MECHANISM OF 5-HYDROXYTRYPTAMINE RELEASE INDUCED BY BACTERIAL LIPOPOLYSACCHARIDE FROM BLOOD PLATELETS

I. A POSSIBLE PARTICIPATION OF PROTEOLYTIC ENZYME IN PLASMA*

HIROSHI TAKAGI, ISAMI KURUMA AND YASUYUKI NOMURA

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto

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Recently it was shown by Kuruma *et al.* (1) and Takagi and Kuruma (2) that a single injection of bacterial pyrogenic lipopolysaccharide (LPS) into rabbit caused a marked reduction of 5-hydroxytryptamine (5-HT) content in brain stem and spleen, without affecting on the content of noradrenaline and the time course of 5-HT decrease in brain stem is closely correspond with that of rise in rectal temperature.

This observation prompted us to study the mechanism of 5-HT release induced by LPS using platelet since it has been shown that the use of platelets is a valuable experimental procedure for study of the 5-HT release mechanism (3).

Although 5-HT releasing effect of LPS on platelet has been reported previously by Shimamoto *et al.* (4, 5), Des Prez (6-8) and Davis *et al.* (9, 10), its mechanism is remains to be investigated.

METHODS

Rabbits of either sexes and weighing 2.0 to 3.5 kg were used. All glassware with which platelets came into contact was coated with silicon, in order to minimize spontaneous release of 5-HT and histamine.

Blood was obtained by cutting the carotid artery and mixed with 1/10 volume of 100 units/ml heparin in saline solution and centrifuged at 100 g for 20 minutes at room temperature.

The supernatant plasma which contained a large amount of platelets was carefully pipetted off. This solution is referred as platelet rich plasma (PRP).

Furthermore platelet was isolated from PRP and suspended in isotonic Krebs solution (pH 7.4) by following procedures (Fig. 1): After centrifugation at 130 g for 20 minutes of PRP at 4°C, supernatant (platelet poor plasma) was removed and pellet were washed once with modified Krebs solution containing 10 units/ml heparin, separated again by centrifugation and resuspended in a modified Krebs solution containing 10 units/ml he-

高木 博司・車 勇・野村 靖幸

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FIG. 1. The isolation procedure of blood platelets from the whole blood.

parin corresponding to the original plasma volume. This solution is referred to below as the platelet-Krebs solution.

Isotonic modified Krebs solution (pH 7.4) has following composition: NaCl 6.90 g; KCl 0.35 g; CaCl_2 0.28 g; MgCl_2 0.11 g; NaHCO_3 1.00 g; NaH_2PO_4 0.14 g; Glucose 2.00 g in 1,000 ml glass distilled water.

In some experiments, a tissue culture medium No. 199 (Difco) was used instead of isotonic modified Krebs solution. This medium contains various kinds of amino acids, vitamins, pyrimidine bases and purine bases.

Platelet rich plasma or platelet-Krebs solution was divided into 4.5 ml samples which were transferred to 30 ml of Erlenmyerflask. Drugs dissolved in 0.5 ml of saline solution were added to the samples and were incubated for 60 to 180 minutes at 37°C under nitrogen gas with 60–70 shakings per minute. Platelet rich plasma or platelet-Krebs solution without drug was added to the control.

Then samples were centrifuged at 1,200 g for 30 minutes at 4°C, and platelets pellet was washed with saline solution. The 5-HT content in the platelet (pellet) and in the medium (supernatant) were determined by a spectrophotofluorimetrically by the method of Bertler (11). In this determination, activation and fluorescence wave length were 310 μ and 550 μ (uncorrected instrumental values) respectively.

Materials used were: lipopolysaccharide of *E. coli* (Dr. Kanoh), lipopolysaccharide of *Pseudomonas fluorescens* ("T.T.G.", Fujisawa Pharmaceutical Co., Ltd.), Leucocytic pyrogen (LP) which was extracted rabbit polymorphonuclear Leucocytes by Dr. S. Kanoh *et al.* (12) reserpine (Serpasil-Chiba), tetrabenazine methanesulphonate (Eisai Co., Ltd.), serotonin creatinine sulfate (Merck), diisopropylfluorophosphate (DFP, Sumitomo

Chemical Co., Ltd.) and *p*-toluene sulfonyl-*l*-arginine methylester hydrochloride (TAME, Nakarai Chemicals Ltd.).

