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BILIRUBIN IS OXIDIZED IN RATS TREATED WITH ENDOTOXIN AND ACTS AS A PHYSIOLOGICAL ANTIOXIDANT SYNERGISTICALLY WITH ASCORBIC ACID *IN VIVO*

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SUMMARY: We examined the possibility that bilirubin physiologically acts as an antioxidant by using scurvy-prone ODS-*od/od* rats treated with endotoxin (lipopolysaccharide: LPS). Recently, bilirubin oxidative metabolites were isolated from human urine and named biotripyrrin-a and biotripyrrin-b. The LPS injection markedly increased bilirubin oxidative metabolites in urine of rats fed an ascorbic acid-free diet. This increase was supressed by feeding an adequate amount of ascorbic acid, a physiological antioxidant. The concentrations of biotripyrrin-a and -b in urine collected 6.5-10 h after the LPS injection were lower in rats fed an ascorbic acid-supplemented diet than in rats fed an ascorbic acid-free diet. Moreover, feeding with ascorbic acid suppressed the elevation of hepatic mRNA level of heme oxygenase-1, the rate-limiting enzyme of bilirubin biosynthesis, in rats injected with LPS. These findings suggest that bilirubin is oxidized in rats treated with LPS and acts as a physiological antioxidant synergistically with ascorbic acid *in vivo*.

Free and albumin-bound bilirubin at 2% oxygen pressure has been reported to have antioxidant activity *in vitro*, and this compound suppresses the oxidation more than physiological antioxidants, such as α-tocopherol (1,2). Bilirubin is biosynthesized from heme, catalyzed by heme oxygenase (EC 1.14.99.3)(3) and biliverdin reductase (EC 1.3.1.24)(4,5). Bilirubin may act as a physiological antioxidant against oxidative stresses.

Recently, Yamaguchi et al. (6) isolated the seven oxidative metabolites of bilirubin, which are recognized by an anti-bilirubin monoclonal antibody designated 24G7 (7), from human urine. The two chemical structures of seven metabolites were determined, and these were new oxidative metabolites of bilirubin named biotripyrrin-a and biotripyrrin-b, and were novel tripyrrole

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biocompounds. These bilirubin oxidative metabolites (BOM) in urine were found to increase in a patient who had undergone laparotomy that had taken a long time (6).

Heme oxygenase (HO), a rate-limiting enzyme of bilirubin biosynthesis, are composed of two isozymes, heme oxygenase-1 (inducible) and heme oxygenase-2 (constitutive). Heme oxygenase-1 activity is significantly increased by heme, endotoxin (lipopolysaccharide: LPS), heavy metals, heat shock, interleukin-1, and hydrogen peroxide (8-11). These observations suggest that the biosynthesis of bilirubin is evoked by loading of oxidative stresses.

The purpose of this study was to examine whether bilirubin acts as a scavenger of reactive oxygen species (ROS) *in vivo* or not. LPS treatment causes inflammation and may stimulate ROS production (12). The results demonstrate that the LPS treatment increase the oxidative metabolites of bilirubin in urine in rats. Moreover, the possibility that vitamin C (ascorbic acid: AsA), the physiological antioxidant, suppresses the production of BOM induced by the LPS treatment was examined by using scurvy-prone ODS-*od/od* rats which have a hereditary defect in AsA biosynthesis (13).

