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Effects of Endotoxin and Some Non-Steroid Anti-Inflammatory Drugs on Liver Tryptophan Oxygenase Activity in Mice

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Introduction

It has been known that high levels of volatile indoles, one of the tryptophan metabolites, are contained in the urine in the patients with rheumatoid arthritis[8]. It has also been proved that these metabolites induce pathologic changes similar to rheumatoid arthritis[17]. Agarwal et al.[2] have observed that endotoxin lowered the tryptophan oxygenase (TO) activity and prevented hydrocortisone induction of this enzyme, in the isolated, perfused rat liver. Berry and Smythe[6] have shown that liver TO activity rises temporarily 5 hours after the administration of LD_{50} of endotoxin and decreases thereafter in mice, and further have suggested that TO may play an important role in protecting mice against lethality of endotoxin, from some facts observed. 5-Hydroxytryptamine, one of tryptophan metabolites, is said to sensitize animals against endotoxin[15], and Nakoneczna et al.[17] and Reinicke et al.[18] also have suggested the relation between tryptophan metabolism and inflammation.

Thus, it is anticipated that a close relation exists between tryptophan metabolism and inflammation response. It is known that TO, one of the main enzymes in tryptophan metabolism, is one of the enzymes whose activity is significantly raised by the stimulation of hypothalamus[19], so the participation of the hypothalamo-hypophyseal-adrenocortical axis is also expected in the induction of TO by endotoxin as well as the anti-inflammatory drugs besides the hypophyseal-adrenocortical axis. From these points of view, the present study was designed to investigate effects of endotoxin which is pyrogenetic by acting the hypothalamus as well as some non-steroid anti-inflammatory drugs known to inhibit the biosynthesis of prostaglandins (PGs) on mouse liver TO.

Materials and Methods

1. Animals The same as the previous report[16].
2. Assay of liver TO The same as the previous report[16].
3. Assay of liver xanthine oxidase activity
   Assayed according to Litwack et al.[13].
4. Drugs used and the administration method of them
   (1) Lipopolysaccharide (DIFCO)
   Preparation purified from *Salmonella equi* by Westphal method was dissolved in saline. It was injected intraperitoneally (8 mg/kg).
   (2) 2, 4-Dinitrophenol (Nakarai Chemicals, LTD.)

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2, 4-Dinitrophenol (DNP) was diluted with distilled water and neutralized with NaOH solution after being dissolved in ethanol. 10 mg/kg i.p.

(3) Phenylbutazone (SIGMA)

Dissolved in NaOH solution and neutralized with HCl. 10 mg/kg i.p.

(4) Endomethacin (Indacin R [Banyu Pharmaceutical Co., LTD.])

One capsule contains 25 mg of indomethacin. This was suspended in 1% methylcellulose solution (Methylcellulose 100 [Nakarai Chemicals, LTD.]). Two drugs below also were similarly treated. 3 mg/kg i.p.

(5) Flufenamic acid (ARLEF 100 [Sankyo Co., LTD.])

One capsule contains 100 mg of flufenamic acid. 40 mg/kg i.p.

(6) Mefenamic acid (PONTAL Capsule 250 [Sankyo Co., LTD.])

One capsule contains 250 mg of mefenamic acid. 340 mg/kg i.p.

In the control mice, 10% ethanol, 10% methylcellulose-solution and saline were injected in the case of DNP; indomethacin, flufenamic acid and mefenamic acid; and other drugs, respectively.

Results

1. Time-course of TO activity after the injection of endotoxin

LD_{50} was considered as a dose of lipopolisaccharide (LPS), but since the LD_{50} of this preparation was obscure, whole TO activity was assayed 4 hours after the injection of 10 mg/kg of this preparation for trial. However, as this dose killed 8 animals of 9 for 48 hours after the injection, it was decided to administer the dose of 8 mg/kg.

