

Preventive Effects of *Impatiens balsamina* on the Hen Egg-White Lysozyme (HEL)-Induced Decrease in Blood Flow

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Monitoring the blood flow of unanesthetized mice was found to be a reliable and effective method for studying their anaphylactic responses, in addition to the known method of monitoring blood pressure. Hen egg-white lysozyme (HEL)-specific anaphylaxis in mice was estimated by monitoring the decrease in blood flow with a Doppler blood flow meter. This method is convenient for searching for both anaphylaxis and anti-anaphylactic substances from natural products. Using this system, we estimated the anti-anaphylactic effects of the 35% ethanol extract (IB) of petals of *Impatiens balsamina* L., as well as those of anti-allergic agents currently used. Kaempferol 3-rutinoside and lawsone from IB significantly inhibited the decrease of blood flow. We also found that platelet-activating factor (PAF) and serotonin participate in decreasing the blood flow, but histamine does not.

Key words anaphylaxis; blood flow monitoring; murine anaphylaxis; *Impatiens balsamina* L.; kaempferol 3-rutinoside; lawsone

The search for anti-allergic substances from natural products has attracted much interest in recent years. Previously, we developed an *in vivo* assay system for quantitatively estimating mouse anaphylaxis, including fatal shock.¹⁾ The method of monitoring blood pressure in unanesthetized mice enabled us to study the dynamics of the anaphylactic response in the same individual animals without killing them.²⁾ Hence, we investigated the participation of nitric oxide in mouse anaphylactic hypotension,³⁾ and the anti-anaphylactic effects of a 35% ethanol extract (IB) of white petals of *Impatiens balsamina* L., from which were isolated anti-anaphylactic compounds.^{4,5)} Some of the antianaphylactic mechanisms of IB were also characterized using a blood pressure monitoring method, with administration of exogenous histamine⁶⁾ or platelet-activating factor (PAF).⁷⁾

The mortality rate due to the shock of Hen egg-white lysozyme (HEL)-anaphylaxis is very high.¹⁾ Ohtsuka *et al.*⁸⁾ observed a decrease in the blood flow 30 min after an oral challenge with antigen in mouse intestine. This observation suggested that not only hypotension but also a decrease in the blood flow is involved in severe HEL-anaphylaxis, and thus led us to quantitatively determine anaphylaxis by blood flow.

This paper describes the monitoring of blood flow as a reliable and useful method for quantitating murine anaphylaxis and evaluating the effects of *I. balsamina*. The results with currently used anti-allergic agents, diphenhydramine hydrochloride (DPH), 3-{2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl}-2,4 (1*H*,3*H*)-quinazolinedione tartrate (Ketanserine),⁹⁾ disodium cromoglycate (DSCG)¹⁰⁾ and *rac*-3-(*N*-*n*-octadecylcarbamoyloxy)-2-methoxy-propyl 2-thiazoliethyl phosphate (CV-3988)¹¹⁾ are also reported.

MATERIALS AND METHODS

Animals Male ddY mice (SPF grade), 5 weeks old, were obtained from Japan SLC (Shizuoka, Japan) and housed at 24±2 °C. Food and water were available *ad libitum*.

Materials DPH was purchased from Nakalai Tesque; Ketanserine was from Funakoshi Co., Ltd.; DSCG was from Molecular Biology Resources, Inc.; CV-3988 was from Bio-

mol Research Laboratories, Inc. These agents were dissolved in 10 µl saline/10 g body weight.

Plant Materials and Extraction *I. balsamina* L. was planted in our medicinal plant garden and the white flowers were collected in August. Fresh flowers (2.5 kg) were freeze-dried (200 g), extracted with cold 35% EtOH for 24 h and concentrated *in vacuo*, giving a residue (designated IB) of 8.47 g. IB was dissolved in 10 µl saline/10 g body weight before use in biological experiments.

Isolation of kaempferol 3-rutinoside (1) and lawsone (2-hydroxy-1,4-naphthoquinone) (2) from IB was done as previously reported.⁴⁾ These compounds were dissolved in 100 µl saline/10 g body weight.

HEL Sensitization and Challenge Immunization with HEL was performed as previously described.²⁾ Male ddY mice, 5 weeks of age, were sensitized intraperitoneally (i.p.) on day 0 with 50 µg of HEL (Sigma, 6 times recrystallized) emulsified in Freund's complete adjuvant (DIFCO). On day 9, each mouse was challenged intravenously (i.v.) with 100 µg of HEL in 30 µl saline.