RESULTS

1. 5-HT release from platelets at various concentrations of LPS, reserpine tetrabenazine and LP

Platelet rich plasma was incubated with various drugs at 37°C for 60 and 180 minutes.

A definite release of 5-HT from platelet was observed at concentrations such as 10^{-5} and 10^{-4} g/ml of LPS (*E. coli*) in a medium which contains 10 units/ml of heparin. A time course of 5-HT release was shown in Fig. 2. In this experimental condition a 5-HT releasing potency of LPS (10^{-4} g/ml) was approximately equivalent to that of reserpine (10^{-6} g/ml). However, as described below, LPS induced 5-HT release was affected by the concentrations of heparin added to the medium. In a medium containing low concentration of heparin such as 1 unit/ml which is enough to prevent clotting, LPS (10^{-6} g/ml) showed almost same 5-HT releasing activity as that of reserpine (10^{-6} g/ml).

Sometimes LPS induced a slight aggregation of platelet but no correlation between aggregation and 5-HT release was observed and no clotting was observed.

A 5-HT releasing action of LPS was observed even after heating of LPS to 98.5°C for 10 minutes.

Tetrabenazine, at a concentration of 4×10^{-4} g/ml, released about 50 per cent of 5-HT for 180 minutes.

Leucocytic pyrogen (LP) showed no 5-HT release from platelets.

The release of 5-HT from platelets by saline was usually 6 to 7 per cent of the total 5-HT appeared in supernatant.

When the incubation temperature was lowered to 4°C, neither LPS nor reserpine induced 5-HT release from platelets.

LPS of *Ps. fluorescens* induced also 5-HT release, but its effect was somewhat potent than that of *E. coli* (Fig. 3).

FIG. 2. Time courses of 5-HT releases from platelets in the presence of various concentrations of LPS (*E. coli*), reserpine (Re), tetrabenazine (Tb), leucocytic pyrogen (LP) and saline.

FIG. 3. Comparison of 5-HT releasing effects of LPS of *Ps. fluorescens* (10^{-4} g/ml) and *E. coli* (10^{-4} g/ml). Incubation: 60 minutes at 37°C.

2. Inhibitory effect of EDTA on 5-HT release by LPS and its reverse by calcium ion

Platelet rich plasma was used and incubation was continued for 60 minutes at 37°C. LPS of *E. coli* was used.

Ethylendiaminetetraacetic acid disodium salt (EDTA-Na) in concentration of 2.5 mM inhibited 5-HT release from platelet by LPS, but did not affect on release by reserpine (Fig. 4). In addition, calcium salt of EDTA (EDTA-Ca) in concentration of 2.5 mM did not cause this inhibitory effect.

The inhibitory effect by EDTA-Na on LPS-induced 5-HT release was reversed by addition of Ca^{2+} (2.5 mM) but unaffected by additions of Mg^{2+} (2.5 mM) or Ba^{2+} (2.5 mM).

FIG. 4. Effect of EDTA on the 5-HT releasing actions of LPS (*E. coli*) and reserpine. Incubation : 60 minutes at 36°C.

3. Combined effects of LPS and reserpine or tetrabenazine

The combined effect of LPS (*E. coli*) and reserpine or tetrabenazine on 5-HT release from platelet rich plasma were investigated at 37°C for 180 minutes. When reserpine (10^{-6} g/ml) or tetrabenazine (4×10^{-4} g/ml) with LPS (10^{-5} to 10^{-4} g/ml) were simulta-

TABLE 1. Combined effect of two drugs on 5-HT release from platelet. Incubation was performed at 37°C for 180 minutes.

