STUDIES ON THE PHARMACOLOGICAL BASES OF FETAL TOXICITY OF DRUGS (IV). EFFECT OF ENDOTOXIN AND STARVATION ON SERUM PROTEIN BINDING OF SALICYLIC ACID IN PREGNANT RATS

Takafumi ITAMI and Seizaburo KANOH
Department of Pharmacology, National Institute of Hygienic Sciences, Osaka Branch, Osaka 640, Japan
Accepted July 7, 1983

Abstract—Our previous paper reported that the fetotoxic effects of aspirin (ASA) were enhanced by bacterial endotoxin (LPS), and the effects of ASA were attributed to its major metabolite, salicylic acid (SA), as indicated by high SA concentrations in fetus and placenta. In order to clarify the mechanisms of the enhancement by LPS, serum total protein, albumin and free fatty acid (FFA) levels and SA-binding capacity of serum protein were investigated in pregnant rats. The following results were obtained: 1) FFA levels increased steadily after day 16 of pregnancy, and SA-binding capacity of serum protein decreased gradually after day 18, as the pregnancy proceeded to full term. 2) LPS injection decreased total protein and albumin levels in normal and starved rats on day 15 of pregnancy. 3) Starvation and/or LPS injection potentiated the increase of FFA level and reduced significantly SA-binding capacity of serum protein in the rats on day 15 of pregnancy. 4) Serum protein showing low SA-binding capacity from LPS-treated rats recovered normal SA-binding capacity when FFA was removed from serum protein by charcoal treatment. These data suggested the decrease of the SA-protein binding in serum by the increased level of FFA, an inhibitor of the binding, and the decreased level of albumin as a possible mechanism for the potentiation of the fetotoxicities of ASA by LPS.

The authors earlier reported (1) that the toxicity of aspirin (Acetylsalicylic acid, ASA) was enhanced by injection of bacterial endotoxin (Lipopolysaccharide, LPS) in both normal and pregnant rats on day 15 of pregnancy. In the pregnant rats, ASA-induced fetal toxicities including fetal weight decrease, fetal death, resorption and skeletal anomalies were observed, and it was concluded that the effects were due to salicylic acid (SA), the hydrolyzed product of ASA, by estimating the tissue concentration of SA and ASA after administration of various doses of ASA. These toxicities were enhanced by LPS injection in the rats fasted overnight for administration of ASA.

The pronounced alteration in the intensity and duration of the pharmacological effects of drugs is known to be caused by changes in protein binding (2). Many reports have dealt with the protein binding; it has been shown that the acidic drugs bind to serum albumin (3, 4), and the binding is inhibited by free fatty acid (FFA) (3, 5). It has been also demonstrated that the pharmacological effects of highly bound drugs can be potentiated by FFA (6, 7).

The present report focused on the relation between the binding of SA to protein and the levels of protein and FFA in the serum of
pregnant rats which were starved and injected with LPS.

Materials and Methods

Animal experiments: Virgin female Wistar rats weighing 240–260 g were caged with male rats of the same strain overnight. The day on which a vaginal plug was found was taken as day 0 of pregnancy. All pregnant females were housed individually throughout the gestation period, and the body weight and general symptoms were checked daily. The pregnant females were allowed the commercial rat chow (LaboMR standard, Nippon Nosan Kogyo Co., Tokyo) ad libitum till 3 hr before sacrifice. The blood samples were collected from the jugular vein at 3:00 pm, and the collection was done on day 15 in the both experiments of starvation and LPS injection. The sera were stored at -20°C and assayed within 24 hr after the blood collection. The other details were presented in the previous paper (1).

Biochemical analysis: Preparation of E. coli endotoxin (LPS) and injection of LPS were described previously (1). Serum protein was analysed by the method of Lowry et al. (8) using bovine serum albumin (Sigma) as the standard. Serum free fatty acid (FFA) and albumin were estimated by clinical test kits (Wako Pure Chemical Co.) based on the methods by Shimizu et al. (9) and Doumas et al. (10), respectively. ASA and SA analytical methods were described in the previous report (1).

