

Induction of hypoglycaemia and accumulation of 5-hydroxytryptamine in the liver after the injection of mitogenic substances into mice

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- 1 Various mitogenic substances (concanavalin A, pokeweed mitogen, polyI:polyC and a phorbol diester), as well as lipopolysaccharides (LPS or endotoxins), produced hypoglycaemia after being injected into mice. However, non-mitogenic immuno-stimulants (zymosan, carrageenan, an adjuvant peptide and interferon) did not induce hypoglycaemia.
- 2 All of the mitogenic substances also induced an increase in 5-hydroxytryptamine (5-HT) in liver, but the non-mitogenic substances did not have this effect.
- 3 The time course of the development of hypoglycaemia was similar to that of the increase in liver 5-HT.
- 4 The dose-dependence of the hypoglycaemia induced by LPS was similar to that of the increase in liver 5-HT.
- 5 In C3H/HeJ mice, the macrophages and/or lymphocytes of the mice are known to be less responsive to LPS, and both the LPS-induced hypoglycaemia and increase in 5-HT were less in these mice than in control mice (C3H/He and ddI mice).
- 6 These results suggest that macrophages and/or lymphocytes may participate in the induction of hypoglycaemia and the increase in 5-HT induced by mitogenic substances and LPS. A possible correlation between hypoglycaemia and the increase in hepatic 5-HT is discussed, although the relationship is not substantiated.

Introduction

Lipopolysaccharides (LPS) from gram-negative bacteria (i.e., endotoxins) are well known to produce hypoglycaemia in experimental animals. The major mechanisms underlying the hypoglycaemia are a decrease in hepatic gluconeogenesis (Filkins & Cornell, 1974) and an increase in glucose utilization by peripheral tissues (Wolf *et al.*, 1977). Insulin (Yelich & Filkins, 1980), the insulin-like action of LPS itself (Witek-Janusek & Filkins, 1980) and other humoral factors (Goodrum & Berry, 1979; Filkins, 1980) have been considered to participate in the LPS-induced hypoglycaemia.

Administration of a large amount of tryptophan (Smith & Pogson, 1977) and lower doses of 5-hydroxytryptophan (Wilson & Furman, 1982), a direct precursor of 5-hydroxytryptamine (5-HT), can produce hypoglycaemia. Although, a correlation between hypoglycaemia and 5-HT formation is not indicated, and the mechanism and the site of action of these aminoacids are not clear, pharmacological

studies suggest that the hypoglycaemia may be mediated by 5-HT (Furman, 1974; Smith & Pogson, 1977; Wilson & Furman, 1982). In addition, gluconeogenesis in isolated hepatic cells has been shown to be inhibited by some tryptophan metabolites including 5-HT (Smith *et al.*, 1979).

Recently, I found that administration of LPS and concanavalin A (Con A) to mice produced a marked increase in 5-HT especially in the liver (Endo, 1983). There was not such a marked increase in 5-HT in other tissues including the brain. The 5-HT increase was suggested to be due to a stimulation of the hydroxylation of tryptophan. Both LPS and Con A are inflammatory agents with mitogenic activity to lymphocytes, but the former is a B-cell mitogen and the latter a T-cell mitogen. Therefore, this study was undertaken to examine whether other mitogenic substances could increase the content of 5-HT in the liver, and also, whether they could induce hypoglycaemia.

Methods

Determination of 5-HT and blood glucose

Mice were kept under conditioned light and dark (7 p.m. to 7 a.m.) and fed *ad libitum*. All agents were injected to fed mice, if not mentioned, between 9 a.m. and 12 noon. Mice were decapitated and blood was collected in a glass tube, which was weighed before use, weighed and stored in a dry ice box. Livers were removed rapidly and stored also in a dry ice box. The 5-HT content was determined as previously described (Tadano *et al.*, 1980). The blood collected from each mouse was homogenized in ice-cold 0.4 M HClO₄ (1 ml 0.1 g⁻¹ blood) in the tube with an Ultra Turrax homogenizer (Janke & Kunkel Co., West Germany), and the supernatant obtained by centrifugation (5000 g, 5 min) was neutralized to pH 6.5–7.5 with 2 M KOH in an ice bath with magnetic stirring. The precipitate was removed by centrifugation (5000 g, 5 min) and the supernatant was used for the determination of glucose. Glucose concentration was routinely measured with glucose oxidase and peroxidase using a kit of Wako Glucose C-Test (Wako Pure Chemical Ind., Osaka, Japan) and expressed as mg % (w/w).

