PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 155, 184-188 (1977)

Effects of *Salmonella enteritidis* Endotoxin on the Excretory Function of the Isolated Perfused Rat Liver (39770)

RICCARDO UTILI,[1] CHARLES O. ABERNATHY,[2] AND HYMAN J. ZIMMERMAN

Liver Research Unit, Veterans Administration Hospital, Washington, D. C. 20422

The pathogenesis of cholestatic jaundice associated with nonhepatic gram-negative bacterial infections (1-4) has not yet been elucidated. We have recently reported that *Escherichia coli* lipopolysaccharide (LPS) exerts dose-dependent cholestatic effects on the isolated perfused rat liver (IPRL). It was also found to decrease the excretion of sulfobromophthalein (BSP) and indocyanine green (ICG) in that model (5). A subsequent study has shown that inhibition of the bile salt-independent fraction (BSIF) of bile appears to be responsible for the decrease in biliary secretion (6). These observations suggested that impairment of hepatic excretory mechanisms at the canalicular level by bacterial LPS might play a role in the development of the cholestasis observed during infections with gram-negative bacteria (5-7). In order to examine the general relevance of this phenomenon, we have extended our investigations to study the effects of a different endotoxin on hepatic function in the IPRL. The LPS from *Salmonella enteritidis* was selected as this bacteria is a common pathogen of rats and its LPS has been reported to increase BSP retention in rats (8).

Methods and materials. Purified endotoxins were purchased from Difco Laboratories (Detroit, Mich.). Endotoxin extracted with trichloracetic acid (Boivin type) was used in the experiments on ICG clearance and bile acid excretion, whereas a phenol-water extracted type (Westphal) was used in the experiments measuring BSP transport. Both types of LPS were dissolved in pyrogen-free saline immediately prior to use.

Male CD rats were purchased from Charles River Laboratories (N. Wellington, Mass.) and were maintained in facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care. Isolation and perfusion of the rat liver were conducted using a slight modification of the method of Penhos and co-workers (9). The perfusing medium consisted of a cell-free Krebs-Henseleit buffer (pH 7.45) supplemented with 240 mg of glucose, 1000 U of heparin, and 2 g of bovine serum albumin/100 ml. Sodium taurocholate (0.5 μmole/min) was infused into the recirculating medium during the entire experimental period to replace the bile salts normally present in the enterohepatic circulation of intact animals. The bile and perfusate flow rates were monitored as described previously (5).

ICG experiments. After an initial 30 min equilibration period, Boivin type LPS was added to the perfusate to final concentrations of either 40 or 60 μg/ml (saline only in controls). Fifteen minutes later, a bolus (4 mg) of ICG was added to the medium and the experiment was continued for 45 min. Samples of bile (20 μl) and perfusate (50 μl) were taken at 5-min intervals to estimate the levels of ICG in each (5).

Bile salts excretion. After allowing time for the preparation to equilibrate (30 min), three 20-μl bile samples, at 5-min intervals, were taken to estimate bile flow and biliary concentration of bile acids. Boivin type LPS was added at 50 min (from the initiation of the experiment) at a final concentration of 40 μg/ml. Twenty minutes later, a second series of three bile samples, 5 min apart, were obtained to estimate bile flow and bile salts excretion after the LPS treatment. Agreement of individual values within 5%

[1] Present address: Clinica delle Malattie Infettive, I Facolta di Medicina, Universita di Napoli, Via Cotugna 1, Napoli 80135, Italy.

[2] Please address reprint requests to: Dr. Charles O. Abernathy, Liver Research Unit (151W), V. A. Hospital, 50 Irving St., N.W., Washington, D. C. 20422.

ISSN 0037-9727

was used as an acceptable criterion of steady-state bile flow. In conjunction with these experiments, a series of control experiments were run adding saline only.

