MDA equivalents. NOx (nitrite/nitrate), the products of NO oxidation, in the liver tissue was assayed using the above-described NO assay kit. SOD and MPO, an index of tissue neutrophil infiltration,[29,30] in the liver tissue were assayed by the methods of Oyangui[31] and Suzuki *et al.*,[32] respectively. For the assays of NOx, SOD, and MPO, livers were homogenized in 9 volumes of ice-cold 0.05 M Tris-HCl buffer (pH 7.4). After sonication on ice for 20 s using a Handy Sonic model UR-20P (Tomy Seiko Co., Tokyo, Japan), the homogenate was centrifuged at 4ºC (10,000 *g*, 20 min). The resultant supernatant was used for the assay of NOx. For the assays of SOD and MPO, the supernatant was further dialyzed against 100 volumes of the same buffer at 4ºC for 1 h using a microdialysis device (molecular weight cut-off 3500 Da; Bio-Tec International Inc., Belleuve, WA, USA). SOD activity was determined at 37ºC by the xanthine oxidase–NH_2OH method using purified bovine erythrocyte SOD (5000 units/mg solid) as a standard. This activity is expressed as the amount of the erythrocyte SOD showing activity equivalent to the determined activity. MPO activity was determined as follows: the dialyzed supernatant was incubated at 60ºC for 2 h to increase the recovery of MPO in liver tissues according to the method of Schierwagen *et al.*[30] MPO activity in the heat-treated liver tissue sample was assessed by measuring the H_2O_2-dependent oxidation of TMB (dissolved in dimethylsulfoxide) at 37ºC. This TMB oxidation was measured spectrophotometrically at 650 nm. One unit of this enzyme activity is expressed as the amount of enzyme causing a change in absorbance of 1.0 per min at 650 nm. Hepatic protein was measured by the method of Lowry *et al.*[33] using bovine serum albumin as a standard.

