A Processed Grain Food Inhibits Hepatic Injury in Endotoxemic Rats*

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Summary Although various antioxidants have been tested as therapeutics for endotoxemic subjects, the results of their efficacy are conflicting. Antioxidant biofactor (AOB) is a unique processed grain food that exhibits strong antioxidant activity (Minamiyama et al, *J Nutr Sci Vitaminol*, 40: 467–477, 1994). The present study was carried out to test the effect of AOB on hepatic injury in rats induced by lipopolysaccharide (LPS). Intravenous administration of LPS induced liver injury with a concomitant increase in hepatic generation of nitric oxide (NO) and 4-hydroxy-2-nonenal (HNE) modified proteins in the control group. The administration of AOB significantly inhibited the LPS-induced hepatic injury and generation of HNE-modified proteins and increased the survival rate of endotoxemic rats without affecting NO generation and plasma levels of tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ). AOB scavenged superoxide radicals without affecting NO production by LPS-stimulated macrophage cell line J 774.2 cells. AOB also inhibited lipid peroxidation induced by LPS in the cells. These results suggested that AOB might scavenge superoxide radicals and decrease toxic metabolites including HNE, thereby inhibiting liver injury in endotoxemic rats.

Key Words antioxidant, rat, free radical, endotoxin, hepatic injury

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Abbreviations: LPS, lipopolysaccharide; NO, nitric oxide; Hb, hemoglobin; ESR, electron spin resonance; HNE, 4-hydroxy-2-nonenal; TBS, Tris-buffered saline; MOF, multiple-organ failure; TNF- α , tumor necrosis factor- α ; IFN- γ , interferon- γ ; SOD, superoxide dismutase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NO_x, NO₂⁻ + NO₃⁻; GSH, reduced glutathione; GSSG, oxidized glutathione.

Multiple-organ failure (MOF) associated with sepsis is a major cause of death in patients with severe infection. The cascade leading to septic MOF involves endotoxin-induced secretion of various cytokines, including tumor necrosis factor- α (TNF- α) and others, by monocytes and formation of reactive oxygen species by polymorphonuclear leukocytes. Oxidative stress induced by these toxic substances decreased the endogenous levels of antioxidants such as vitamin C in patients (1) and animals with sepsis (2). Endotoxin also decreased hepatic levels of coenzyme Q10, α -tocopherol, and reduced glutathione (GSH). Thus an imbalance between endogenous antioxidants and generated reactive oxygen species seems to occur in patients with endotoxemia (1, 3–5). Based on such a concept, various antioxidants have been tested as possible therapeutics for septic shock. Some, such as black tea extract (6), N-acetylcysteine (7), and L-cysteine (8), decreased tissue injury in animals with endotoxin shock and MOF. However, the therapeutic potential of N-acetylcysteine was not apparent in patients with severe sepsis (9). Endotoxin-induced liver injury and increased levels of plasma transaminases in animals were only slightly suppressed by treating with either superoxide dismutase (SOD; EC 1.15.1.1), catalase (EC 1.11.1.6), α -tocopherol, or xanthine oxidase inhibitor allopurinol (10). Furthermore, endotoxin-dependent tissue injury also occurred in transgenic mice overexpressing Cu/Zn-SOD to an extent similar to that in control animals (11). Since various intermediates of reactive oxygen species react with different target molecules, it might be important to inhibit different steps of tissue injury caused by reactive intermediates by means of a combination of various antioxidants. Although the effects of individual antioxidants on endotoxemic tissue injury have been studied extensively, the effects of a combined use of antioxidants remain to be elucidated.

Antioxidant biofactor (AOB) is a commercially available unique processed grain food that contains extracts from wheat germ, soybeans, rice bran, tear grass, sesame, wheat, citron, green tea, green leaf extract, and malted rice (12). A chemical analysis of AOB shows the presence of various phenolic compounds, trace elements, and vitamins with antioxidant properties, such as α -tocopherol and vitamin C (Table 1). AOB has been known to scavenge superoxide and hydroxyl radicals generated by activated neutrophils and to inhibit lipid peroxidation. It also improved clastogenic factors in plasma from Chernobyl liquidators (13). Based on these properties, AOB inhibits tissue injury caused by reactive oxygen species both in vitro and in vivo (14). The present work describes the effect of AOB on liver injury induced by endotoxin in rats.