Bile acid determination. The biliary concentration of bile acids was estimated using a purified hydroxysteroid dehydrogenase (Worthington Biochemicals, Freehold, N. J.) by the method of Paumgartner *et al.* (10). A 20-μl sample of bile was diluted to 200 μl with absolute methanol. Then, 20 μl of this solution was incubated with 1.0 ml of glycine buffer (pH 9.4) containing 5.6 μmole of EDTA and 0.4 mmole of hydrazine sulfate, 0.1 ml of a NAD solution (5.4 mmol/ml), and 0.1 ml of the enzyme solution (0.7 U/ml). The reaction mixture was incubated for 45 min at 26°. Then, the absorbance at 340 nm was determined and corrected by using a bile acid blank.

BSP transport maximum (Tm). In these studies, the Westphal preparation of endotoxin (20 μg/ml) was added 25 min after the start of the perfusion (saline only in controls). Fifteen minutes later, BSP (1.19 mg/min) was infused for 20 min to saturate the BSP transport processes. Subsequently, from 60 to 90 min, the dye was infused at a rate of 0.40 mg/min. The BSP Tm was determined as described previously (6, 11).

Samples of the perfusate were taken every 15 min and the level of aspartate aminotransferase (GOT) was determined in each (12). At the termination of each experiment, all nonhepatic tissue was carefully removed. Then, the liver was blotted on gauze pads, weighed, and placed in an oven (80°), and the dry weight was determined. There were no differences in the percentage dry weights of the control (29.1 ± 0.4) and the LPS (28.3 ± 0.3) groups. Student's *t* test was used to determine differences between means (13), and the 5% level was taken to be significant. All data are expressed as means ± SE.

Results. The *S. enteritidis* endotoxin, at concentrations of 40 and 60 μg/ml, reduced bile flow by 18 and 25%, respectively ($P < 0.01$; Table I). In control experiments, ICG was excreted at a rate of 5.43 ng/min/g of liver. ICG, at the dose employed in these experiments, had no effect on the rate of bile flow. Endotoxin caused a dose-dependent reduction in the rate of ICG excretion at 40 μg/ml (3.75 ng/min/g of liver; $P < 0.01$) and at 60 μg/ml (3.47 ng/min/g of liver; $P < 0.01$). LPS treatment also decreased ICG concentration in the bile, but did not affect the uptake ("half-life") of ICG from the perfusate (Table I).

The effects of the LPS (Boivin type) on the excretion of bile acids and bile flow are shown in Table II. Initially, the rate of bile secretion was the same in both control and LPS experiments. After the addition of the LPS, bile flow decreased from 1.30 to 1.14 μl/min/g of liver ($P < 0.01$) and a similar reduction was observed in the excretion of bile acids. However, the concentration of bile acids in the bile was unchanged.

In the experiments measuring the BSP Tm, maximal excretion of BSP was observed 30 to 35 min after initiation of the dye infusion and remained at the same level throughout the experiment (Fig. 1). The bile flow, during infusion of the BSP, was 1.84 μl/min/g of liver in the controls. Endotoxin (Westphal type), at a concentration of 20 μg/ml, caused a significant reduction in bile flow (1.48 μl/min/g of liver; $P < 0.01$) and in the excretion of BSP (15.4 vs 20.1 μg/min/g of liver for controls; $P < 0.05$). The BSP Tm was reduced by 23% ($P < 0.05$) by the LPS, but the concentration of

TABLE I. THE EFFECTS OF *S. enteritidis* ENDOTOXIN (BOIVIN) ON BILE FLOW AND INDOCYANINE GREEN (ICG) CLEARANCE IN THE ISOLATED PERFUSED RAT LIVER.