Whole (refer to the previous report[16]), total and holo- TO activities are shown in Table 1. Further, the ratio of apoenzyme to holoenzyme also was shown in connection with apoenzyme in Table 1. Whole TO activities rose by about 50% 4 hours after endotoxin, decreased thereafter, reached minimum level after 24 hours and were somewhat restored after 48 hours. Although this change of activity is similar to that of holoenzyme acitvity in Berry and Smythe[6], it differed in the

Table 1. The change of liver tryptophan oxygenase activity after the intraperitoneal injection of *S. abortus equi* lipopolysaccharide (8 mg/kg)

Hours after the injection	TO activity (μM kynurenine/g/h)			Holo/apo	Holo/whole
	Whole enzyme	Total enzyme	Holoenzyme		
0	8.74±1.67 (5)	2.62±0.67 (4)	2.36±0.52 (4)	9.1	0.27
2.5	11.21±1.47 (5)	1.76±0.31 (8)*3	1.65±0.37 (8)*4	15.0	0.15
4	14.15±1.97 (4)*2	1.78±0.65 (8)	1.74±0.76 (8)	43.5	0.12
6	8.31±0.72 (5)	2.00±0.46 (5)	2.08±0.55 (5)	—	0.25
10	6.79±1.19 (5)	0.91±0.36 (5)*1	1.07±0.57 (5)*3	—	0.16
14	6.49±1.34 (4)	0.81±0.15 (5)*1	1.06±0.21 (5)*1	—	0.16
24	5.89±1.09 (4)*4	—	—	—	—
48	7.53±1.40 (4)	—	—	—	—

Each value is the mean±standard deviation for the number of amimals shown in parentheses.

*1: Sinficantly different from the 0 hour ($P<0.005$)

*2: Significantly different from the 0 hour ($P<0.01$)

*3: Significantly different from the 0 hour ($P<0.02$)

*4: Significantly different from the 0 hour ($P<0.05$)

point that the minimum value decreased below the activity in the adrenalectomized animals as mentioned below, that is, the average whole TO activity was 7.40 ± 1.44 and was not significantly different from the control value 4 hours after LPS in the adrenalectomized mice (The data are not tabulated.).

Total and holo- TO activities significantly decreased from the begining in spite of the first rising of whole TO activity, continued to be decreasing thereafter and was less than half the control level after 14 hours. The fall of total TO activity was particularly significant. The ratio of apoenzyme to holoenzyme also increased gradually, and it was found that no apoenzyme existed after 6 hours. The slight rising of total and holo- activities after 6 hours appears to be incidental. That the ratio of holo- to whole enzyme falls by 40 to 50% after 2.5 hours in comparison with the value at 0 hour is considered to show that the active part is more decreased toward whole enzyme quantity.

2. Effects of LPS on xanthine oxidase activities in mouse livers

It is suggested that TO comes to have a regulatory mechanism via two steps, that is, the synthesis of inactive TO molecules and the activation of these molecules by xanthine oxidase (XO)[10]. It is also suggested that the existence of substrate[12] and XO activity[7,10,11] are related to the activation of inactive forms of TO in the liver. XO activities were assayed from the consideration that the lowering of total and holoenzyme activities by LPS may be due to the earlier depression of XO activity brought by LPS.

As shown in Fig. 1, XO activities decreased as early as 1 hour and did by 50% 2 hours after LPS. This approximately agreed with the proportion of the fall of the ratio of holoenzyme to whole

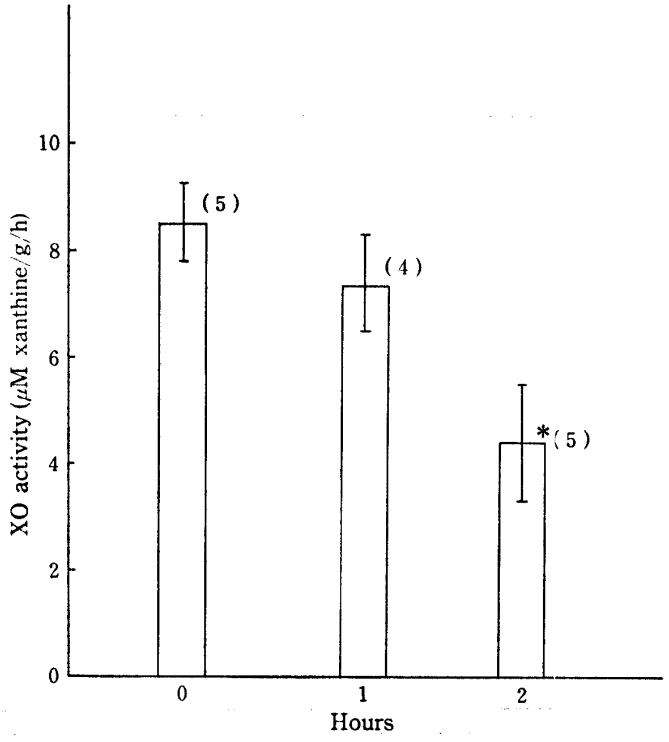

Fig. 1. Liver xanthine oxidase activity in mice after the intraperitoneal injection of *S. abortus equi* lipopolysaccharide (8 mg/kg).