MATERIALS AND METHODS

Chemicals. LPS (*E. coli* 055:B5) was obtained from DIFCO (Detroit, MI). AOB was provided by AOA Japan (Kobe). Other reagents used were of analytical grade available from Wako Chemicals (Osaka). Mouse TNF- α ELISA kit was obtained from Genzyme (Cambridge, MA); the specific antibody cross-reacted

Table 1. Chemical analysis of AOB ingredients.

	Content (mg/100 g on a dry weight basis)	
	AOB	Basal diet
Trace element		
Potassium	1,400	870
Phosphorus	760	830
Magnesium	321	240
Iron	10.0	15.4
Calcium	203	1,110
Sodium	55.5	240
Zinc	6.6	5.1
Phenolic compounds		
Rutin	33.0	—
Genistin	31.0	—
Daidzin	24.0	—
Hyperin	9.9	—
Isoquercitrin	4.6	—
Daidzein	3.0	—
Genistein	2.4	—
Kaemperol	n.d.	—
Quercetin, quercitrin	n.d.	—
Vitamins		
Ascorbic acid	282	4
Tocopherols	24.5	9.4
Retinol (IU)	n.d.	2,117
Carotene	2.3	—
Vitamin B6	0.97	0.82
Vitamin B12	n.d.	5.1

These constituents were analyzed by Japan Food Research Laboratories and were authorized by the Japanese government. AOB was evaporated to dryness. All values were expressed as mg per 100 g on a dry weight basis extract of AOB; n.d., not detectable.

with rat TNF- α (15). Rat IFN- γ ELISA kit was purchased from Gibco BRL (Gaithersburg, MD).

Animals. Male Wistar rats, 200–230 g, were purchased from SLC (Shizuoka). Under light ether anesthesia, lipopolysaccharide (LPS, 10 mg/kg) was intravenously injected into rats. Before the experiments, each rat was housed in a separate cage and the control group was allowed free access to a basal diet (Oriental Yeast MF, 15 g·d⁻¹). In the AOB-treated group, an AOB-containing diet (14 g·d⁻¹ of basal diet and 1 g·d⁻¹ of AOB) was given for 3 d. The experimental design was approved by the Committee of Osaka City University for Care and Use of Laboratory Animals. Under pentobarbital anesthesia (50 mg/kg, i.p.), blood samples were obtained from the abdominal aorta by using heparin-containing syringes. Plasma

samples were prepared by centrifugation at $3,000 \times g$ and 0°C for 10 min and were stored on ice until assay.

Analysis of plasma samples. Aspartate aminotransferase (AST; EC 2.6.1.1) and alanine aminotransferase (ALT; EC 2.6.1.2) levels in plasma were determined by a Hitachi 7450E autoanalyzer (Hitachi, Ibaraki). Plasma levels of $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ were measured by using ELISA kits.

Histological evaluation of hepatic injury. Excised liver tissues were fixed with 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Measurement of nitrosylhemoglobin (NO-Hb). The blood samples ($400\ \mu\text{L}$) were collected in tubes for electron spin resonance spectrometry (ESR), quickly frozen in liquid nitrogen, and analyzed at 110 K using a JES-RE1X apparatus (JEOL, Tokyo) with 100 kHz field modulation. ESR analysis was conducted with the microwave power of 8 mW at a frequency of 9.108 GHz, $325 \pm 250\ \text{mT}$ field, 1 min sweep time, 0.63 mT modulation amplitude, and 0.03 s time constant. The double integrated signal for NO-hemoglobin complex (NO-Hb, $g = 2$) was compared with that for CuSO_4 -EDTA reference solution, previously described (16).