Endotoxin concentration in perfusate (μg/ml)	Bile flow (μl/min/g of liver)	ICG excreted (μg/min/g of liver)	ICG bile (μg/ml)	ICG half-life in perfusate (min)
0 (5)[a]	1.40 ± 0.03	5.43 ± 0.16	3.89 ± 0.15	25 ± 1
40 (5)	1.15 ± 0.02*	3.75 ± 0.24*	3.25 ± 0.19**	27 ± 1
60 (5)	1.05 ± 0.05*	3.47 ± 0.30*	3.20 ± 0.14**	24 ± 2

[a] Numbers in parentheses are the number of experiments in each group. All data are given as mean ± SE.
* Significantly different from controls ($P < 0.01$).
** Significantly different from controls ($P < 0.05$).

TABLE II. EFFECTS OF *S. enteritidis* LPS ON THE EXCRETION OF BILE ACIDS.[a]

Time of perfusion (min)	Controls			Endotoxin		
	Bile flow (μl/min/g of liver)	Bile acid excretion (μmole/min/g of liver)	Bile Acid concentration in bile (μmole/ml)	Bile flow (μl/min/g of bile acid liver)	Bile acid excretion (μmole/min/g of liver)	Bile acid concentration in bile (μmole/ml)
35 to 45[b]	1.27 ± 0.02	67.5 ± 4.6	53.2 ± 3.2	1.30 ± 0.02	70.3 ± 2.7	53.5 ± 4.8
70 to 80[c]	1.29 ± 0.03	64.9 ± 3.5	49.8 ± 2.0	1.14 ± 0.01*	54.0 ± 0.8*	47.7 ± 1.9

[a] Four experiments in each group. All data expressed as mean ± SE.
[b] Basal steady-state period prior to any treatment.
[c] Steady-state period after treatment with saline (controls) or endotoxin (Boivin type, 40 μg/ml).
* $P < 0.05$.

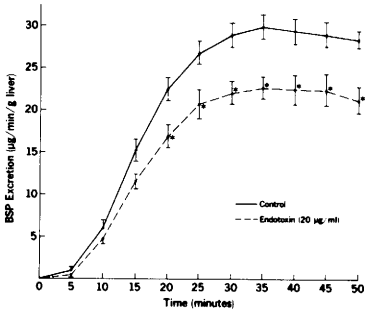

FIG. 1. Effects of *S. enteritidis* endotoxin (Westphal type) on the sulfobromophthalein (BSP) transport maximum in the isolated perfused rat liver. Four experiments were conducted at each treatment level. (*) denotes significance at the 5% level.

the dye in the bile was not changed whether calculated during the period of maximum transport or over the entire experimental period (Table III).

At the termination of the experiments, the GOT level in the recirculating perfusate of controls was 34 ± 4 IU/liter. LPS concentrations of 20 (45 ± 6), 40 (43 ± 4), and 60 μg/ml (45 ± 3) did not cause significant increases in the levels of this enzyme in the medium. The perfusate flow for all experiments, during the equilibrium period, ranged from 54 to 60 ml/min. By the end of the control experiments, there was a 7% average reduction in the rate of flow. There were 11, 10, and 11% average reductions in the endotoxin experiments using 20, 40, and 60 μg/ml, respectively.

Discussion. The results of the present investigation, using the *S. enteritidis* LPS, resembled those of our previous studies with the *E. coli* endotoxin (5, 6) as both LPS impaired hepatic excretory processes in the IPRL. The concentrations of LPS utilized in the present study are slightly higher than those used in the earlier study and suggest that the rat is less sensitive to the effects of this LPS than to the *E. coli* endotoxin. These concentrations, however, are similar to those employed by others to study the effects of endotoxin on hepatic function in rodents, dogs, and baboons (8, 14–17).

The *S. enteritidis* LPS, like the *E. coli* LPS, led to dose-dependent decreases in bile flow and organic anion excretion (Table I). Inhibition of ICG excretion seemed to be due to both a reduction in bile flow and a decrease in the concentration of the dye in the bile. The LPS, however, did not affect the uptake of the dye, as judged by the half-life of ICG in the perfusate. Inhibition of hepatic conjugation mechanisms could not have accounted for the observed results, as ICG is excreted as the parent compound (18). The results suggested that the major defect is on the excretory processes of the hepatocyte.