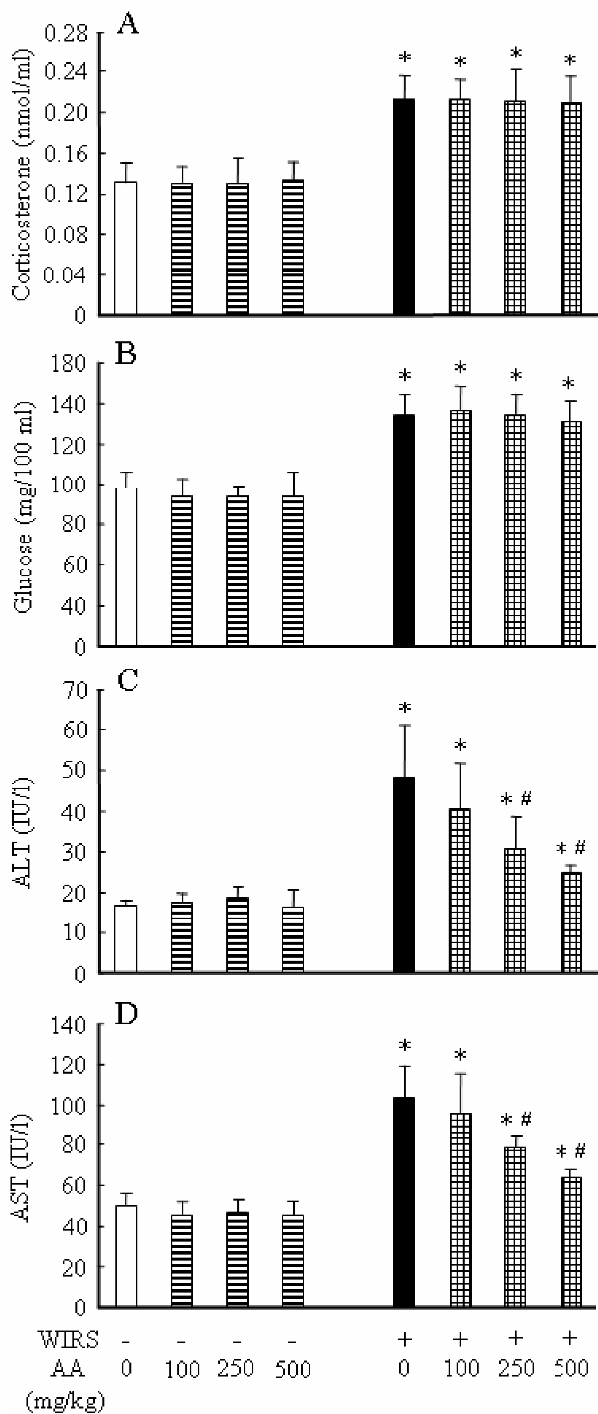

Figure 1 **Effect of pre-administered AA on changes in corticosterone (A) and glucose (B) concentrations and ALT (C) and AST (D) activities in the serum of rats subjected to WIRS for 6 h. Either AA (100, 250 or 500 mg/kg) or vehicle was orally administered to fasted rats with and without 6 h of WIRS at 0.5 h before the onset of the stress. Serum corticosterone, glucose, ALT, and AST were assayed in serum separated from blood collected at 6 h of WIRS. Each value is a mean ± SD (*n* = 5 for unstressed groups; *n* = 8 for stressed groups). *Significantly different from the unstressed control group, *P* < 0.05; #significantly different from the group with WIRS alone, *P* < 0.05**

Statistical analysis

All results are expressed as mean values ± SD. The statistical analyses of the results were performed using a computerized statistical package (StatView). Each mean value was compared by one-way analysis of variance (ANOVA) and Fisher's protected least significance (PLSD) for multiple comparisons as the *post-hoc* test. The significance level was set at $P < 0.05$.

Results and discussion

The plasma concentrations of corticosterone and glucose are known to increase to the responses of the HPA axis to acute emotional stress such as immobilization stress and WIRS.[20,21] Serum corticosterone and glucose concentrations in rats subjected to WIRS for 6 h were significantly higher than those in the serum of control rats without stress (Fig. 1A,B). The increased serum corticosterone and glucose concentrations did not change at all in the stressed rats administered orally with AA (100, 250 or 500 mg/kg) at 0.5 h before the onset of stress (Fig. 1A,B). These results indicate that pre-administered AA has no effect on the response of the HPA axis to WIRS.

The activities of ALT and AST, indices of liver cell damage, in the serum of rats with 6 h of WIRS were significantly higher than those of unstressed control rats (Fig. 1C,D), as reported previously.[8,10] The increases in serum ALT and AST activities were significantly inhibited by AA pre-administered at a dose of 250 or 500 mg/kg, but not 100 mg/kg, and the inhibitory effects occurred in a dose-dependent manner (Fig. 1C,D). The serum ALT and AST activities in stressed rats pre-administered with 500 mg/kg of AA were 1.48- and 1.29-fold higher than those in unstressed control rats, respectively, while the serum ALT and AST activities in stressed rats not given AA were 2.88- and 2.08-fold higher than those in unstressed control rats, respectively (Fig. 1C,D). Thus, AA pre-administered at high doses was found to be able to protect against cell damage in the liver of rats with WIRS.

AA can be synthesized in the liver of rats.[34] In rats, the liver releases AA to the bloodstream to maintain the plasma level of the vitamin.[35] AA is known to be consumed by reaction with ROS, such as $O_2^{\bullet-}$ and H_2O_2.[12,14] We have reported that AA concentration in the liver of rats with WIRS decreases 3 h after the onset of stress and further decreases at 6 h.[10] Hepatic T-AA and AA concentrations in rats with 6 h of WIRS were significantly lower than those in unstressed control rats, while hepatic DHA concentration and DHA/AA ratio in the stressed group were