Analysis of NO metabolites in plasma and urine. Plasma and urine samples were deproteinized by acidification with 6% sulfosalicylic acid (SSA) followed by centrifugation at $10,000 \times g$ for 15 min. The supernatant fractions ($100\ \mu\text{L}$) were mixed with $150\ \mu\text{L}$ of 5% NH_4Cl and $30\ \mu\text{L}$ of 5% NaOH . Nitrate in the samples was reduced by a cadmium column to NO_2^- , which reacted with Griess reagent to form a purple azo dye (17). The total amounts of NO_2^- ($\text{NO}_2^- + \text{NO}_3^-$, NO_x) were determined by high performance liquid chromatography. The reaction was developed in a 60°C water bath, and the absorbance at 546 nm was detected by a flow-through spectrophotometer.

Determination of glutathione and thiols in the liver. Total glutathione (reduced glutathione and oxidized glutathione: $\text{GSH} + 2\text{GSSG}$) and thiol levels in the liver were determined by the methods of Tietze (18) and Ellman (19), respectively. Under urethane anesthesia (1 g/kg, i.p.), the liver was perfused through the abdominal aorta with 50 mL of ice-cold saline. The excised liver was immediately homogenized in 3 volumes of ice-cold 5% SSA. After centrifugation at $12,000 \times g$ for 15 min, concentrations of thiols and total glutathione in the acid-soluble fractions were determined.

Measurement of glutathione reductase and glutathione peroxidase. Fresh liver (200 mg) was homogenized in 1.5 mL of ice-cold 0.01 M potassium phosphate buffer, pH 7.4, containing 30 mM KCl. The supernatant was obtained after centrifugation of the homogenate at $16,000 \times g$ at 0°C for 30 min. Glutathione reductase (EC 1.6.4.2) and glutathione peroxidase (EC 1.11.1.9) activities were determined by the methods of Bergmeyer (20) and Günzler et al (21), respectively.

Western blot analysis. 4-Hydroxy-2-nonenal (HNE) and its protein adducts have been demonstrated to be good markers for lipid peroxidation induced by reactive oxygen species (22). For the estimation of HNE-modified proteins in the

liver, the homogenates from control and LPS-treated rats were incubated in Laemmli sample buffer for 3–5 min at 100°C (23). The incubated samples (10 µg protein) were subjected to polyacrylamide gel electrophoresis (PAGE) in SDS. The gel was transblotted to Immobilon PVDF membrane. The membrane was incubated with 2% BSA in Tris-buffered saline containing 10% Tween 20 (TBS/Tween) for blocking, washed with TBS/Tween, then treated with the monoclonal antibody (HNEJ-2) specific for HNE-histidine adduct (24). This procedure was followed by reacting with horseradish peroxidase-conjugated rabbit antimouse IgG (diluted 1:2,000). Chemiluminescent enhancers (Amersham, Buckinghamshire, England) were used for visualization by autoradiography. The mean density of the band area was measured by using NIH Image (version 1.56) after obtaining a black-and-white image as PICT files by an image scanner.

Generation of NO, O_2^- and lipid peroxidation in J 774.2 cells. The murine macrophage cell line J 774.2 was purchased from JCRB cell bank (Osaka) and maintained in DMEM culture medium (Gibco, BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 20 mM glucose. Cells were cultured for 48 h at 37°C in an atmosphere of 5% CO₂ in 12-well Costar plates at 2×10^6 cells per 2 mL of DMEM. To each well was added 2 mL of control medium (control) or medium containing 10 µg/mL LPS in the presence or absence of various concentrations of AOB. Superoxide generation was determined 6 h after LPS treatment. Concentration of NO₂⁻ in the medium was determined 6 h after LPS treatment. Superoxide was determined by the cytochrome *c* reduction method (25). Generation of lipid peroxidation in J 774.2 cells was determined 6 h after LPS treatment. The collected cells were lysed by repeating the freeze/thaw procedure 3 times in distilled water. Lipid peroxidation in the cells was measured by using a colorimetric assay kit (BIOXYTECH LPO-586, OXIS International, Portland, OR) and expressed as a sum of as malonaldehyde and 4-hydroxyalkenals.