MATERIALS AND METHODS

Animals and diet-----Male ODS-*od/od* rats weighing about 170 g (8-week old) were purchased from Japan Clea (Tokyo). They were housed individually in a temperature-controlled (24°C) room with a 12-h light:12-h dark cycle. They were allowed free access to water and a purified diet. The composition (g/kg diet) of the basal diet was as follows: casein, 250; carbohydrate (sucrose-starch; 1:2), 612; AIN76 mineral mixture (14), 35; AIN76A vitamin mixture (AsA-free)(15); 10; AsA, 1; choline chloride, 2; corn oil, 50; cellulose powder, 40.
Experimental design-----Thirty-three rats were fed the AsA-free diet, which was depleted AsA from the basal diet, for 14 d (the AsA-deficient group), and another 33 rats were fed the basal diet for 14 d (the AsA-fed group). On d 14, three rats in each group were killed at 1000 h. Then, 15 rats in each group were injected intraperitoneally with LPS(1 mg/kg body weight, dissolved in saline), and 15 rats in each group were injected intraperitoneally with saline. Rats (3 rats each) injected with LPS or saline were killed at 3 h, 5 h, 7.5 h, 14 h, and 24 h after the injection. Urine was collected from each rat immediately after the injection with LPS or saline. Livers and spleens were taken and stored at - 80°C.
Enzyme-linked immunosorbent assay (ELISA) ----The procedure, described previously (16), was slightly modified to obtain more precise data.
Preparation of BOM in rat urine-----Rat urine (about 5-10 ml) was mixed with acetonitrile of the same volume and was centrifuged at 8000 x g for 10 min. The supernatant was lyophilized immediately. The crude urinary specimen obtained was separated on a Sep-Pak C18 ENV (Waters) to give seven fractions (Fr. 1, 0%; Fr. 2, 10%; Fr. 3, 20%; Fr. 4, 30%; Fr. 5, 35%; Fr. 6, 40%; Fr. 7, 50%; Fr. 8, 100%: acetonitrile/5% acetic acid, v/v). The compounds reactive to 24G7 were especially rich in Fr. 5 (about 70% of yield) and were analyzed by means of HPLC.
Analysis of BOM by HPLC-----Fr. 5 separated by Sep-Pak C18 ENV was analyzed by HPLC on the reverse-phase column as described previously by Yamaguchi et al. (6). The analysis was performed on a Waters 600E-LC system. HPLC was performed as follows; the column (μBondasphere; ϕ 3.9 mm x 15 cm, 5μ C18-100Å, Waters) was equilibrated with 30% acetonitrile in 0.1% trifluoroacetic acid

(TFA) at a flow rate of 1 ml/min and specimen was injected into the column. Then it was eluted with a 25-min linear gradient from 30 to 50% acetonitrile in 0.1% TFA. Each peak of bilirubin-related compounds was detected from its absorbance at both 436 nm and 254 nm.

HO-1 cDNA-----A cDNA (nucleotides 70-833) encoding a rat heme oxygenase 1 (HO-1) was synthesized using a polymerase chain reaction. Two oligonucleotides, 5'-GTGCACATCCGTGCAGAGAATTCT-3' and 5'-AGGAAACTGAGTGTGAGGACCCAT-3', corresponding to nucleotides 70-90 and the inverse complement of nucleotides 814-833 of the published rat HO-1 cDNA (17) with the addition of Bam HI site and Xho I site, respectively, were used as primers. The oligonucleotide (500 ng) complementary to nucleotides 814-833 was annealed to 1 μg of rat spleen poly (A)+ RNA obtained by oligo(dT)-cellulose column chromatography (18). Subsequent first-strand conversion of RNA to DNA and polymerase chain reaction were done as described previously (19).

RNA extraction and Northern blot analysis-----Total RNA was extracted from the liver and spleen according to the method of Chomczynski and Sacchi (20). RNA (20 μg) was separated by electrophoresis on a 1% agarose gel containing 6.6% (w/v) formaldehyde, and immobilized on Hybond N+ membranes (Amersham) in 10 x SSC (1 x SSC is 150 mM NaCl and 150 mM sodium citrate). The membranes were baked at 80°C for 2 h, and then hybridized with probe (2 x 10^6 cpm/ml ^{32}P-labeled cDNAs) specific for HO-1 mRNA at 42°C overnight in a solution containing 50% (w/v) formamide, 5 x Denhardt's solution, 0.1 % (w/v) sodium dodecyl sulfate, 5 x SSC, 50 mM sodium phosphate, and 0.05% (w/v) denatured salmon sperm DNA. The blots were washed twice with 0.1 x SSC containing 0.1% (w/v) sodium dodecyl sulfate at 55°C for 15 min. The washed blots were autoradiographed, after which the radioactivity on the band was measured with a Radioanalytic Imaging System apparatus (AMBIS System Inc.).