Vertical bars represent the standard deviation of the mean for the number of animals shown in parentheses.

*: Significantly different from the 0 hour ($P<0.005$)

enzyme 2.5 hours and later than those after LPS. Accordingly, although XO acitivities were not assayed 2 hours and later than those after the administration, it is expected that they are going to show the similar fall until about 14 hours after LPS.

3. Effects of DNP on TO activities

In order to confirm whether the change of whole TO activity by LPS (Table 1) is due to a general effect of pyrogenetic substance or the specific action of LPS, changes of whole TO activities were investigated in mice after the injection of DNP, which may be one of the peripheral pyrogenetic

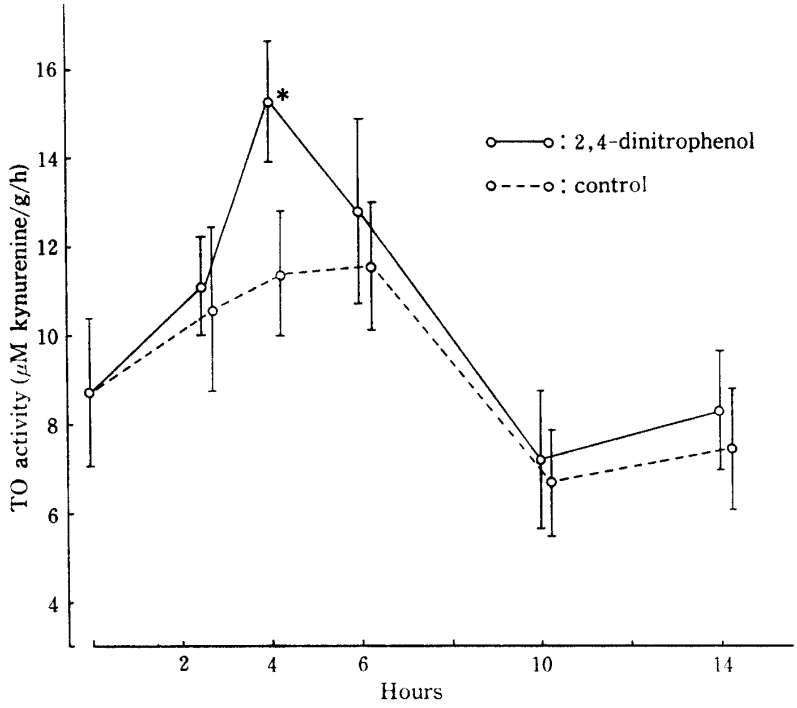

Fig. 2. Time cours of liver whole tryptophan oxygenase activity in mice after the intraperitoneal injection of 2, 4-dinitrophenol (10 mg/kg).

Vertical bars represent the standard deviation of the mean for 5 animals.

*: Significantly different from the control ($P<0.01$)

Table 2. Effect of 2, 4-dintrophenol on liver tryptophan oxygenase activity in adrenalectomized mice 4 hours after the intraperitoneal injection (10 mg/kg)

Treatment	Whole TO activity (μM kynurenine/g/h)
Control	5.76±0.86 (4)*
2, 4-dinitrophenol	5.64±1.22 (5)*
Saline	7.64±1.06 (6)

2, 4-Dinitrophenol was dissolved in ethanol and neutralized with 1/10N NaOH. The ethanol concentration of this solution was 10%.
Controls were injected with 10% ethanol.
Each value is the mean±standard deviation for the number of animals shown in parentheses.
*: Significantly different from the saline ($P<0.05$)

substances. As DNP was dissolved in ethanol, the ethanol solution of the same concentration was given in the control animals. The whole TO activity was already elevated after 2.5 hours of the treatment, showed the highest value of 15.25 after 4 hours, and began to fall thereafter. This change was the same as that after LPS. In the control animals treated with ethanol, the activity somewhat rose until 6 hours after the administration and thereafter showed the same trend as that in the animals treated with DNP (Fig. 2). No difference was found between the animals treated with DNP and the control ones treated with ethanol in the adrenalectomized animals (Table 2). This control TO activity in the adrenalectomized animals treated with ethanol was lower by about 25% than that in the adrenalectomized ones without ethanol. From these results, it is assumed that DNP only increases whole TO activity via the adrenal.