Statistical analysis. All values in the figures are expressed as means $\pm$ SE and analyzed by using 2-way ANOVA followed by the Fischer *t*-test. The difference between AOB and control groups was considered significant at $p < 0.05$.

RESULTS

Survival and hepatic injuries of LPS-treated rats

Figure 1 shows the survival rate of LPS-treated rats. Fifty percent of LPS-treated rats died within 24 h of treatment. The administration of AOB significantly decreased the mortality of rats. To evaluate hepatic injury, plasma AST and ALT levels were measured. These levels markedly increased in LPS-treated rats by some mechanism inhibited by AOB (Fig. 2).

Figure 3 shows that AOB inhibits the progression of LPS-induced damage. Microscopic examination of the liver from LPS-treated rats revealed the presence of focal necrosis and infiltration of neutrophils (control). On the other hand, such

Fig. 1. Effect of AOB on the survival rate of LPS-treated rats. AOB was orally given for 3 d (1 g/d) with basal diet before the experiments. LPS (10 mg/kg) was intravenously administered into rats, of which the survival rate was observed over 24 h. Ten rats were used for each group. Closed circle, LPS-treated group; open circle, AOB-treated group (LPS treatment in AOB-prefed rats). * Significantly different at $p < 0.05$ from LPS-treated group.

Fig. 2. Effect of AOB on plasma activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). LPS and AOB were administered as described in Fig. 1. Under light ether anesthesia, plasma samples were collected via the abdominal aorta. Values are means $\pm$ SE ($n = 5-10$). Closed circle, LPS-treated group; open circle, AOB-treated group. ** $p < 0.01$ as compared with LPS-treated group.

pathological findings were not observed in livers from AOB-treated rats.

NO metabolites and cytokines in plasma

Kinetic analysis by ESR revealed that NO-Hb levels in blood markedly increased and reached a maximum 8 h after LPS injection (Fig. 4). Plasma and urinary levels of NO_x ($\text{NO}_2^- + \text{NO}_3^-$) also increased after LPS treatment. The administration of AOB did not affect the blood levels of NO-Hb and NO_x .

Fig. 3. Histology of liver specimens from normal, LPS-treated (Control), and LPS + AOB-prefed rats. The liver from LPS-treated rats shows necrosis of hepatocytes and infiltration of neutrophils (arrows). The liver from the AOB-treated group shows no abnormal finding. Magnification: $\times 100$.

Fig. 4. Plasma levels of NO metabolites in LPS-treated rats. LPS and AOB were administered as described in Fig. 1. Levels of urinary NO_x were corrected by creatinine levels. Values are means $\pm$ SE ($n = 5-10$). a, NO-Hb in blood; b, nitrite + nitrate (NO_x) in plasma; c, urinary NO_x . Closed circle, LPS-treated group; open circle, AOB-treated group.

As shown in Table 2, the plasma levels of $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ markedly increased after LPS treatment, and AOB failed to inhibit the increase.

Hepatic glutathione status

The hepatic levels of free thiols and glutathione decreased 8 h after LPS administration (Fig. 5). Although AOB itself had no appreciable effect on the

Table 2. Effect of AOB on cytokine production levels in plasma.

		Time (h)			
		0	1.5	3	8
TNF- α (ng/mL)	Control	0.08 $\pm$ 0.01	6.39 $\pm$ 0.06	0.58 $\pm$ 0.03	n.d.
	AOB	0.09 $\pm$ 0.02	6.45 $\pm$ 0.08	0.40 $\pm$ 0.01	n.d.
INF- γ (ng/mL)	Control	0.05 $\pm$ 0.01	n.d.	56.9 $\pm$ 6.1	20.7 $\pm$ 2.6
	AOB	0.08 $\pm$ 0.01	n.d.	49.1 $\pm$ 5.5	16.9 $\pm$ 3.5

At the indicated times after LPS administration (10 mg/kg), plasma TNF- α and INF- γ were determined as described in the text. Values are means $\pm$ SE ($n=4-5$). n.d., not determined.