significantly higher than those in the control group (Fig. 2). AA pre-administered at a dose of 100, 250 or 500 mg/kg inhibited significantly the decreases in hepatic T-AA and AA concentrations and the increases in hepatic DHA concentration and DHA/AA ratio in stressed rats in a dose-dependent manner (Fig. 2). In addition, there were no significant differences in hepatic T-AA, AA, and DHA concentrations and DHA/AA ratio between stressed rats pre-administered with AA (500 mg/kg) and unstressed control rats (Fig. 2). AA administered to rats without WIRS at a dose of 100, 250 or 500 mg/kg increased the hepatic T-AA and AA concentrations in a dose-dependent manner but did not affect the hepatic DHA concentration and DHA/AA ratio (Fig. 2). Thus, hepatic AA status was found to be disrupted in rats with 6 h of WIRS. However, this disruption in hepatic AA status induced by WIRS was found to be prevented by pre-administered AA.

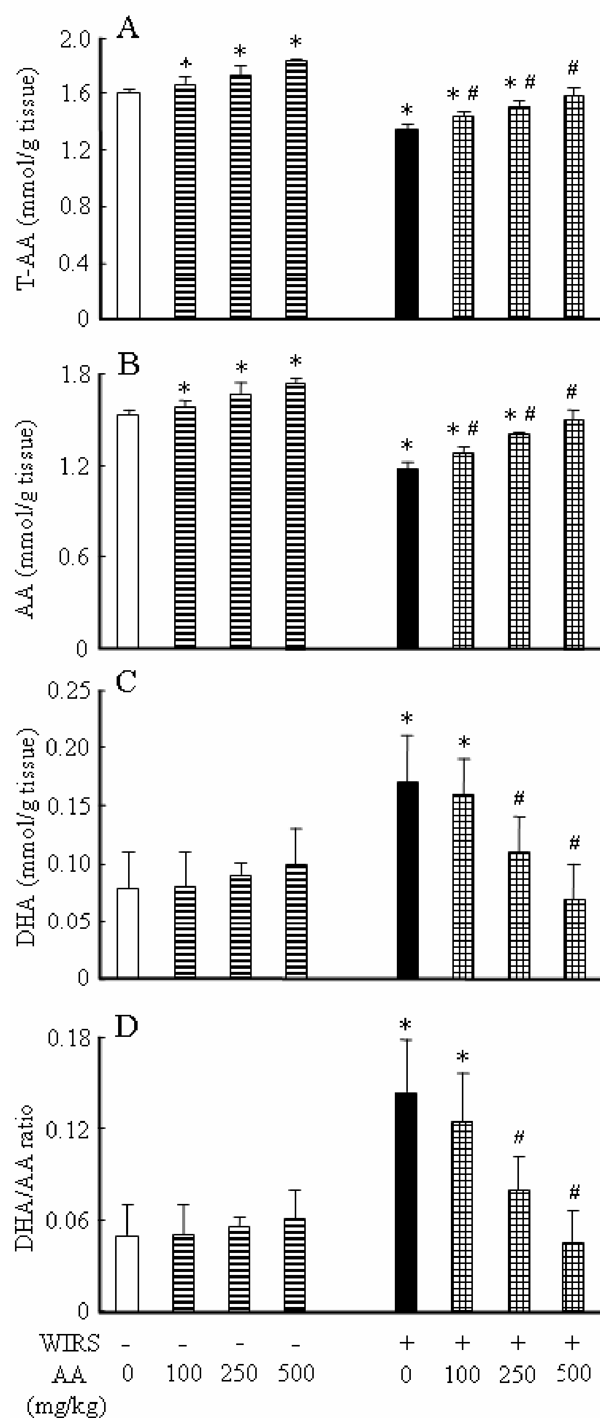

Figure 2 Effect of pre-administered AA on changes in T-AA (A), AA (B), and DHA (C) concentrations and DHA/AA ratio (D) in the liver of rats subjected to WIRS for 6 h. See the caption to Figure 1 for details