Since ICG, at concentrations saturating the transport mechanisms for the dye, exerts a profound cholestatic effect (19, 20), we measured the effects of the LPS on the Tm of BSP. A different LPS extract (Westphal), however, was used for these studies to exclude the possibility that contaminants present in the Boivin type LPS could account for the adverse effects on the liver function. This preparation, at 20 μg/ml, decreased the BSP Tm (Fig. 1), but did not affect the concentration of BSP in the bile (Table III). These data suggest that, at lower concentrations, the impairment of dye transport is the result of decreased bile secretion, but, at higher concentrations, it may also reduce

TABLE III. INHIBITION OF SULFOBROMOPHTHALEIN (BSP) TRANSPORT BY *S. enteritidis* ENDOTOXIN (WESTPHAL TYPE) IN THE PERFUSED RAT LIVER.[a]

Treatment	Bile flow (μl/min/g of liver)	BSP excretory rate (μg/min/g of liver)	BSP/Bile (mg/ml)	BSP Tm μg/min/g of liver)	Maximal concentration of BSP in bile (mg/ml)
Controls (4)	1.85 ± 0.05	20.1 ± 1.1	10.9 ± 0.6	29.5 ± 1.3	14.4 ± 0.8
Endotoxin (4) (20 μg/ml)	1.48 ± 0.08*	15.4 ± 1.0**	10.4 ± 0.3	22.6 ± 1.5**	14.2 ± 0.2

[a] Numbers in parentheses are number of experiments in each group. All data given as mean ± SE.
* Significantly different from controls ($P < 0.01$).
** Significantly different from control ($P < 0.05$).

the concentration of the dye in the bile. It is not possible, of course, to exclude the possibility that, at the lower concentration, the LPS exerted a dual effect, of the same magnitude, on both bile flow and BSP transport. Since we used two types of LPS preparations, it appeared that the adverse effects of *S. enteritidis* on hepatic function are a biological property of the endotoxin and not the result of contamination. The Westphal method of extraction (phenol-water) yields a LPS with less protein contamination than does the Boivin method (trichloroacetic acid) (21) and this might account for the effects of the Westphal preparation at a lower concentration.

Some effects of the *S. enteritidis* LPS were found to be different than those observed with the *E. coli* LPS. Although the *E. coli* endotoxin caused a slight decrease in the net biliary bile acid excretion, the biliary concentration of bile acids rose. This suggested that the main effect of the *E. coli* LPS was on the BSIF (6). In the present study, the *S. enteritidis* LPS, at a concentration twice that used in the *E. coli* study, decreased bile acid excretion significantly, but did not affect the concentration of the bile acids in the bile (Table II).

Bacterial endotoxins exhibit many similarities in their biological activities (22). This phenomenon has been attributed to the close structural similarity, among the LPS from different gram-negative bacteria, of the lipid A moiety (21–23) which is considered to be responsible for the primary toxicity of the molecule. However, from the present study, it is not possible to deduce whether the lipid A moiety is responsible for the effects of the LPS.

Endotoxins exert a number of effects on hepatic function. They include alterations in carbohydrate metabolism, oxygen utilization, enzyme induction, and biliary secretion (7). Impairment of BSP removal from blood by *S. enteritidis* has been reported in rats (8) and we have found increased BSP retention in guinea pigs using an *E. coli* LPS (unpublished results). Similar results have been described for *Serratia marcescens* (24) and *E. coli* (16) endotoxins in rabbits and baboons, respectively. These reports and studies from our laboratory (5, 6), including the present study, have suggested that impairment of hepatic excretory mechanisms is a biologic activity of these LPS. The above observations lend support to the hypothesis (6) that circulating endotoxins play a role in the pathogenesis of the cholestasis observed during gram-negative bacterial infections.