Drugs(g/ml)	No. of experiments	5-HT released (%) mean values S.E.
RP 10^{-6}	4	45.0±5.8
Tb 4×10^{-4}	4	51.5±6.2
LPS 10^{-4}	4	46.0±4.8
LPS 10^{-5}	4	28.5±4.7
RP 10^{-6} and Tb 4×10^{-4}	3	45.7±5.7
RP 10^{-6} and LPS 10^{-4}	3	97.0±2.2*
RP 10^{-6} and LPS 10^{-5}	3	59.4±6.4*
Tb 4×10^{-4} and LPS 10^{-4}	3	77.1±4.7*
Tb 4×10^{-4} and LPS 10^{-5}	3	69.8±1.0**

RP : Reserpine, Tb : Tetrabenazine, LPS : Lipopolysaccharide (*E. coli*)

* : significant synergism ($P < 0.01$)

** : significant synergism ($P < 0.05$)

neously added to the platelet rich plasma, synergistic effects were observed, while combinations of reserpine and tetrabenazine showed no synergistic effect (Table 1).

4. 5-HT releases by drugs from platelets in various suspending media

Platelets were isolated and resuspended in an isotonic modified Krebs solution or a tissue culture medium (No. 199) as described under the METHODS. LPS of *E. coli* was used. In both suspending media, LPS (10^{-4} g/ml) did not induce 5-HT release from platelet, whereas reserpine (10^{-6} g/ml) induced a definite release such as seen in platelet suspended in plasma (Figs. 5 and 6).

FIG. 5. Time courses of 5-HT releases from platelets suspended in isotonic modified Krebs solution by LPS (*E. coli*) and reserpine. Incubation : 60 minutes at 37°C.

FIG. 6. 5-HT releases from platelets suspended in various media by LPS (*E. coli*) and reserpine. Incubation : 60 minutes at 37°C.

In order to make clear the role of the heat sensitive component of plasma in releasing 5-HT, isolated platelets were suspended in a solution which contained heat-treated plasma (56°C, 30 minutes) and modified Krebs solution and effects of drugs were investigated. In this medium, a 5-HT releasing effect of LPS (10^{-4} g/ml) was completely blocked, while that of reserpine (10^{-6} g/ml) was unaffected (Fig. 6).

5. Inhibition of LPS-induced 5-HT release by heparin

In this experiment, EDTA-Na was used as an anticoagulant.

The collected whole blood was mixed with 1/10 volume of 1% EDTA-Na and then platelet rich plasma was separated by the procedure described in Fig 1.

Ca^{2+} (3 mM) was added to the platelet poor plasma, and mixture was incubated at 37°C. After formation of fibrin clotting, serum was separated by filtration.

When platelet was resuspended in serum without addition of drugs, a marked 5-HT release (100% of control) was observed. However, this release was completely blocked by addition of 1 to 10 units/ml of heparin.

When platelet was suspended in a serum containing 1 unit/ml heparin, LPS of *Ps. fluorescence* showed a potent 5-HT release as shown in Fig. 7. In a serum containing 10 units/ml of heparin, LPS-induced 5-HT release was reduced, and it was completely blocked in a concentration of 100 units/ml of heparin, which induced the platelet aggregation (Fig. 7).

Reserpine-induced 5-HT release was unaffected at various concentrations of heparin (Fig. 7).

FIG. 7. Effect of heparin on the 5-HT releasing actions of LPS (*Ps. fluorescence*) and reserpine. Incubation : 30 minutes at 37°C.

6. Effect of DFP and TAME on LPS-induced 5-HT release

Platelet suspended in a serum which contains 10 units/ml of heparin and LPS of *Ps. fluorescence* were used.

FIG. 8. Effects of DFP and TAME on the 5-HT releasing actions of LPS (*Ps. fluorescence*) and reserpine. Incubation : 30 minutes at 37°C.

When DFP 10^{-4} M was added to a sample, LPS-induced 5-HT release was markedly inhibited, while reserpine-induced 5-HT release was unaffected by DFP (Fig. 8).

It was also shown that 10^{-3} g/ml of TAME inhibited LPS-induced 5-HT release whereas reserpine-induced 5-HT release was unaffected (Fig. 8).