Fig. 5. Effect of AOB on hepatic glutathione levels of LPS-treated rats. Glutathione and free SH levels in the liver were determined with control and AOB-treated rats 8 h after intravenous injection of 10 mg/kg of LPS. Data show means $\pm$ SE ($n=5$). □, free thiols; ■, glutathione. ## $p < 0.01$ as compared with normal. * $p < 0.05$ as compared with LPS-treated group.

concentrations of glutathione and free thiols in the liver of normal rats, it inhibited the LPS-induced decrease of hepatic thiols. Under the same conditions, LPS increased glutathione reductase activity in the liver (Fig. 6). AOB did not affect the LPS-induced change in hepatic glutathione reductase activity. LPS also increased hepatic activity of glutathione peroxidase 3 h after administration. The elevated activity returned to the initial level 3 h after LPS treatment. The increased activity of glutathione peroxidase remained high in the AOB-treated group even 8 h after LPS treatment.

LPS-induced changes in HNE-modified proteins

Figure 7 shows the effect of AOB on the LPS-induced changes in hepatic levels of HNE-modified proteins. Kinetic analysis using anti-HNE antibody revealed that several proteins were modified by HNE. Among various proteins, 29 kDa protein was most sensitive to modification by HNE. As a result of densitometry, the amount

Fig. 6. Effect of AOB on glutathione reductase and glutathione peroxidase in the liver. LPS and AOB were administered to rats as described in Fig. 1. Hepatic glutathione reductase and glutathione peroxidase were measured at 0, 3, and 8 h after LPS treatment. Data show means $\pm$ SE ($n=5$). Closed circle, LPS-treated group; open circle, AOB-treated group. # $p < 0.05$ as compared with normal group. * $p < 0.05$ as compared with LPS-treated group.

Fig. 7. Effect of AOB on hepatic levels of HNE-modified proteins. At the indicated times, rats were treated with LPS (10 mg/kg). AOB was administered as described in Fig. 1. HNE-modified proteins in liver cytosol were analyzed by using anti-HNE antibody. Densitometric analysis of HNE-modified proteins (29 kDa) was performed. Inserted figure shows Western blots of HNE-modified proteins. Lanes 1, 3, 5, 7, and 2, 4, 6, 8, show samples from control and AOB-treated groups, respectively. Data show means $\pm$ SE ($n=5$). Closed circle, LPS-treated group; open circle, AOB-treated group. ** $p < 0.01$ as compared with LPS-treated group.

of HNE-modified 29 kDa protein proved to increase 3- and 4-fold 3 and 8 h after LPS treatment, respectively. AOB depressed the formation of these proteins.

Generation of O_2^- , NO, and lipid peroxidation in J 774.2 cells

LPS treatment generated both superoxide and NO_2^- in J 774.2 cells. Figure 8 and Table 3 show the effect of AOB on the LPS-induced generation of superoxide

Fig. 8. Effect of LPS on the superoxide generation by J 774.2 cells. After incubation of 10^5 cells with $10 \mu\text{g/mL}$ of LPS and 0.05 mg/mL of AOB, superoxide generation in the medium was determined. Concentrations of O_2^- in the medium were determined at 6 h after LPS treatment. Data show means $\pm$ SE ($n=4$). * $p < 0.05$, ** $p < 0.01$, as compared with LPS-treated rats. Closed circle, LPS-treated group; open circle, AOB-treated group.

Table 3. Effect of AOB on the generation of O_2^- and NO_2^- after LPS treatment by J 774.2 cells.

AOB (mg/mL)	O_2^- (nmol/h/ 10^5 cells)	NO_2^- (nmol/6 h/ 10^5 cells)
0	7.94 ± 0.89	5.13 ± 0.85
0.03	$3.87 \pm 0.56^*$	4.73 ± 0.86
0.1	0	4.81 ± 0.54
1.0	0	4.56 ± 0.68
10.0	0	3.78 ± 1.03

After the incubation of 10^5 cells with $10 \mu\text{g/mL}$ of LPS and varying concentrations of AOB, the generation of O_2^- and the levels of NO_2^- in the medium were determined. Concentrations of O_2^- in the medium were determined for 1 h between 5–6 h after LPS treatment. Concentrations of NO_2^- in the medium were determined at 6 h after LPS treatment. Data show mean $\pm$ SE. * $p < 0.05$ as compared with LPS-treated.

and NO_2^- in J 774.2 cells. LPS increased the cellular production of both superoxide and NO_2^- . Table 4 shows the effect of AOB on lipid peroxidation 6 h after LPS treatment. AOB strongly inhibited the generation of superoxide and lipid peroxidation, but not of NO_2^- .