Protein-binding experiments: Serum protein-binding of SA was determined by equilibrium dialysis with isotonic phosphate buffer (0.02 M phosphate, 0.15 M NaCl, pH 7.4). Serum samples (in duplicates) were incubated with a double volume of 2 mM SA solution for 4.5 hr at 37°C. Aliquots were then taken for SA assay. When FFA-free serum was necessary, the serum was delipidized by charcoal according to the method of Chen (11).

The data obtained were subjected to curve fitting by means of the program developed by Dr. Zenei Taira (Faculty of Pharmacy, University of Bunri Tokushima, Tokushima) using a microcomputer, PC-8801 (Nippon Electric Co., Tokyo). Two binding sites resolution was accomplished by a non-linear least-square fit to the following equation in which C is the independent variable:

\[ r = \frac{n_1 K_1 C}{1 + K_1 C} + \frac{n_2 K_2 C}{1 + K_2 C} \]

where \( K_1 \) and \( K_2 \) are the association constants corresponding to \( n_1 \) and \( n_2 \), the number of primary and secondary binding sites; \( C \) is the free drug concentration; and \( r \) is the molar ratio of bound drug to binding protein.

Results

Change of rat serum components during normal pregnancy: Serum was withdrawn at day 10, 12, 14, 15, 16, 18 or 20 of pregnancy; and total protein, albumin and serum FFA were estimated, and SA-binding capacity to serum protein was measured. The results are shown in Figs. 1 and 2.

The concentrations of total protein and albumin decreased gradually from day 17 of pregnancy and attained 88% of the control.
at term. On the other hand, the concentration of FFA increased from day 16 and attained 2.8-fold the normal value at term. The SA-binding of serum protein obtained from the above samples was estimated in vitro. As shown in Fig. 2, the capacity decreased gradually from day 18 to day 20.

Dose-response and time course of change in serum components and SA-binding of serum protein by LPS injection of pregnant rats: Previously, the authors studied the abortive effect of LPS on pregnant rats in terms of dose-response and time of injection (12). Further study of the effect of LPS on the change of serum components and the SA-binding of serum protein in pregnant rats was done with dose-response and time course after injection of LPS on day 15 of pregnancy. Data are presented in Table 1.

After injection of LPS (20 μg/kg, i.v.), total protein and albumin were decreased significantly, except albumin at 5 hr after LPS (20 μg/kg) injection. However, FFA increased significantly and attained the maximum level (1.12±0.01 μeq/ml) at 3.5 and 5 hr after injection, and then it returned to the normal level (0.58±0.04 μeq/ml) at 24 hr after injection.

The dose-related response of FFA was estimated at 3.5 hr after injection of 5, 20 and 60 μg/kg of LPS. Both total protein and albumin levels decreased in all cases, but the FFA level increased by LPS injection at doses of 20 and 60 μg/kg.

The SA-binding of serum protein decreased gradually and was lowest at 3.5 and 5 hr after injection, then recovered to the normal level within 24 hr.

Effect of starvation on serum components and SA-binding of serum protein in non-treated and LPS-treated pregnant rats: The same experiments with the above cited items were carried out in pregnant rats starved for 8, 20 or 40 hr; in addition, 20 μg/kg of LPS (i.v.) was administered. Data are shown in Table 2.

Total protein and serum albumin remained unchanged, but FFA increased significantly and attained 2.8-fold the normal value at term.

![Fig. 2. Salicylic acid (SA) binding of maternal serum protein. Vertical bar represents the S.E. of four to eight rats. *P<0.05 as compared to rat of day 0.](image)

Table 1. The concentrations of total protein, albumin and free fatty acid and the salicylic acid (SA) binding of serum protein after LPS injection in pregnant rats