Serum T-AA and AA concentrations in rats with 6 h of WIRS were significantly higher than those in control rats without WIRS, while there were no significant differences in serum DHA concentration and DHA/AA ratio between rats with and without stress (Fig. 3). Thus, T-AA and AA concentrations increased in the serum of rats with 6 h of WIRS, although there were no changes in serum DHA concentration and DHA/AA ratio. These results suggest that the decreases in hepatic T-AA and AA concentrations in rats with 6 h of WIRS could occur due to both the enhanced release of AA from the liver tissue into the bloodstream and the conversion of AA to DHA by oxidative stress in the liver tissue. AA pre-administered to rats with 6 h of WIRS at a dose of 500 mg/kg, but not 100 or 250 mg/kg, caused further increases in the serum T-AA and AA concentrations and a significant increase in serum DHA concentration (Fig. 3A–C). Any dose of AA pre-administered to stressed rats had no significant effect on serum DHA/AA ratio (Fig. 3D). AA given to rats without WIRS at a dose of 250 or 500 mg/kg increased serum T-AA and AA concentrations significantly, although its dose of 100 mg/kg tended to increase the serum T-AA and AA concentrations (Fig. 3A,B). Any dose of AA given to rats without WIRS had no effect on serum DHA concentration and DHA/AA ratio (Fig. 3C,D). Furthermore, the serum T-AA and AA concentrations in unstressed rats administered with AA (100, 250 or 500 mg/kg) were not significantly different from those in stressed rats pre-administered with the corresponding doses of the vitamin ($P > 0.05$; (Fig. 3A,B). These results suggest that pre-administered AA could be taken up by the liver of rats with 6 h of WIRS, resulting in its utilization to maintain AA status in the liver tissue. These results also suggest that AA pre-administered to rats with WIRS might inhibit the release of AA from the liver tissue into the bloodstream.

Hepatic GSH concentration and SOD activity in rats with 6 h of WIRS were significantly lower than those in control rats without the stress, while there was

no difference in hepatic VE concentration between the two groups (Fig. 4). These results were in good agreement with those shown in our previous report.[10] Pre-administration of AA at a dose of 250 or 500 mg/kg, but not 100 mg/kg, inhibited significantly the decreases in hepatic GSH concentration and SOD activity in stressed rats in a dose-dependent manner (Fig. 4A,C). In addition, pre-administered AA (500 mg/kg) suppressed the decrease in hepatic GSH concentration in stressed rats completely (Fig. 4A). The hepatic SOD activity in stressed rats pre-administered with 500 mg/kg of AA was 87% of that in unstressed control rats, while the hepatic SOD activity in stressed rats not pre-administered with AA was 72% of that in unstressed control rats (Fig. 4A). Any dose of pre-administered AA did not affect the hepatic VE concentration in stressed rats (Fig. 4B). The same doses of AA given to rats without WIRS in the same manner did not affect the hepatic GSH and VE concentrations and SOD activity (Fig. 4). GSH is consumed by its reaction with ROS such as $O_2^{\bullet-}$ and H_2O_2 *in vitro*.[36] AA is recycled from DHA by GSH in a non-enzymatic or an enzymatic manner.[16] These findings suggest that pre-administered AA could restore GSH in the liver of rats with 6 h of WIRS by