Animals

Male ddI mice were obtained from the Mouse Centre of our university. C3H/HeJ mice were kindly donated by R. Yoshida (Department of Medical Chemistry, Kyoto University, Kyoto, Japan) and bred conventionally in our laboratory. C3H/He and ddY mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All the mice used in these experiments were male and about 6 weeks old.

Agents

A lipopolysaccharide (LPS) derived from *Escherichia coli* 055:B5, prepared from the Boibin method, was obtained from Difco Lab. (Detroit, MI, USA). Pokeweed mitogen (PWM) was from E-Y Lab. (San Mateo, CA, USA). A synthetic double stranded polyI:polyC (pIpC) was from P-L Biochemical Inc. (Milwaukee, Wis., USA). Carrageenan (Seakem No 202) was supplied from Marine Colloid Inc. (Springfield, NJ, USA). Zymosan (from *Saccharomyces cerevisiae*), concanavalin A (Con A), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) and bovine insulin were purchased from Sigma Chemical Company (St Louis, MO, USA). Mouse interferon (β) was a kind gift from Professor K. Kumagai of this school (Department of Microbiol-

ogy). Agents, except for TPA, were dissolved or suspended in saline and injected into mice intravenously (i.v.) or intraperitoneally (i.p.) (0.05–0.1 ml 10 g⁻¹ of body weight). TPA was dissolved in ethanol, mixed with 6 volumes of saline and injected (i.p., 0.1 ml per mouse). Other agents were used as described previously (Endo, 1983).

Results

Time course of the development of increase in liver 5-HT and hypoglycaemia induced by LPS and Con A

Time courses of the increase in liver 5-HT and the decrease in blood glucose induced by LPS and Con A are shown in Figure 1. It was found that, in addition to LPS, Con A also induced hypoglycaemia. The

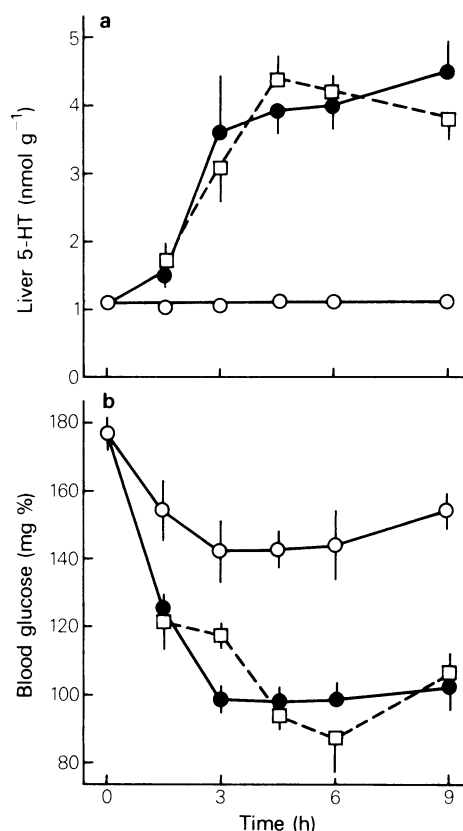


Figure 1 Time-course of the increase in liver 5-hydroxytryptamine (5-HT) (a) and the decrease in blood glucose (b) following the injection of lipopolysaccharide (LPS) (40 μ g kg⁻¹, i.v.) (●), concanavalin A (Con A) (5 mg kg⁻¹, i.v.) (□) and saline (○). Each value is the mean \pm s.d. of 4 mice. All values for the mice treated with LPS and Con A are statistically significant vs saline injection mice ($P < 0.01$, Student's *t* test).

development of the increase in 5-HT and hypoglycaemia was similar with LPS and Con A. Also, the increase in 5-HT and the decline in blood glucose occurred concurrently and reached maximum levels at 3 to 6 h after the injection of either LPS or Con A.

Effects of various immuno-stimulants on blood glucose and liver 5-HT

LPS is a B-cell mitogen and Con A is a typical T-cell mitogen (Greaves & Janosy, 1972). Therefore, the following mitogenic substances were tested. PWM and pIpC are known as B-cell mitogens (Greaves & Janosy, 1972; Scher *et al.*, 1973; Keightley *et al.*, 1976). TPA, a component of croton oil, is known to induce skin tumours and to stimulate lymphocytes (Nagel *et al.*, 1982). All these agents are inflammatory substances. Therefore, inflammatory agents without mitogenic activity, such as zymosan (Humes *et al.*, 1977) and carrageenan (Sugio & Tsurufuji, 1981), were also tested. Since LPS has adjuvant activity, MDP, a minimum unit of BCG vaccine with adjuvant activity (Chedid *et al.*, 1978), was tested. In addition, interferon was also examined, because LPS and pIpC are known to produce interferon. In *in vitro* and *in vivo* experiments, these agents are usually used at doses of the order of $\mu\text{g ml}^{-1}$ or g^{-1} body weight, therefore, the doses used in this experiment seemed reasonable to examine their effects. Since the hypoglycaemia and the increase in liver 5-HT reached maximum levels at 3 to 6 h after the injection of LPS and Con A, the effects of the various agents were tested at 4.5 h after injection.