| Dose (μg/kg, i.v.) | Time after LPS injection (hr) | Serum concentration | SA binding (nmol/mg protein) |
|-------------------|-----------------------------|---------------------|-----------------------------|
|                   |                             | Total protein (mg/ml) | Albumin (mg/ml) | Free fatty acid (μeq/ml) | |
| 0                 | —                          | 86.0±2.6            | 55.1±2.1         | 0.43±0.06               | 15.4±0.8          |
| 20                | 2                          | 75.1±2.0*           | 46.1±1.7*        | 0.83±0.09*              | 10.9±1.3*         |
| 20                | 3.5                        | 64.7±1.1*           | 41.8±0.7*        | 1.12±0.01*              | 5.8±1.1*          |
| 20                | 5                          | 72.4±2.2*           | 49.5±1.2         | 1.12±0.01*              | 5.8±0.7*          |
| 20                | 8                          | 67.1±3.8*           | 42.8±1.9*        | 0.96±0.13*              | 3.9±2.1*          |
| 20                | 24                         | 78.3±3.8*           | 44.9±0.7*        | 0.58±0.04               | 13.7±0.7          |
| 5                 | 3.5                        | 71.1±3.1*           | 46.1±0.9*        | 0.56±0.04               | 11.0±1.4*         |
| 60                | 3.5                        | 73.8±1.0*           | 43.0±0.6*        | 1.46±0.14*              | 4.8±0.5*          |

Rats were used on day 15 of pregnancy. *: See "Materials and Methods". Each value represents the mean±S.E. of three to twelve rats. *: P<0.05.
Table 2. Effect of starvation and/or LPS on the concentrations of total protein, albumin and free fatty acid in serum and the salicylic acid (SA) binding of serum protein in pregnant rats

| Treatment | Serum concentration | SA binding\(^*\) |
|-----------|---------------------|------------------|
|           | Total protein (mg/ml) | Albumin (mg/ml) | Free fatty acid (\(\mu g/ml\)) | (nmol/mg protein) |
| Starvation (hr) | LPS (\(\mu g/kg\), i.v.) |           |             |                  |
| —         | —                   | 86.0±2.6        | 55.1±2.1    | 0.43±0.06        | 15.4±0.8         |
| —         | 20                  | 75.1±2.0*       | 46.1±1.7*   | 0.83±0.09*       | 10.9±1.3*        |
| 8         | —                   | 84.9±0.5*       | 50.0±1.1    | 0.78±0.17*       | 10.5±0.9*        |
| 20        | —                   | 84.9±1.4        | 52.4±0.8    | 0.78±0.06*       | 11.6±0.8*        |
| 20        | 20                  | 73.9±1.5*       | 47.3±0.6*   | 1.35±0.20*       | 9.5±1.0*         |
| 40        | —                   | 84.7±0.8        | 52.2±2.2    | 0.84±0.15*       | 10.7±1.1*        |

Rats were used on day 15 of pregnancy. The serum was obtained 2 hr after injection of LPS or saline. Each value represents the mean±S.E. of three to twelve rats. \(^*\) See "Materials and Methods". \(^\circ\) P<0.05.

Table 3. Data obtained from Scatchard plots

| LPS (\(\mu g/kg\)) | Time after LPS injection (hr) | Charcoal treatment | Binding constants |
|-------------------|-------------------------------|-------------------|------------------|
|                   |                               |                   | \(n_1\) | \(n_2\) | \(K_1\times10^3\) \(M^{-1}\) | \(K_2\times10^3\) \(M^{-1}\) |
| —                 | —                             | —                 | 0.9    | 2.8    | 14.3     | 0.5    |
| 20                | 3.5                           | —                 | 0.2    | 1.0    | 10.5     | 0.6    |
| 20                | 24                            | —                 | 1.0    | 2.8    | 13.2     | 0.5    |
| —                 | —                             | +                 | 0.9    | 3.4    | 17.8     | 0.5    |
| 20                | 3.5                           | +                 | 1.0    | 3.1    | 13.9     | 0.4    |
| 20                | 24                            | +                 | 1.0    | 3.5    | 16.7     | 0.4    |

See "Materials and Methods" and Fig. 3.

Almost constant during the 40 hr starvation. However, the administration of 20 \(\mu g/kg\) of LPS (i.v.) significantly decreased total protein and albumin in the rats starved for 20 hr, the same as in the non-starved rats. FFA increased with starvation even for 8 hr. The FFA increase was potentiated by LPS in rats starved for 20 hr. SA-binding of serum protein was decreased significantly in every case, and the combination of starvation and LPS reduced the SA-binding to 62% of the control value.