Measurement of AsA in serum and tissues-----Blood collected by decapitation was centrifuged for 15 min at 1,600 x g, and AsA in the supernatant (serum) was measured by the method of Kodaka et al. (21). The liver or spleen was homogenized in ice-cold 5% metaphosphoric acid and centrifuged for 10 min at 1,600 x g. The AsA in the supernatant was measured by a modification of the dinitrophenylhydrazine method (22), in which the oxidation of AsA was accomplished with 2,6-dichlorophenolindophenol.

Assay of HO activity in liver-----Heme oxygenase was assayed as described by Maines and Kappas (23). The assay was carried out on the microsomal fraction isolated from liver. The production of bilirubin in this mixture was measured spectrophotometrically at 450 nm. The molecular extinction coefficient for bilirubin used was 45 $mM^{-1}cm^{-1}$. Microsomal protein was determined by the method of Lowry et al. (24).

Statistical analysis-----The means and SE are reported. Data were analyzed by a two-way ANOVA. When the *F* value for the AsA effect or the LPS effect was significant ($P<0.05$), the differences between means were analyzed at $P<0.05$ by Duncan's multiple range test (25).

RESULTS

Fig. 1 compares the time course of urinary BOM levels in LPS-treated rats and non-LPS-treated (saline-treated) rats in both the AsA-fed group and the AsA-deficient group.

When the BOM levels were measured using ELISA with 24G7 during the first 24-hour period after treatment with LPS or drug-free saline, the BOM level after saline treatment was less than about 2 μM in both the AsA-fed group and the AsA deficient group, without any significant inter-group difference. However, the BOM

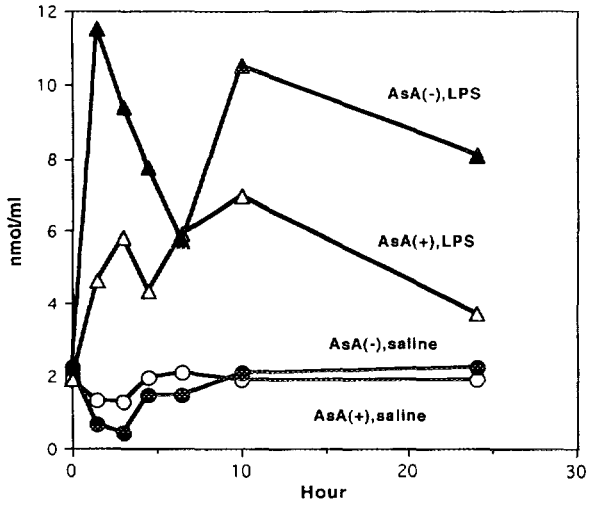

Fig. 1. Time course of the concentration of BOM in urine after injection with LPS or saline in rats.

level measured in the AsA-fed group at 3 hours after LPS treatment was about 4.4 times the level measured in the same group at 3 hours after saline treatment. The BOM level measured in the AsA-deficient group at 3 hours after LPS treatment was about 20.8 times the level measured in the same group at 3 hours after saline treatment. AT ten hours after LPS treatment, the BOM level in the AsA-fed group was 7 μM, which was about 3.5 times that for saline-treated rats from the same group, and the BOM level in the AsA-deficient group was 10.5 μM, which was about 5 times that for saline-treated rats from the same group.

Thus in both the AsA-fed group and the AsA-deficient group, the LPS-treated rats showed a two-peak increase in urinary BOM levels (one peak at the 3rd hour and the other at the 10th hour). However, the BOM level continued to be lower in the AsA-fed group than in the AsA-deficient group until 24 hours after LPS treatment.