4. Effects of non-steroid anti-inflammatory drugs on TO activities

The experiment was scheduled to carry out on mefenamic acid, flufenamic acid, indomethacin, phenylbutazone and acetylsalicylic acid which were available. However, it was impossible to inject with salicylate, because a large quantity was required to dissolve it in water or ethanol, and the crystal of salicylate in 1% methylcellulose was too large to pass the needle. Since it was eventually ascertained to be impossible to administer salicylate except for the oral administration, the use of salicylate was discontinued. As each drug was treated as noted in Table 3, the same volume of 1%

Table 3. Effects of some anti-inflammatory drugs on mice liver tryptophan oxygenase activity

Injected substance	Whole TO activity (μM kynurenine/g/h)
Saline	9.00±1.21 (6)
1% Methylcellulose solution	8.62±1.18 (6)
Mefenamic acid (340 mg/kg)	14.11±1.96 (5)*
Flufenamic acid (90 mg/kg)	13.14±1.37 (5)*
Indomethacin (3 mg/kg)	9.07±1.38 (7)
Phenylbutazone (50 mg/kg)	14.71±1.31 (5)*

Activities were assayed 4 hours after the injection.
Mefenamic acid, flufenamic acid and indomethacin were suspended in 1% methylcellulose solution. Phenylbutazone was dissoluved in 1/10N NaOH and neutralized with HCl.
Each value is the mean±standard deviation for the number of animals shown in parentheses.
*: Significantly different from the value of vehicle ($P<0.001$)

Table 4. Effects of some anti-inflammatory drugs on liver trypophan oxygenase activity in adrenalectomized mice

Injected substance	Whole TO activity (μM kynurenine/g/h)
Saline	7.40±1.68 (4)
1% Methylcellulose solution	7.00±1.33 (4)
Mefenamic acid (340 mg/kg)	7.68±1.84 (4)
Flufenamic acid (90 mg/kg)	6.54±1.25 (5)
Phenylbutazone (50 mg/kg)	7.05±1.24 (5)

Notes are the same as in table 3.

methylcellulose or saline was administered in the control animals. The doses of drugs were decided according to Reinicke et al.[18] and the whole TO activities were assayed 4 hours after the injection of them.

As shown in Table 3, whole TO activities were increased by 50 to 60% after mefenamic acid, flufenamic acid and phenylbutazon, which were significantly different from the control. No difference was found after indomethacin. These increases were not observed in the adrenalectomized mice after 3 drugs in which the rise of activities was observable (Table 4).

Discussion

The increase of whole TO activity was observed in the early stage after LPS (Table 1). This increase is thought to be via the adrenal because this failed to occure in the adrenalectomized mice. Although whether the hypophysis also takes part on this effect or not was not investigated in this experiment, the possibility may be present as it is found that blood ACTH level is elevated after LPS[14]. Holo- and total activities decreased from the first after LPS in spite of the temporal increase of whole TO activity. Therefore, this increase of whole TO activity is thought to be due to the activation of the inactive form of holoenzyme. Berry and Smythe[6] have observed a temporal rising of holo- TO activity in livers of mice, following a single injection of LD_{50} dose of heat-killed *S. typhimurium*. Although this observation is different from the present result, whether this discrepancy is due to the difference of bacterial species or not is not clear in this stage.

A role of prostaglandins (PGs) has been suggested on the action of pyrogen, from the fact that the concentration of PGE like substance is elevated in cerebrospinal fluid when a body temperature rises by pyrogen, and this concentration is returned to normal level when the rise of body temperature is depressed by some inhibitory substances of PG synthesis[9]. Accordingly, the rise of PGs in brain (particularly hypothalamus) may play some roles on the increasing effect of whole TO activity by LPS, even if LPS per se is incapable of passing the blood-brain barrier.

As mentioned above, it has been suggested that TO comes to have a regulatory mechanism via two steps besides the activation by hematin[10]. It may be reasonable to postulate that the fall of total and holo- TO activities in the early stage after LPS, as well as the fall of whole TO activity 10 hours and later than those after LPS, may be owing to the reduction of activation by XO of inactive TO molecules synthesized, because XO activity decreased to nearly one-half the activity at 0 time (Fig. 1).

Berry and Smythe[6] have presumed that the depression of TO activity by endotoxin may be due to the reason that endotoxin antagonizes the action of glucocorticoid to permit normal TO level. In mice in this experiment, however, so much participation of the adrenal as the one observed by them was not recognized on the maintenance of normal TO level. Further, TO activity after LPS decreased below the activity in the adrenalectomized mice. Consequently, the inhibition of TO by endotoxin is thought not to be associated with glucocorticoid alone. Since Balis et al.[5] have observed that the necrosis in liver parenchyma cells is induced by the administration of *Escherichia coli* or its endotoxin and depressed by methylpredonisolone, the TO inhibition by LPS may be associated with the degeneration or necrosis of liver cells.