SA-binding of serum protein treated with or without charcoal in vitro: The above experiments revealed a possible gross correlation between the decrease of SA-binding of serum protein and the increase of FFA by starvation and/or LPS injection.

Scatchard plots of SA-binding of serum protein treated with or without charcoal were done for the serum withdrawn at 3.5 and 24 hr after LPS injection (20 \(\mu g/kg\), i.v.), and the binding constants were calculated. The data are shown in Fig. 3 and Table 3.

Before charcoal treatment of the sera, SA-
binding of serum obtained 3.5 hr after LPS injection markedly decreased in comparison with that of the control or the serum obtained at 24 hr after LPS injection. However, it recovered the normal binding capacity by charcoal treatment.

Discussion

In our previous paper (1), it was found that ASA fetotoxicity was enhanced by LPS injection in the rats which were fasted overnight before ASA administration on day 15 of pregnancy, and the mechanism partially depended on the increased fetal and maternal tissue concentrations of SA, a main metabolite of ASA. Wilson et al. (13) reported that the fetal concentration of SA after ASA administration was closely paralleled by the concentration of unbound SA in maternal plasma. Thus it was postulated that the enhanced fetal concentration might depend on the decreased binding capacity of serum protein after LPS injection. Therefore, in the present experiments we focused our attention on the change of serum protein binding by LPS and starvation in rats on day 15 of pregnancy.

The present results showed that the protein binding of SA decreased by starvation or LPS injection and decreased still more by LPS injection in combination with starvation (Tables 1 and 2). These results indicate that the decrease of protein binding was closely related to the increase of the fetal toxicities of ASA in LPS-injected rats (1). The binding of SA to protein has been well studied, and it was determined that albumin plays a main role in serum protein binding of SA (4) and has two or more types of binding sites (14). Interaction between FFA and protein has been studied by Chen (11), and FFA was known as a potent inhibitor of SA-binding (3, 5). In the present experiments, the decrease of protein binding was shown to be related to the increase of FFA concentration after LPS injection (Tables 1 and 2), and normal protein binding capacity was recovered when FFA was removed by charcoal treatment (Fig. 3 and Table 3). Thus the present results indicate that the inhibition of protein binding of SA was related to the FFA level. It was also known that the actions of thiopental (6) and warfarin (7) were potentiated by the increasing level of FFA, and these phenomena were considered to be related to the decrease of protein binding of the drugs. It was suggested that the fetal toxicity of ASA was potentiated by LPS through the increase of FFA level.

In this study, the increase of FFA level was observed in starved, LPS-injected and late pregnant rats (Tables 1 and 2, Fig. 1). It has been already shown that the plasma FFA arises primary from a lipolysis of triglyceride in adipose tissue (15, 16), and the release of FFA from the tissue is stimulated by starvation and by “lipolytic hormones” such as catecholamine and glucagon (15–17). Rats in late pregnancy are known to become in the starved state easily because of the great supply of glucose to the fetus (18). Sakaguchi and Sakaguchi (19) observed that the activity of hormone-sensitive lipase in the adipose tissue of mice was elevated after LPS injection. Spizer (20) reported that LPS injection can induce epinephrine from dog adrenal gland (21). Thus the increased lipolysis in adipose tissue by LPS might be include these mechanisms, but the details have not yet been clarified.

The present results also demonstrated that decrease of albumin level was observed in late pregnant and LPS-injected rats. Hønger (22) reported that the plasma volume was significantly higher in pregnancy, but the intravascular mass of albumin was normal,
and the extravascular mass of albumin was reduced. These observations show that the low concentration of albumin in late pregnancy was due to the increase of plasma volume. However, this mechanism and that for the decrease of albumin level by LPS are still unclear.

The present experiments suggested the inhibition by FFA of SA-binding to serum protein and the decrease of serum albumin level as possible mechanisms for the potentiation by LPS of the fetal toxicities of ASA.

Acknowledgements: The authors wish to thank Dr. Zenei Taira, University of Bunri Tokushima, for kindly offering us the use of his computer program for this study.