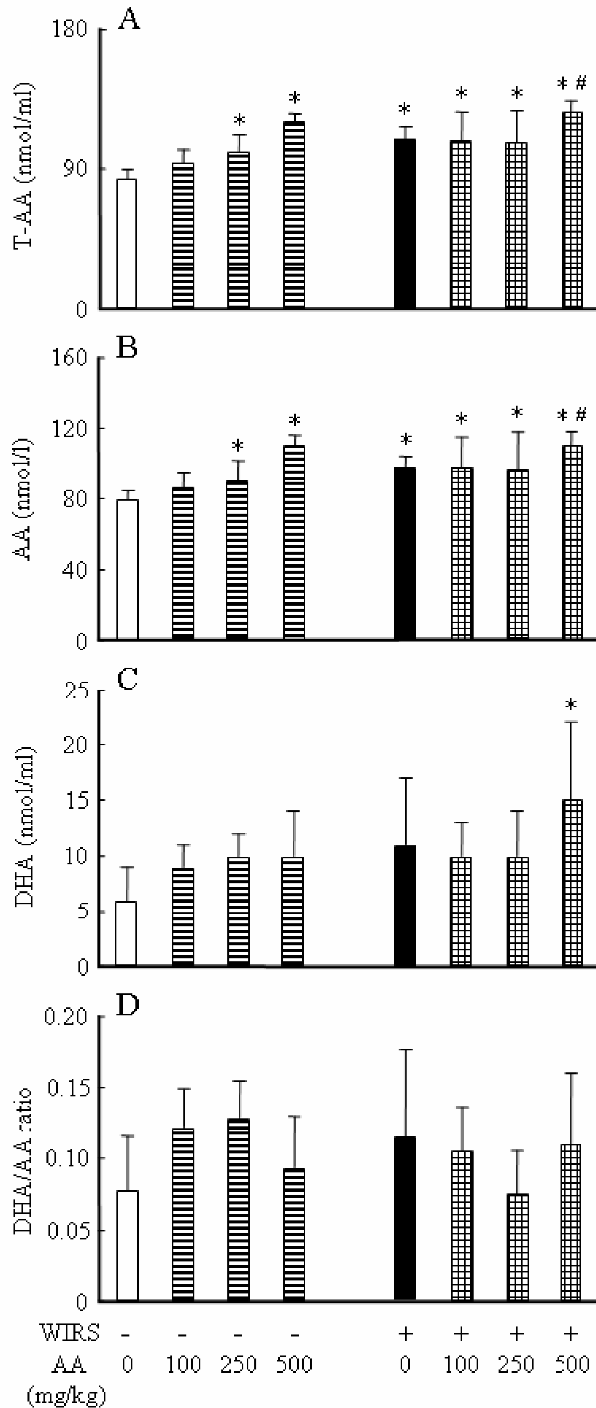

Figure 3 **Effect of pre-administered AA on changes in T-AA (A), AA (B), and DHA (C) concentrations and DHA/AA ratio (D) in the serum of rats subjected to WIRS for 6 h. See the caption to Figure 1 for details**

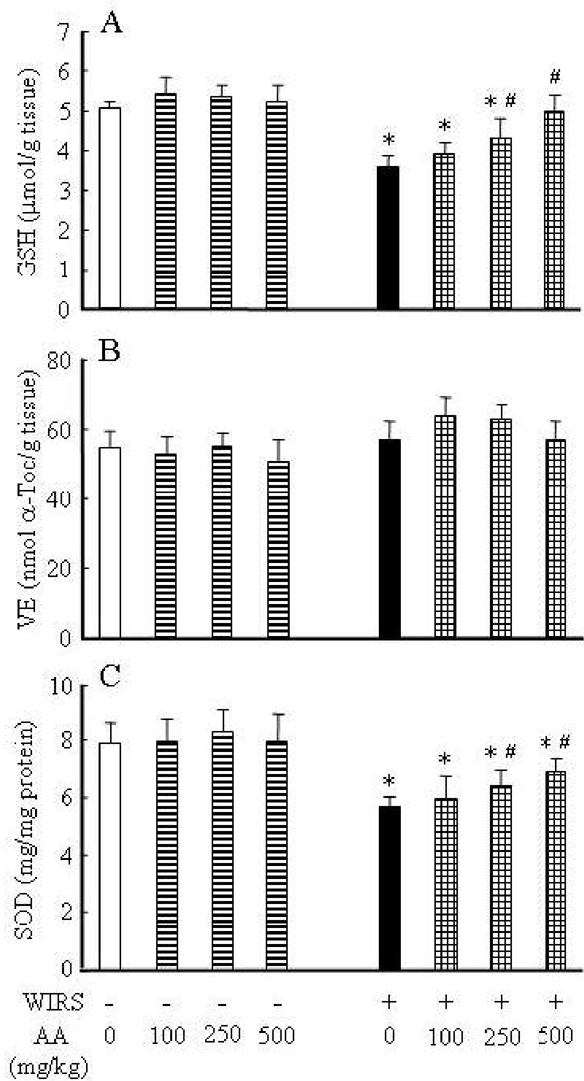

Figure 4 **Effect of pre-administered AA on changes in GSH (A) and VE (B) concentrations and SOD activity (C) in the liver of rats subjected to WIRS for 6 h. See the caption to Figure 1 for details**