During the period from the 6.5th to 10th hour after administration of LPS or drug-free saline, urinary BOM in LPS-treated and saline-treated rats from the AsA-deficient group were separated into 8 fractions, using a Sep-Pak C18 ENV. The amounts of BOM in each faction were measured, using ELISA. The BOM level in each of the 8 fractions was higher in LPS-treated rats than in saline-treated rats. The difference was particularly marked in the BOM recovery from Fraction 5.

Fig. 2a shows an HPLC pattern of BOM for crude Fraction 5 separated from healthy human urine using a Sep-Pak C18 ENV. Fig. 2b shows an HPLC pattern of BOM for Fraction 5 isolated from the urine of saline-treated rats during the period from the 6.5th to 10th hour after saline treatment, using a Sep-Pak C18 ENV. X1 and X2 are oxidative metabolites of bilirubin. X1 corresponds to biotripyrrin-a,

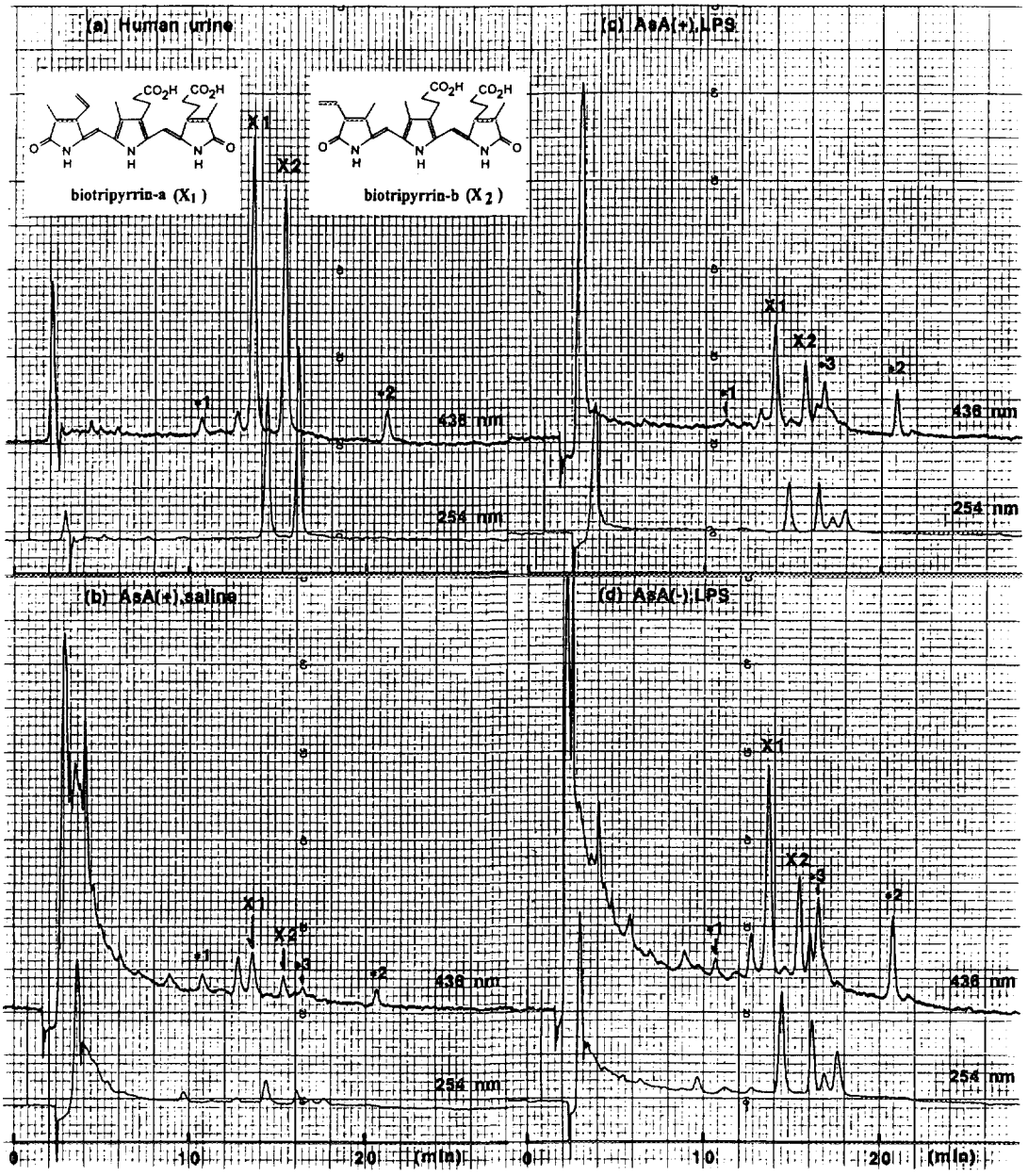

Fig. 2. HPLC elution profile of Fr. 5 samples separated by Sep-Pak C18 ENV of human urine (a) and urine (b, c, and d) from 6.5 to 10 h after injection with LPS in rats. Fr. 5 separated by Sep-Pak C18 ENV was analyzed by HPLC on a reverse-phase column as described in "Materials and Methods". Biotripyrrin-a (X1) and biotripyrrin-b (X2) are shown in Fig. 2(a). Peak *1, *2, and *3 indicate the unidentified BOM reactive to 24G7 by means of ELISA.

whereas X2 corresponds to biotripyrrin-b (Fig. 2) (6). Peaks 1, 2 and 3 indicate unidentified 24G7-reactive bilirubin metabolites. It has been confirmed that these metabolites are oxidative products derived from bilirubin by reaction with hydrogen