As shown in Table 1, all of mitogenic substances induced both the increase in liver 5-HT and hypoglycaemia, but none of non-mitogenic substances produced these responses and LPS appeared to be the most potent inducer of these responses.

Dose-dependent increase in liver 5-HT and hypoglycaemia induced by LPS in different strains of mice

C3H/HeJ mice are known to be bad responders to LPS in various immunological tests, and lymphocytes and/or macrophages from these mice have been shown to be less responsive to LPS (Glode *et al.*, 1976; Ryan *et al.*, 1979; Nowakowski *et al.*, 1980).

The induction of the increase in liver 5-HT and hypoglycaemia by LPS in C3H/HeJ mice were shown clearly to be less responsive than in ddI mice (Figure 2). In ddI mice, a measurable increase in 5-HT had occurred after $2 \mu\text{g kg}^{-1}$ of LPS and its maximum effect was observed at $200 \mu\text{g kg}^{-1}$. The blood glucose level declined in almost the same dose-dependent manner as the increase in liver 5-HT. While, in C3H/HeJ mice, the 5-HT increase was slight even at $200 \mu\text{g kg}^{-1}$ and a clear increase was only seen after 2 mg kg^{-1} . Blood glucose in C3H/HeJ mice, also, declined in a comparable dose-dependent manner to the increase in 5-HT. The dose-dependency in C3H/He, a normal strain of C3H/HeJ, was similar to that in ddI (data now shown).

In order to further confirm the similarity of the dose-dependency between the increase in liver 5-HT and hypoglycaemia, LPS was injected intravenously into another strain of mice (ddY) (Table 2). Maximum effects of LPS on both liver 5-HT and blood glucose were produced by as little as $2 \mu\text{g kg}^{-1}$ of LPS. The LD_{50} for 24 h in this strain of mice was about 40 mg kg^{-1} (i.v.).

Another noticeable result in Table 2 is that the effects of LPS show little dose-dependence over a wide range of doses ($2 \mu\text{g}$ to 20 mg kg^{-1}). This ineffectiveness of LPS on blood glucose was in contrast to the effect of insulin. Insulin, 2 h after its subcutane-

Table 1 Effects of various immuno-stimulants on blood glucose and liver 5-hydroxytryptamine (5-HT) in ddI mice

Agent	Dose (mg kg^{-1} , i.v.)	Blood glucose (mg \%)	Liver 5-HT (nmol g^{-1})
Saline		155 ± 6	1.05 ± 0.11
LPS	0.04	$107 \pm 17^{***}$	$3.36 \pm 0.32^{***}$
Con A	5	$109 \pm 5^{***}$	$3.85 \pm 0.46^{***}$
PWM	5	$96 \pm 3^{***}$	$3.96 \pm 0.29^{***}$
pIpC	5	$110 \pm 6^{***}$	$2.48 \pm 0.16^{***}$
TPA	0.16 (i.p.)	$124 \pm 11^{**}$	$1.61 \pm 0.19^{***}$
Zymosan	5	149 ± 12	1.19 ± 0.05
Carrageenan	5	150 ± 5	1.19 ± 0.11
MDP	5	153 ± 8	1.00 ± 0.05
Interferon	0.7×10^5 (unit/mouse)	149 ± 16	1.02 ± 0.15

Mice were killed at 4.5 h after the injection of each stimulant. Each value is the mean \pm s.d. of 5 mice. Statistical significances vs control were made using Student's *t* test and represented as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