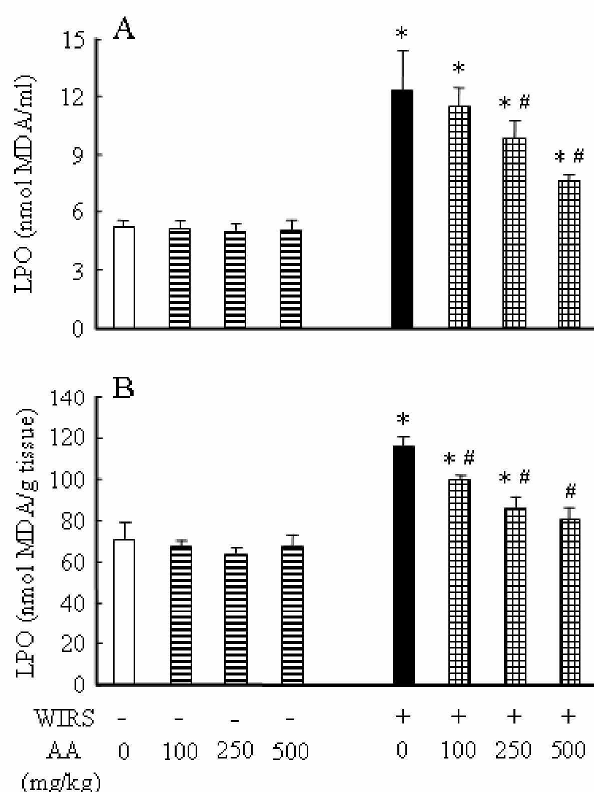

Figure 5 Effect of pre-administered AA on changes in serum (A) and hepatic (B) LPO concentrations in rats subjected to WIRS for 6 h. See the caption to Figure 1 for details

reducing GSH consumption due to scavenging of excessively generated ROS and AA regeneration from DHA generated by oxidative stress in the liver tissue, which could be involved in its protective effect against WIRS-induced oxidative damage in the liver tissue. AA is known to support the chain-breaking action of VE by reduction of VE radical to VE at the liquid/aqueous interface.[15] Therefore, the decreased AA concentration found in the liver of rats with 6 h of WIRS may be enough to support the chain-breaking action of VE in the liver tissue. It has been shown that the activity of Mn-SOD, which is localized in the mitochondria of cells, but not the activity of Cu,Zn-SOD, which is localized in the cytoplasm of cells, is reduced in the liver of rats with 6 h of WIRS.[9] From this finding, it is suggested that pre-administered AA could exert a preventive effect on the inactivation of Mn-SOD in the liver of rats with 6 h of WIRS, which could be involved in its protective effect against WIRS-induced oxidative damage in the tissue.

Serum and hepatic LPO concentrations in rats with 6 h of WIRS were significantly higher than those in control rats without stress (Fig. 5). The increases in serum LPO concentration in stressed rats was significantly inhibited by pre-administration of AA at a dose of 250 or 500 mg/kg, but not 100 mg/kg, while the increases in hepatic LPO concentration in stressed rats was significantly inhibited by pre-administration with each dose of AA (Fig. 5). Pre-administered AA exerted the inhibitory effects on the WIRS-induced increases in serum and hepatic LPO concentrations in a dose-dependent manner (Fig. 5).