Summary. The LPS (Boivin type) from *S. enteritidis* caused dose-dependent reductions in bile secretion and excretion of ICG in the isolated perfused rat liver (IPRL). The transport maximum of BSP was also impaired by this endotoxin (Westphal type). Since two types of LPS preparations were used in these studies, the results suggest that the observed effects were a biological property of the endotoxin and not the result of contaminants left by one of the extraction procedures. This LPS had no effect on either the rate of perfusate flow or on the rate of aspartate aminotransferase release by the IPRL. These data have provided additional evidence that inhibition of hepatic excretory pathways is a biological activity of LPS and are consistent with the theory that impairment of hepatic excretion by bacterial endotoxins might play a role in the pathogenesis of the cholestatic jaundice seen during gram-negative bacterial infections.

1. Bernstein, J., and Brown, A. K., Pediatrics **29,** 873 (1962).
2. Eley, D. F., Hargreaves, T., and Lambert, H. P., Brit. Med. J. **2,** 75 (1965).
3. Rooney, J. C., Hill, D. J., and Danks, D., Amer. J. Dis. Child. **122,** 39 (1971).
4. Escobedo, M. B., Barton, L. L., Marshall, R. E., and Zarkowsky, H., Clin. Pediat. **13,** 656 (1975).
5. Utili, R., Abernathy, C. O., and Zimmerman, H. J., Gastroenterology **70,** 248 (1976).
6. Utili, R., Abernathy, C. O., and Zimmerman, H. J., J. Lab. Clin. Med. **89,** 471 (1977).
7. Utili, R., Abernathy, C. O., and Zimmerman, H. J., Life Sci. **20,** 553 (1977).
8. Di Luzio, N. R., Trejo, R. A., and Crafton, C. G., J. Reticuloendothel. Soc. **11,** 637 (1972).
9. Penhos, J. C., Wu, C. H., Daunas, J., Reitman, B. A., and Levine, R., Diabetes **15,** 740 (1966).
10. Paumgartner, G., Horak, W., Probst, P., and Grabner, G., Naunyn-Schmied. Arch. Pharmak. **270,** 98 (1971).
11. Klaassen, C. D., and Plaa, G., J. Appl. Physiol. **22,** 1151 (1967).
12. Zimmerman, H. J., and Mao, R., Amer. J. Med. **250,** 688 (1965).
13. Snedecor, G. W., *in* "Statistical Methods," 5th ed., p. 237. Iowa State University Press, Ames (1966).
14. Nolan, J. P., and O'Connell, C. J., J. Exp. Med. **122,** 1063 (1965).
15. Filkins, J. P., and Cornell, R. P., Amer. J. Physiol. **227,** 778 (1974).
16. Holper, K., Trejo, R. A., Brettschneider, L., and Di Luzio, N. R., Surg. Gynecol. Obstet. **136,** 593 (1973).
17. Sleeman, H. K., Jennings, P. B., and Hardaway, R. M., Surgery **61,** 945 (1967).
18. Combes, B., and Shenker, S., *in* "Diseases of the Liver" (L. Schiff, ed.), p. 165. J. P. Lippincott, Philadelphia (1969).
19. Groszmann, R. J., Kotelanski, B., Kendler, J., and Zimmerman, H. J., Proc. Soc. Exp. Biol. Med. **132,** 712 (1969).
20. Horak, W., Grabner, G., and Paumgartner, G., Gastroenterology **64,** 1005 (1973).
21. Galanos, C., Z. Immun-Forsch. **149,** 214 (1975).
22. Luderitz, O., Galanos, C., Lehman, V., and Reitschel, E. T., J. Hyg. Epidemiol. Microbiol. Immunol. **18,** 381 (1974).
23. McCabe, W. R., Advan. Intern. Med. **19,** 135 (1974).
24. Hirsch, R. L., McKay, D. G., Travers, R. I., and Skaly, R. K., Lipid Res. **5,** 563 (1964).

Received January 16, 1977. P.S.E.B.M. 1977, Vol. 155.