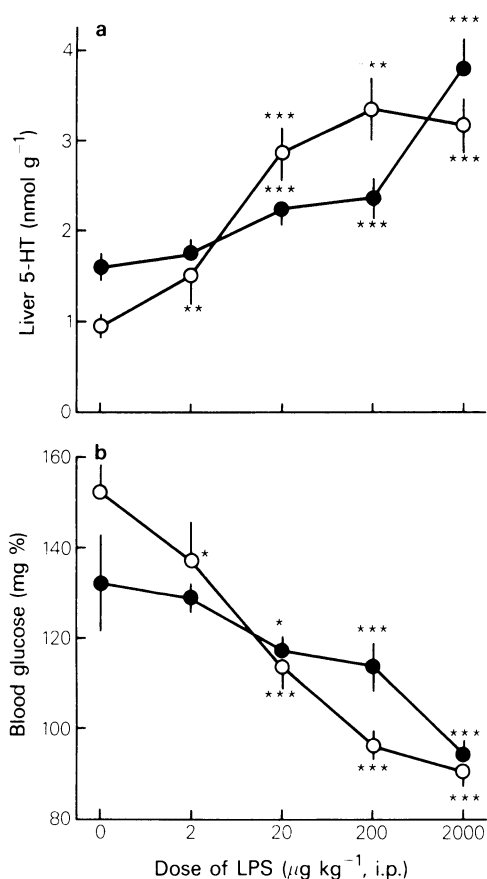


Figure 2 Dose-dependent increase in liver 5-hydroxytryptamine (5-HT) (a) and decrease in blood glucose (b) induced by lipopolysaccharide (LPS) in ddI (○) and C3H/HeJ (●) mice. Each value is the mean \pm s.d. of 5 mice. Control mice were given saline. Statistically significant differences vs control mice are represented as in Table 1.

ous injection to ddY mice, decreased blood glucose profoundly: 0.5, 2 and 10 units kg^{-1} of insulin decreased blood glucose levels to 57, 39 and 19% respectively (mean of 5 mice), in control mice. In addition, in mice starved for 24 h (ddI and ddY), their blood glucose decreased to about 60% of that in fed mice, LPS ($0.2 \mu\text{g}$ to 2 mg kg^{-1} i.v.) did not produce further hypoglycaemia. In the starved mice, however, LPS increased liver 5-HT to a similar extent as in fed mice (results shown in Table 2).

Effects of inhibitors of liver 5-HT synthesis on LPS-induced hypoglycaemia

By the same procedures as described previously (Endo, 1983), the effects of *p*-chlorophenylalanine

Table 2 Dose-dependency of the increase in liver 5-hydroxytryptamine (5-HT) and decrease in blood glucose induced by LPS in ddY mice

Dose of LPS ($\mu\text{g kg}^{-1}$, i.v.)	Blood glucose (mg %)	Liver 5-HT (nmol g^{-1})
Saline	110 ± 10	1.55 ± 0.10
0.2	$89 \pm 8^*$	$3.84 \pm 0.40^{***}$
2	$75 \pm 2^{***}$	$8.25 \pm 1.13^{***}$
20	$73 \pm 10^{***}$	$7.61 \pm 0.87^{***}$
200	$70 \pm 2^{***}$	$6.85 \pm 0.84^{***}$
2000	$68 \pm 4^{***}$	$6.98 \pm 0.51^{***}$
20000	$64 \pm 6^{***}$	$7.27 \pm 0.74^{***}$

Mice were killed at 4.5 h after the injection of various doses of LPS. Each value is the mean \pm s.d. of 5 mice. Statistically significant differences are represented as in Table 1.

(a possible inhibitor of tryptophan hydroxylation), Ro4-4602 (an inhibitor of aromatic amino acid decarboxylase), pargyline (an inhibitor of monoamine oxidase) and dexamethasone on the LPS-induced increase in liver 5-HT and hypoglycaemia were examined. The effects of these agents on the increase in 5-HT were essentially the same as in the previous experiments, i.e., *p*-chlorophenylalanine suppressed the increase in 5-HT but Ro4-4602 and pargyline did not affect the LPS-induced increase in liver 5-HT. Neither *p*-chlorophenylalanine, Ro4-4602 nor pargyline produced a significant effect on the LPS-induced hypoglycaemia. Dexamethasone was a potent inhibitor of the LPS-induced increase in liver 5-HT (Endo, 1983). This agent could clearly suppress the LPS-induced hypoglycaemia at a higher dose (20 mg kg^{-1}): the levels of blood glucose in dexamethasone-treated and non-treated mice were 85 ± 5 and $67 \pm 3\%$ of those in control mice (saline injected) ($n = 5$, $P < 0.001$). Methysergide (an antagonist of 5-HT), was also examined. This was injected (0.5 mg kg^{-1} i.p.) 1.5 h before the LPS injection ($40 \mu\text{g kg}^{-1}$ i.p.), but, did not produce a significant effect on the LPS-induced hypoglycaemia and increase in liver 5-HT.