The hepatic LPO concentration in stressed rats pre-administered with 500 mg/kg of AA was not significantly different from that in unstressed control rats ($P > 0.05$; Fig. 5B). The same doses of AA given to rats without WIRS in the same manner did not affect serum and hepatic LPO concentrations (Fig. 5). The hepatic activity of MPO, an index of tissue neutrophil infiltration,[29,30] in rats with 6 h of WIRS was significantly higher than that in control rats without WIRS (Fig. 6). Thus, rats with 6 h of WIRS had increased hepatic MPO activity, as shown in our previous report.[10] Pre-administration of AA at a dose of 250 or 500 mg/kg, but not 100 mg/kg, inhibited significantly the increase in hepatic MPO activity in stressed rats in a dose-dependent manner (Fig. 6). The hepatic MPO activity in stressed rats pre-administered with 500 mg/kg of AA was 1.69-fold higher than that in unstressed control rats, while the hepatic MPO activity in stressed rats not pre-administered with AA was 2.67-fold higher than that in unstressed control rats (Fig. 6). The same doses of AA given to rats without WIRS in the same manner did not affect the hepatic MPO activity (Fig. 6). Thus, the increase in hepatic MPO activity in rats with 6 h of WIRS was found to be prevented by pre-administered AA. It is known that lipid peroxidation occurs via ROS generated by NADPH oxidase in activated neutrophils.[37] It is also known that MPO present in neutrophils mediates lipid

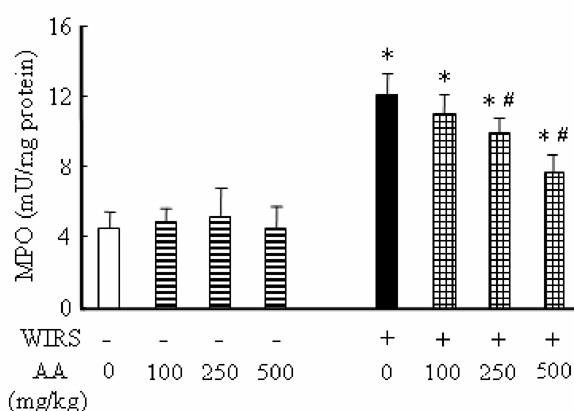

Figure 6 Effect of pre-administered AA on a change in MPO activity in the liver of rats subjected to WIRS for 6 h. See the caption to Figure 1 for details

peroxidation in the presence of H_2O_2 and halide ions.[38] AA is consumed by its reacting with $O_2^{\bullet-}$ and H_2O_2 generated by NADPH oxidase in activated neutrophils and HOCl generated in the presence of H_2O_2 and Cl^- by MPO present in neutrophils *in vitro*.[17,18] The adherence of neutrophils to endothelial cells is inhibited by AA *in vitro*.[19] These findings suggest that pre-administered AA protects against oxidative damage in the liver of rats with 6 h of WIRS by inhibiting lipid peroxidation mediated by ROS being generated in infiltrated neutrophils more effectively than neutrophil infiltration in the liver tissue.

Endogenous NO is known to exert protective and cytotoxic actions under pathophysiological conditions.[39] Serum and hepatic NOx concentrations in rats with 6 h of WIRS were significantly higher than those in control rats without the stress (Fig. 7). These results were consistent with those shown in our previous report.[10] The increase in serum NOx concentration in stressed rats was significantly inhibited by pre-administration of AA at a dose of 250 or 500 mg/kg, but not 100 mg/kg, while the increase in hepatic NOx concentration in stressed rats was significantly inhibited by pre-administration with each dose of AA (Fig. 7). Pre-administered AA exerted the inhibitory effects on the increases in serum and hepatic NOx concentrations in a dose-dependent manner (Fig. 7). The hepatic NOx concentration in stressed rats pre-administered with 500 mg/kg of AA was 1.95-fold higher than that in unstressed control rats, while the hepatic NOx concentration in stressed rats not pre-administered with AA was 4.06-fold higher than that in unstressed control rats (Fig. 7B). The same doses of AA given to rats without WIRS in the same manner did not affect the serum and hepatic NOx concentrations (Fig. 7). Theses results suggest that NO generated excessively in the liver of rats with WIRS could contribute to oxidative damage in the liver tissue through its cytotoxic action. Nitric oxide synthase II, *i.e.* inducible nitric oxide synthase (iNOS), is known to be induced rapidly with a large production of NO in neutrophils of rats with endotoxin-induced inflammation.[40] In rats with 6 h of WIRS, the pattern of the inhibitory effect of pre-administered AA on the increase in hepatic NOx concentration was very similar to that on the increase in hepatic MPO activity. This finding suggests that the excessive NO generation found in the liver of rats with 6 h of WIRS could be caused by iNOS in infiltrated neutrophils in the tissue and that pre-administered AA could reduce the excessive generation of NO via iNOS in the liver tissue by inhibiting infiltration of neutrophils into the liver tissue.