DISCUSSION

Free radicals have been postulated to underlie the mechanism of liver injury

Table 4. Effect of AOB on LPS-induced lipid peroxidation by J 774.2 cells.

AOB (mg/mL)	After LPS administration (h)	
	0	6
0	24.5 ± 1.70	46.2 ± 8.28
0.05	25.1 ± 3.05	30.3 ± 2.66*
0.1	25.2 ± 3.98	27.7 ± 4.11*

After incubation of 10^5 cells with 10 $\mu\text{g/mL}$ of LPS and varying concentrations of AOB, levels of lipid peroxidation in the cells were determined at 6 h after LPS treatment. Data show mean $\pm$ SE. * $p < 0.05$ as compared with control (6 h).

in endotoxemic subjects. The present work clearly demonstrates that AOB markedly inhibited liver injury and increased the survival rate of LPS-treated endotoxemic rats.

Although AOB inhibited liver injury by LPS, it failed to suppress the enhanced generation of NO, NO-Hb, and toxic cytokines. Since xanthine oxidase (EC 1.1.3.22) is markedly activated in the liver of endotoxemic rats, this enzyme has been postulated to be involved in the generation of toxic metabolites. However, allopurinol, a potent inhibitor of the enzyme, increased the survival rate of LPS-treated rats only slightly (10). Thus the enzyme may not play an important role in the pathogenesis of liver injury in endotoxemic rats. Oxygen radicals are derived from activated macrophages and neutrophils. AOB scavenged the superoxide radical without affecting its generation by xanthine oxidase (12) and activated neutrophils (14). The present work also demonstrates that AOB scavenges the superoxide radical generated by LPS-stimulated J 774.2 without affecting the cellular production of NO. Since superoxide rapidly reacts with NO ($K = 6.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$), it is likely that AOB inhibits the generation of toxic peroxynitrite in LPS-treated rats.

AOB also inhibited the LPS-induced decrease in GSH levels in the liver. Because glutathione reductase and peroxidase after LPS administration remained high in the AOB-treated group, AOB may have enhanced the regeneration of GSSG and the GSH-dependent degradation of lipid hydroperoxides. Consistent with this notion, the present finding showed that AOB also suppressed the formation of HNE-protein adducts in the liver and lipid peroxidation in J 774.2 cells. Reactive oxygen species initiate free radical chain reaction and induce lipid peroxidation. This chain reaction generates various aldehydes, including HNE. Although LPS enhanced the formation of HNE-protein adducts with different molecular weights, this modification was characteristic of 29 kDa protein(s). AOB strongly decreased the formation of HNE-modified proteins, including 29 kDa protein. The biochemical properties of 29 kDa protein(s) remain to be further studied.

N-Acetylcysteine has been successfully used as an antioxidant therapeutic. However, the intravenous administration of N-acetylcysteine (3 g/d) had no ap-

preciable effects either on the plasma levels of glutathione and lipid peroxides or on the prognosis of patients with endotoxemia (26). Because different species of reactive oxygen metabolites react with different molecules and appear at different steps of inflammation, the administration of a single type of antioxidant may not be sufficient for the treatment of patients with severe endotoxemia. And because AOB contains various types of antioxidants with both hydrophobic and hydrophilic natures that scavenge different types of free radicals in different cellular compartments and inhibit lipid peroxidation, these components may synergistically function in decreasing oxidative stress elicited in patients with endotoxemia. Thus AOB may have a therapeutic potential to inhibit liver injury and to increase the survival rate of patients with severe endotoxemia.

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