peroxide. Fig. 2c shows an HPLC pattern of Fraction 5 isolated from the urine of the AsA-fed group during the 6.5-10 h period after LPS treatment. The sum total of X1 and X2 levels in Fig. 2c is 3.1 times that in Fig. 2b. Fig. 2d shows an HPLC pattern of urinary BOM in the AsA-deficient group during the period from 6.5th to 10th hour after LPS treatment. The sum total of X1 and X2 levels in Fig. 2d is 6.1 times that in Fig. 2b. The amounts of unidentified bilirubin metabolites (peaks 1 and 2) also tended to be greater for LPS-treated rats than for saline-treated rats in both the AsA-fed group and the AsA-deficient group.

Fig. 3a shows the time course of liver HO-1 mRNA levels after LPS treatment. In the AsA-fed group, the HO-1 mRNA level at five hours after LPS treatment was about 6.5 times its level measured at five hours after drug-free saline treatment. In the AsA-deficient group, the level at five hours after LPS treatment was about 13 times the level measured at 5 hours after drug-free saline treatment. These results indicate that the liver HO-1 mRNA level after LPS treatment differs between the AsA-fed group and the AsA-deficient group. When a similar analysis was made for splenic HO-1 mRNA levels, neither the AsA-fed group nor the AsA-deficient group showed any increase in HO-1 mRNA levels following LPS treatment.

Fig. 4 shows the time course of HO activity after LPS treatment. HO activity began to rise at 5 hours after LPS treatment and remained elevated until 24 h. It was significantly higher in the AsA-deficient group than in the AsA-fed group.