Discussion

The present investigation demonstrates that various mitogenic but not non-mitogenic substances to lymphocytes induce an increase in 5-HT in the liver of mice. In C3H/HeJ mice, which have lymphocytes and/or macrophages that are less responsive to LPS, the increase in 5-HT to LPS was poor compared to other strains of mice. Of all the mitogenic substances tested, LPS was the most potent inducer of an increase in liver 5-HT. In addition to its mitogenic

activity on lymphocytes (B-cells), LPS is also known to be a potent stimulator of macrophages. Macrophages have been shown to be an absolute requirement for the activation of lymphocytes by various mitogens as well as to Con A (a T-cell mitogen) (Rosenstreich *et al.*, 1976; Rosenstreich & Mizel, 1978). Therefore, in addition to a direct interaction of mitogens with responder cells in the liver, the results described above suggest that the stimulation of macrophages and/or lymphocytes may be involved in the increase in liver 5-HT induced by mitogens.

In isolated normal liver cells of rats, it has been shown that 5-HT may not be formed even at high concentrations of tryptophan (Smith *et al.*, 1980). I also could not observe any increase in liver 5-HT at 4 h after the injection of tryptophan (200 mg kg⁻¹ i.p.) to ddI mice. On the other hand, an administration of 5-hydroxytryptophan (200 mg kg⁻¹, i.p.) increased liver 5-HT about 2.5 fold 4 h later as shown by Udenfriend *et al.* (1957). Therefore, the hydroxylation of tryptophan, a rate limiting step in the formation of 5-HT, may be stimulated in the liver cells of mitogen-treated mice as has been previously suggested (Endo, 1983). I have no data at present indicating the cell type in which the 5-HT increase occurs.

In the present study it was also found that hypoglycaemia was induced concomitantly with the increase in liver 5-HT. Goodrum & Berry (1979) have proposed a factor(s) derived from macrophages as a possible mediator of LPS-induced hypoglycaemia. The factor, termed glucocorticoid antagonizing factor (GAF), has been shown to inhibit the induction of phosphoenolpyruvate carboxykinase, a key enzyme in gluconeogenesis, although there is no direct evidence that GAF produces hypoglycaemia. Filkins (1980) also suggested the participation of insulin-like substances derived from macrophages. Vignaux & Gresser (1981) demonstrated that several viral and non-viral inducers (such as pIpC) of interferon cause hypoglycaemia, but, in agreement with my results, interferon was found not to be responsible for this effect. Taken together, these observations and results presented here suggest that the induction of hypoglycaemia by mitogenic substances, as well as LPS, may occur through the activation of macrophages and/or lymphocytes leading to the production of some humoral factors other than interferon, although the effect of insulin-like action of mitogens like LPS

cannot be ruled out (Witek-Janusek & Filkins, 1980).

The LPS-induced hypoglycaemia was not so drastic as the hypoglycaemia induced by insulin. Also, LPS did not produce a further decline in blood glucose levels in 24 h starved mice. Such an ineffectiveness of LPS has been also observed in starved rats (Buchanan & Filkins, 1976; Lloyd *et al.*, 1982). Although the reason is not clear, these results may indicate that LPS-induced hypoglycaemia differs from that induced by insulin, tryptophan and 5-hydroxytryptophan.

The increase in liver 5-HT and hypoglycaemia were quite similar to each other in terms of the effect of various mitogenic substances, time course of development and dose-dependency on LPS. In addition to these results, inhibition of gluconeogenesis in isolated hepatic cells by 5-HT (Smith *et al.*, 1979) and production of hypoglycaemia by tryptophan and 5-hydroxytryptophan, probably mediated through 5-HT, (Smith & Pogson, 1977; Furman, 1974; Wilson & Furman, 1982) support the idea that the increase in 5-HT in the liver may inhibit gluconeogenesis and contribute to the production of hypoglycaemia. In fact, in the present study, it was observed that, under the conditions described above, 5-hydroxytryptophan increased 5-HT in the liver and induced hypoglycaemia, but tryptophan (at a lower dose than those used by other investigators) neither increased liver 5-HT nor induced hypoglycaemia.

Dexamethasone suppressed both LPS-induced increase in liver 5-HT and hypoglycaemia. However, in contrast to the hypoglycaemia induced by tryptophan and 5-hydroxytryptophan, neither *p*-chlorophenylalanine, Ro4-4602, pargyline nor methysergide showed any significant effect on the LPS-induced hypoglycaemia. In addition, in starved mice, LPS increased liver 5-HT but did not produce further hypoglycaemia. It seems, therefore, that more direct evidence is necessary to confirm a correlation between the increase in liver 5-HT and hypoglycaemia induced in fed mice by LPS and other mitogenic substances. The mechanism and the site of action of tryptophan and 5-hydroxytryptophan induced hypoglycaemia need clarification.

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