References
1) Itami, T. and Kanoh, S.: Studies on the pharmacological bases of fetal toxicity of drugs. (I). Relation of fetal toxicity and tissue concentration of acetylsalicylic acid with pyrogen in pregnant rats. Folia Pharmacol. Japon. 79, 357-367 (1982) (Abs. in English)
2) Levy, G.: Effect of plasma protein binding of drugs on duration and intensity of pharmacological activity. J. Pharm. Sci. 65, 1264-1265 (1976)
3) Solomon, H.M., Schrogie, J.J. and Williams, D.: The displacement of phenylbutazone-14C and warfarin-14C from human albumin by various drugs and fatty acids. Biochem. Pharmacol. 17, 143-151 (1963)
4) Short, C.S. and Tumbelesson, M.E.: Binding of drugs to plasma proteins of swine during the perinatal period. Toxicol. Appl. Pharmacol. 24, 612-624 (1973)
5) Rudman, D., Bixler, T.J., II and Del Rio, A.E.: Effect of free fatty acids on binding of drugs by bovine serum albumin, by human serum albumin and by rabbit serum. J. Pharmacol. Exp. Ther. 176, 261-272 (1971)
6) Ohmiya, Y., Ohshika, H. and Nakai, K.: Mechanism of potentiating action of epinephrine on thiopental anesthesia. Japan. J. Pharmacol. 20, 577-584 (1970)
7) Laliberté, R., Chakrabarti, S. and Brodeur, J.: The influence of fasting and stress on the response of rats to warfarin. J. Pharmacol. Exp. Ther. 196, 184-203 (1976)
8) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275 (1951)
9) Shimizu, S., Yasui, K., Tani, Y. and Yamada, H.: Acyl-CoA oxidase from Candida tropicalis. Biochem. Biophys. Res. Commun. 91, 108-113 (1979)
10) Doumas, B.T., Watson, W.A. and Biggs, H.G.: Albumin standards and the measurement of serum albumin with bromcresol green. Clin. Chim. Acta 31, 87-96 (1971)
11) Chen, R.F.: Removal of fatty acids from serum albumin by charcoal treatment. J. Biol. Chem. 242, 173-181 (1967)
12) Kanoh, S. and Ema, M.: Effects of bacterial endotoxin on pregnant rats and their offspring. Cong. Anom. 20, 151-155 (1980)
13) Wilson, J.G., Ritter, E.J., Scott, W.J. and Fradkin, R.: Comparative distribution and embryotoxicity of acetylsalicylic acid in pregnant rats and rhesus monkeys. Toxicol. Appl. Pharmacol. 41, 67-78 (1977)
14) Kragh-Hansen, U.: Molecular aspects of ligand binding to serum albumin. Pharmacol. Rev. 33, 17-53 (1981)
15) Hagen, J.H.: Effect of glucagon on the metabolism of adipose tissue. J. Biol. Chem. 236, 1023-1027 (1961)
16) Steinberg, D.: Catecholamine stimulation of fat mobilization and its metabolic consequences. Pharmacol. Rev. 18, 217-235 (1966)
17) Shapiro, B.: Adipose tissue. In Lipid Metabolism in Mammals, Edited by Snyder, F., p. 287-316, Plenum Press, New York (1977)
18) Gabbe, S.G. and Quilligan, E.J.: Fetal carbohydrate metabolism: Its clinical importance. Am. J. Obstet. Gynecol. 127, 92-103 (1977)
19) Sakaguchi, O. and Sakaguchi, S.: Alterations of lipid metabolism in mice injected with endotoxin. Microbiol. Immunol. 23, 71-85 (1979)
20) Spizer, J.A.: Endotoxin-induced alterations in isolated fat cells: Effect on norepinephrine-stimulated lipolysis and cyclic 3',5'-adenosine monophosphate accumulation. Proc. Soc. Exp. Biol. Med. 145, 186-191 (1974)
21) Egdahl, R.H.: The differential response to the adrenal cortex and medulla to bacterial endotoxin. J. Clin. Invest. 38, 1120-1125 (1959)
22) Hanger, P.E.: Albumin metabolism in normal pregnancy. Scand. J. Clin. Lab. Invest. 21, 3-9 (1968)