Fig. 5c shows the time course of serum AsA levels after LPS or drug-free saline treatment. In the AsA-fed group, the serum AsA level was lower in LPS-treated rats than in saline-treated rats during the period from 7.5th to 24th hour after treatment. In the AsA-deficient group, however, the serum AsA levels remained low (<0.02 μg/ml) in both LPS-treated rats and saline-treated rats. In the AsA-fed group, both

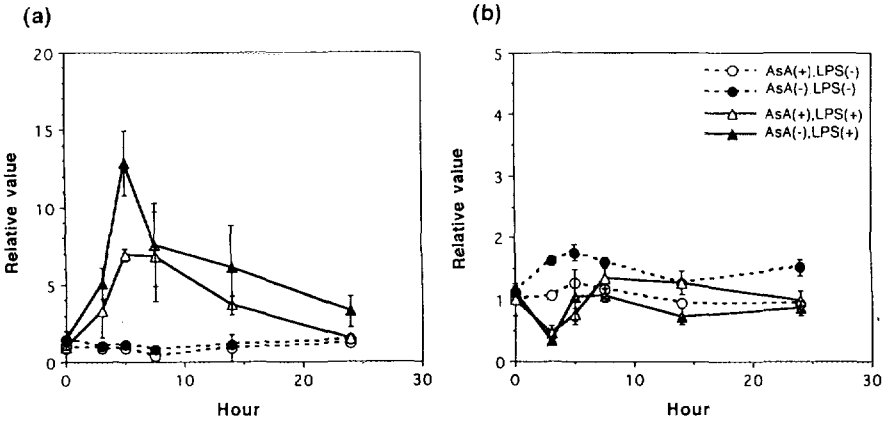

Fig. 3. Time course of HO-1 mRNA level in the liver (a) and spleen (b) after injection with LPS or saline in rats.

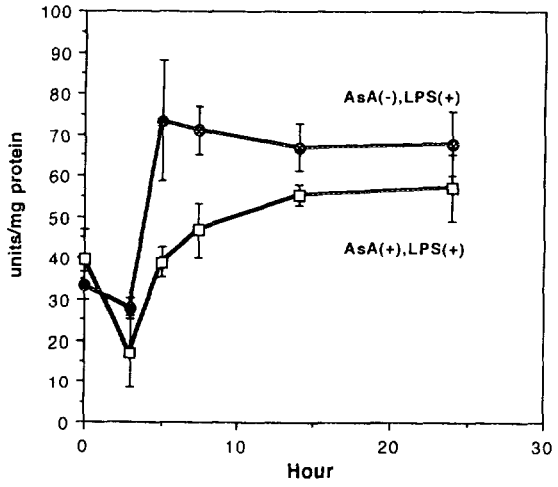

Fig. 4. Time course of heme oxygenase activity in liver microsomes after injection with LPS in rats.

the liver AsA level and the spleen AsA level were lowered immediately after LPS treatment. In the AsA-deficient group, however, neither the liver AsA level nor the spleen AsA level showed any significant difference between LPS-treated rats and saline-treated rats (Fig. 5a and 5b).

DISCUSSION

AsA is known to serve as a physiological antioxidant. Changes in the production of BOM depending on the presence or absence of AsA were analyzed in this study, to provide evidence for the view that bilirubin serves as a scavenger of ROS *in vivo*. LPS, which is an endotoxin, was employed in this study as a source of oxidative stress. Administration of LPS was expected to increase ROS *in vivo* and to be useful in establishing an animal model of inflammation.

HO-1 is induced by the oxidative stress caused by treatment with agents that are oxidants or can generate ROS or by resultant reduction in cellular glutathione levels (26). Recently, Rizzardini et al. suggested that ROS were mediators of LPS-induced HO-1 mRNA accumulation and that glutathione content appeared to be one of the factors regulating this accumulation in the liver (12).

Furthermore, Meister's group has shown that ascorbic acid can serve as an essential antioxidant in the presence of severe glutathione deficiency, and to maintain a normal cellular glutathione level (27).