DISCUSSION

The present results have shown that LPS at concentrations of 10^{-5} and 10^{-4} g/ml releases 5-HT from platelet rich plasma and this release is dependent on the presence of Ca^{2+} and a temperature sensitive plasma component. LPS-induced 5-HT release has been inhibited at 4°C, suggesting that biochemical (enzymatic or metabolic) process might be involved. A 5-HT releasing effect of reserpine from platelets is also blocked at 4°C, but its effect is independent on the presence of Ca^{2+} and plasma. Moreover the synergistic effects observed between LPS and reserpine or tetrabenazine suggest that the site of action of LPS might be different from that of reserpine or tetrabenazine.

LPS-induced 5-HT release from platelet is not related to platelet aggregation of clotting, since platelet aggregation induced by LPS is not paralleled by a 5-HT release and no clotting was observed in the present experimental conditions. This is also confirmed by electron microscopic studies on platelets (Kuruma and Takagi (13)). This result is incompatible with the work of Des Prez (6) that LPS-induced 5-HT release from platelet *in vitro* closely parallels the extent of platelet aggregation. However, this discrepancy may be not so important, since in a succeeding paper, Des Prez (7) has described a discussion that platelet aggregation and release of 5-HT may not necessarily occur together.

Calcium and plasma requirements for 5-HT release by LPS, and inhibition of LPS-

induced 5-HT release by DFP and TAME implies an indirect action, possibly mediated through activation of proteolytic enzyme such as prothrombin, plasminogen, prekallikrein, the first component of complement and others in plasma. In this connection, it is interesting to note the work of Des Prez (7) that LPS-induced 5-HT release is mediated through some mechanism other than thrombin mechanism. The present evidence that LPS is able to release 5-HT from platelet in the presence of the amount of heparin necessary to produce the inhibition of thrombin activity suggests also participation of some proteolytic enzyme other than thrombin.

There are certain similarities between LPS-induced 5-HT release and 5-HT releases induced by antigen, glycogen and dextran from platelet (14-16), since both agents require for the presence of Ca^{2+} and plasma in the medium. A participation of antigen-antibody reaction in 5-HT release induced by LPS is not conceivable, since heating of antiserum to 56°C has been shown to have no great effect on the ability of subsequently formed antigen-antibody precipitates to release histamine from rabbit platelets (17).

A possibility that LPS itself has a proteolytic activity is definitely denied, since the heat-treated LPS does not lose its 5-HT releasing action.

The common chemical structure of LPS, glycogen and dextran is polysaccharide, indicating that polysaccharide may play important role in activation of proteolytic enzyme. Further studies on this proteolytic enzyme are in progress.

Beeson (19) has described that pyrogenic activity of LPS may be attributed to a leucocytic pyrogen (LP) which was released from polynuclear leucocytes after uptake of LPS to them.

In the present results, LP showed no 5-HT release from platelet, indicating that LPS-induced 5-HT release *in vivo* is not mediated through LP.

SUMMARY

1. The mechanism of 5-HT release induced by LPS (*E. coli*) from rabbit platelet was investigated and compared with those of reserpine and tetrabenazine.

2. 5-HT release from platelet by LPS occurred only in the presence of Ca^{2+} and plasma component which is destroyed by preheating (56°C, 30 minutes) and this release is inhibited by DFP and TAME, while reserpine-induced 5-HT release was independent the presence of Ca^{2+} and plasma and not affected by DFP and TAME.

Leucocytic pyrogen showed no 5-HT release.

3. Potentiation of 5-HT release of LPS by reserpine and tetrabenazine were observed.

4. LPS-induced 5-HT release from platelet was observed in a medium which contained concentration of heparin necessary to block thrombin activity, but very high concentration of heparin blocked LPS-induced release.

5. A 5-HT releasing action of LPS was still observed even after its heating to 98.5°C for 10 minutes.

6. From the results obtained above, a participation of activation of some proteolytic enzyme in 5-HT release from platelet induced by LPS was suggested.

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