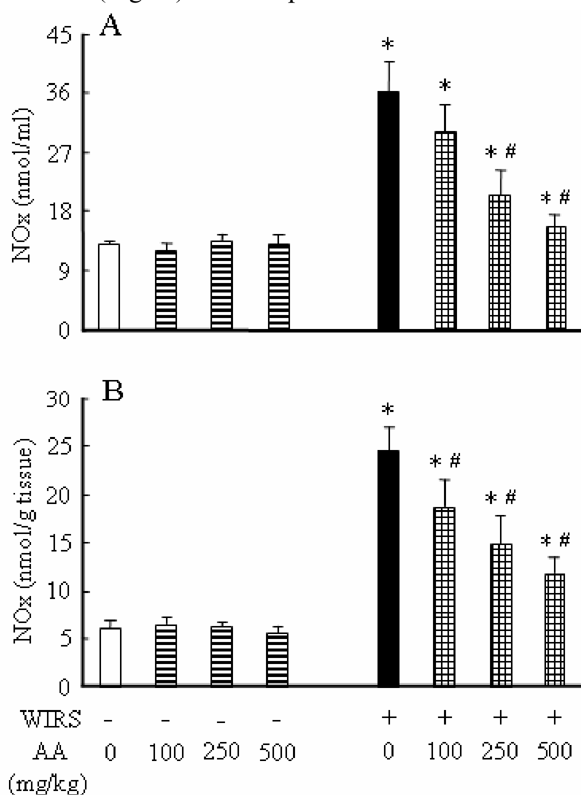

Figure 7 Effect of pre-administered AA on changes in serum (A) and hepatic (B) NOx concentrations in rats subjected to WIRS for 6 h. See the caption to Figure 1 for details

Peroxynitrite ($ONOO^-$), a strong oxidant, is known to be generated by the reaction between NO and $O_2^{\bullet-}$.[41] In the liver of rats with 6 h of WIRS, excessively generated NO may react with $O_2^{\bullet-}$, resulting in the formation of $ONOO^-$. $ONOO^-$ inactivates Mn-SOD and initiates lipid peroxidation *in vitro*.[42,43] AA prevents the interaction of $O_2^{\bullet-}$ and NO at very high physiological concentrations and reacts with $ONOO^-$ *in vitro*.[44,45] Therefore, one can assume that pre-administered AA prevents the decrease in SOD activity in the liver of rats with 6 h of WIRS by inhibiting the inactivation of Mn-SOD by generated $ONOO^-$ and that pre-administered AA inhibits $ONOO^-$-initiated lipid peroxidation in the liver of rats with 6 h of WIRS by scavenging generated $ONOO^-$. These assumable actions of pre-administered AA may be involved in its protective effect against oxidative damage in the liver of rats with 6 h of WIRS.

AA pre-administered at a dose of 500 mg/kg protected against cell damage, assessed by serum ALT and AST activities, in the liver of rats with 6 h of WIRS to a considerable extent, as described above. The same dose of pre-administered AA prevented the disruption of AA status associated with the increase in LPO concentration and the decrease in GSH concentration in the liver of the stressed rats completely, as described above. These findings allow us to think that oxidative stress due to the disruption of AA status in the liver of rats with 6 h of WIRS is closely associated with cell damage in the liver tissue.

Conclusions

The results of the present study indicate that pre-administered AA protects against oxidative damage in the liver of rats with WIRS without affecting the responses of the HPA axis to the stress. These results also suggest that pre-administered AA could exert this protective effect by attenuating disruption of the antioxidant defense system closely associated with disrupted AA status and increases in NO generation and neutrophil infiltration in the liver of rats with WIRS.

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