In the present study, using scurvy-prone ODS-*od/od* rats, urinary BOM levels were increased by LPS treatment in both the AsA-fed group and the AsA-deficient group, but the increase was greater in the AsA-deficient group (Fig. 1). This suggests that AsA, which is a physiological antioxidant, suppresses LPS-induced

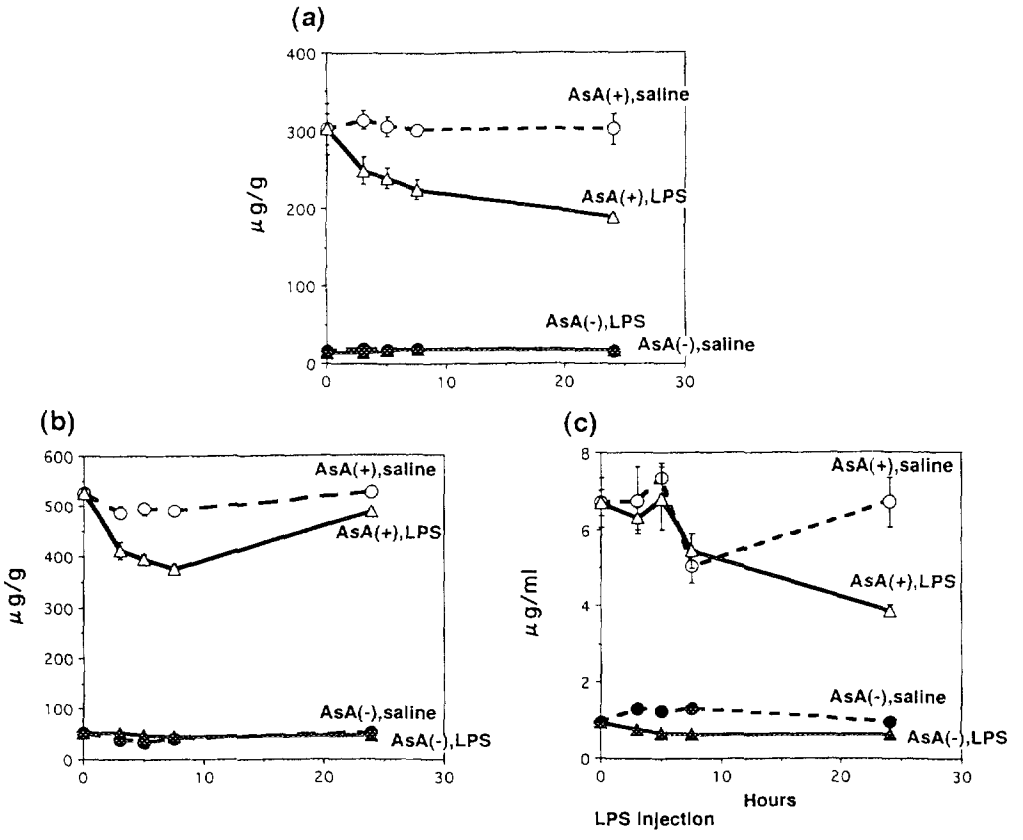

Fig. 5. Time course of the concentration of ascorbic acid in the liver (a), spleen (b), and serum (c) after injection with LPS or saline in rats.

increase in urinary BOM levels (Figs. 1 and 2). The results of this study also suggest that administration of LPS causes oxidative stress, resulting in a decrease in liver AsA levels and an increase in liver heme oxygenase-1 (HO-1) levels (Fig. 4 and 5a). In the AsA-fed group, serum and tissue AsA levels were lowered until 24 h after LPS treatment. As the AsA levels in the liver and spleen began to be lowered immediately after LPS treatment, this results may reflect consumption of AsA by its reaction to ROS (Fig. 5). Feeding AsA suppressed the elevation of hepatic activity and mRNA level of HO-1, the rate-limiting enzyme of bilirubin biosynthesis, in rats injected with LPS. These results suggest that bilirubin is oxidized by reactive oxygen species in rats treated with LPS and acts as a physiological antioxidant synergistically with ascobic acid *in vivo*.