DNP is, so to speak, a peripheral pyrogenetic substance which is an uncoupler of oxidative phosphorylation and causes pyrexia by affecting intracellular metabolisms. Liver whole TO activities were investigated in mice administered with DNP for the comparison with endotoxin. The results were similar to those after LPS (Fig. 2). The lowering of the activities in the latter stage,

however, is thought to be due to a different mechanism from LPS. That is, from the facts that (1) the activities were similar to those in the control treated with ethanol except for the highest activity 4 hours after DNP, (2) the restoration to normal level was faster in the animals treated with DNP than in ones treated with LPS, (3) the activities fell to the same level as those in the control treated with ethanol in the adrenalectomized mice, this lowering of activities may not the effect of DNP but that of ethanol, just as the observation by Badawy and Evans[3,4] that chronic ethanol administration inhibited the activity of rat liver TO. Thus, it is considered that DNP does not have an inhibitory effect on TO activity. From this, although an inhibitory effect by endotoxin is considered to be the inhibition of TO synthesis in the liver, further studies should be undertaken in this regard, because there is also a report that tyrosin transaminase activity increases after endotoxin[1].

Of non-steroid anti-inflammatory drugs used, mefenamic acid, flufenamic acid and phenylbutazone raised significantly liver whole TO activities in the mice injected with relatively large doses of them, but indomethacin was of no effect (Table 3). This result is different from the one got from the holoenzyme activities in rats administered orally by Reinicke et al.[18]. They reported that indomethacin alone raised significantly holoenzyme activity, but all the other drugs showed no effects. Since they assayed holoenzyme activities alone, indomethacin may have had an action of activating inactive form in TO or of increasing heme saturation.

Since the TO induction by these drugs failed to occure in the adrenalectomized mice (Table 4), this action is thought to be via the adrenal. The consideration that a part of these drug's anti-inflammatory effects may be related to the increase of glucocorticoid release from the adrenal seems to be unreasonable, because the antipyretic, analgesic and anti-inflammatory actions are the highest in indomethacin and are not so different in the other drugs in clinical doses. It is difficult, furthermore, to desire a direct relation between the inducing capacity of TO and the doses in thse drugs owing to the fact that effects of drugs are various by their doses, the doses of drugs used were considerably high and a relation between the inducing capacity of TO and the dose was not investigated in this experiment.

It is said that the depressing action of the proliferation in granulation tissue is generally stronger in steriod drugs than in non-steroid ones, but it is stronger in indomethacin than in hydrocortisone, further, this action of indomethacin and mefenamic acid is effective even in the local application of them or in the adrenalectomized animals, and they do not act iva adrenal cortex[20]. Thus, the results in this experiment are uncompatible with this viewpoint as well, at least on mefenamic acid. Although Reinicke et al.[18] have suggested a corelation between anti-inflammatory and TO inducing activity in these drugs, it is unreasonable to expect this corelation, at least from the present result. More detailed study should be carried out, including a relation between the action and doses of these drugs.

Summary

Changes of liver whole, total and holo- activities were investigated in mice injected intraperitoneally with endotoxin and some of nonsteroid anti-inflammatory drugs. LPS increased whole TO activity on the earlier stage after the injection and decreased thereafter. The activity was below the normal value even 48 hours after LPS. This increase of whole TO activity was thought to be via the hypophyseal-adrenocortical axis.

Total and holo- TO activities decreased from the begining after LPS and the fall of the ratio of holoenzyme to whole enzyme, namely the decrease of active form of TO, was recognized con-

tinuously after LPS.

The XO activity, one of factors activating TO, decreased by 50% 2 hours after LPS.

Whole TO activities rose 4 hours after the injection of DNP used for the comparison with LPS, thereafter fell below the normal value, and were restored to normal value after 14 hours. Whole TO activities, other than the activity 4 hours after DNP, were not different from those in the control animals treated with ethanol. The adrenalectomy diminished by about 25% whole TO activities in both the animals treated with DNP and the control ones treated with ethanol.

Whole TO activities were raised by 50 to 60% after mefenamic acid, flufenamic acid and phenylbutazone, but indomethacin was ineffective. Effects of 3 drugs on TO induction failed to occure in the animals adrenalectomized.

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