On the other hand, splenic HO-1 mRNA level showed no marked changes after LPS treatment. We infer that the oxidative stress caused by LPS treatment did not induce further HO-1 production in the spleen, because HO-1 is steadily produced in the spleen and HO-1 is a predominant isozyme of heme oxygenase produced in the spleen (i.e., HO-2 production is very small in the spleen) (Fig. 3b).

Our findings are compatible with the theory that HO-1 induction and subsequent bilirubin biosynthesis have a protective function *in vivo* when the defense mechanism is challenged.

REFERENCES

1. Stocker, R., Yamamoto, Y., McDonagh, A. F., Glazer, A. N., and Ames, B. N. (1987) Science. 235, 1043-1046.
2. Neuzil, J., and Stocker, R. (1994) J. Biol. Chem. 269, 16712-16719.
3. Maines, M. D., Ibrahim, N. G., and Kappas, A. (1977) J. Biol. Chem. 252, 5900-5903.
4. Yamaguchi, T., Komoda, Y., and Nakajima, H. (1994) J. Biol. Chem. 269, 24343-24348.
5. Yamaguchi, T., Komuro, A., Nakano, Y., Tomita, M., and Nakajima, H. (1993) Biochem. Biophys. Res. Commun. 197, 1518-1523.
6. Yamaguchi, T., Shioji, I., Sugimoto, A., Komoda, Y., and Nakajima, H. (1994) J. Biochem. 116, 298-303.
7. Shimizu, S., Izumi, Y., Yamazaki, M., Shimizu, K., Yamaguchi, T., and Nakajima, H. (1988) Biochim. Biophys. Acta. 967, 255-260.
8. Taketani, S., Kohno, H., Yoshinaga, T., and Tokunaga, R. (1989) FEBS Lett. 245, 173-176.
9. Keyse, S. M. and Tyrrell, R. M. (1989) Proc. Natl. Acad. Sci. USA. 86, 99-103.
10. Vile, G. F., and Tyrrell, R. M. (1993) J. Biol. Chem. 268, 14678-14681.
11. Tomaro, M. L., Frydman, J., and Frydman, R. B. (1991) Arch. Biochem. Biophys. 286, 610-617.
12. Rizzardini, M., Carelli, M., Cabello Porras, M. R., and Cantoni, L. (1994) Biochem. J. 304, 477-483.
13. Horio, F., Ozaki, K., Yoshida, A., Makino, S., and Hayashi, Y. (1985) J. Nutr. 115, 1630-1640.
14. American Institute of Nutrition (1977) J. Nutr. 107, 1340-1348.
15. American institute of Nutrition (1980) J. Nutr. 110, 1726.
16. Izumi, Y., Yamazaki, M., Shimizu, S., Shimizu, K., Yamaguchi, T., and Nakajima. H. (1988) Biochim. Biophys. Acta. 967, 261-266.
17. Shibahara, S., Muller, R., Taguchi, H., and Yoshida, T. (1985) Proc. Natl. Acad. Sci. USA. 82, 7865-7869.
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in Molecular Cloning, A Laboratory Manual (2nd Ed.), pp. 7.26-7.29. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
19. Horio, F., Shibata, T., Makino, S., Machino, S., Hayashi, Y., Hattori, T., and Yoshida, A. (1993) J. Nutr. 123, 2075-2084.
20. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
21. Kodaka, K., Inagaki, S., Ujiie, T., Ueno, T., and Suda, H. (1985) Vitamins (Japan). 59, 451-455.
22. Roe, J. H., and Kuether, C. A. (1943) J. Biol. Chem. 147, 399-407.
23. Maines, M. D., and Kappas, A. (1978) J. Biol. Chem. 253, 2321-2326.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
25. Duncan, D. B. (1957) Biometrics. 13, 164-176.
26. Applegate, L. A., Luscher, P. and Tyrrell, R. M. (1991) Cancer Res. 51, 974-978.
27. Mrtensson, J., and Meister, A. (1991) Proc. Natl. Acad. Sci. USA. 88, 4656-4660.