Blood Flow Measurement Subcutaneous blood flow in the mouse tail was monitored using a laser Doppler blood flowmeter of the non-contact type (FLO-N1, Neuroscience, Japan). Each mouse was placed on a holder in a measuring chamber kept at 37 °C throughout the measurement. The blood flow of the venous microcirculation of the tail hypodermic of the unanesthetized mouse was measured every 2 min. It was *ca.* 50% less than normal blood flow under anesthesia. Each mouse was pre-warmed for 10 min. at 37 °C, prior to the experiment. The normal blood flow was measured for 10 min at 20 min before the experiment. The results were expressed as mean±S.E. of percent of the normal blood flow of each mouse, because the individual specificity of the blood flow is large. When a mouse died due to fatal shock after challenge during measurement, the blood flow was converted to 0%.

Natural Whole Blood Clotting Time The whole blood of each sensitized mouse was sampled within 15 s, after the animal was anesthetized with ether on day 9. Natural blood clotting time was measured immediately with an Automatic Blood Coagulometer (Amelung KC 4A). The results are ex-

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pressed as mean $\pm$ S.E. of the natural blood clotting time of a normal mouse and a sensitized mouse ($n=7$).

Statistical Analysis The data were evaluated by Student's *t*-test.

RESULTS

Examination of the Amount of Antigen Sensitization was performed with the same amount used in the blood-pressure method, but the amount of challenge was changed because no significant decrease in blood flow was observed with the challenge using 1 $\mu\text{g}/30\ \mu\text{l}$ /mouse, the same amount used in the blood-pressure method. The dose of 100 $\mu\text{g}/30\ \mu\text{l}$ /mouse was used since a significant difference was observed for doses of 100 $\mu\text{g}/30\ \mu\text{l}$ /mouse. At this level, the mice may die due to anaphylactic shock, but a lower dose would not lead to a significant difference, thus making different the evaluation of drug efficacy.

Monitoring Blood Flow of Control Groups The changes in blood flow of normal unsensitized mice were monitored after intravenous injection with 30 μl of saline or HEL (100 $\mu\text{g}/30\ \mu\text{l}$ saline). As shown in Fig. 1, the blood flow of the normal and saline administration groups was almost stable in every case. The blood flow of the HEL-administered group rose slightly and showed a significant difference from the normal blood flow at 28 to 30 min. This increase may be due to the vasodilator action by PGI_2 or NO produced from vascular endothelial cells activated by the cytokine discharged by HEL-stimulation.¹²⁾

Although mice were kept on a holder in a 37°C chamber for 30 min during the monitoring, this condition did not induce stress responses, and it had little, if any, effect on the net blood flow within the monitoring period.

Decrease in Blood Flow Caused by Antigen-Specific Anaphylaxis The blood flow of ddY mice sensitized with HEL on the 9th day rapidly decreased within 2 min. after i.v. challenge of HEL (100 $\mu\text{g}/30\ \mu\text{l}$), and fell to the minimum value (about 10% of normal blood flow) after 6 min (Fig. 2, —●—). The decreased blood flow did not return to the control level within 30 min, and showed a significant difference ($p<0.001$) from the normal blood flow.

Anti-anaphylactic Activities of Diphenhydramine Ketanserin, Disodium Cromoglycate and CV-3988 DPH, an anti-histaminic agent, Ketanserin, a serotonin 5HT_{2A} receptor antagonist,⁹⁾ DSCG, an anti-degranulate agent,¹⁰⁾ and CV-3988, a PAF-antagonist,¹¹⁾ were examined. DPH, DSCG or CV-3988 at 10 mg/kg each, were administered intravenously 1 h prior to HEL-challenge ($n=7$ per group). DPH did not cause any significant difference (Fig. 2A), while Ketanserin

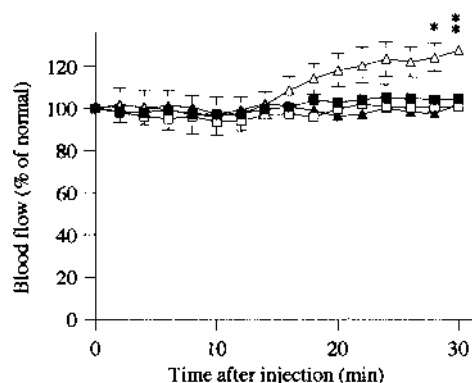


Fig. 1. Monitoring of Blood Flow in Control Groups

■: Untreated normal mice; □: saline; △: HEL; ▲: pretreatment with IB in normal mice. Results are mean $\pm$ S.E., $n=7$ per groups. * $p<0.05$, ** $p<0.01$ compared with normal by Student's *t*-test.

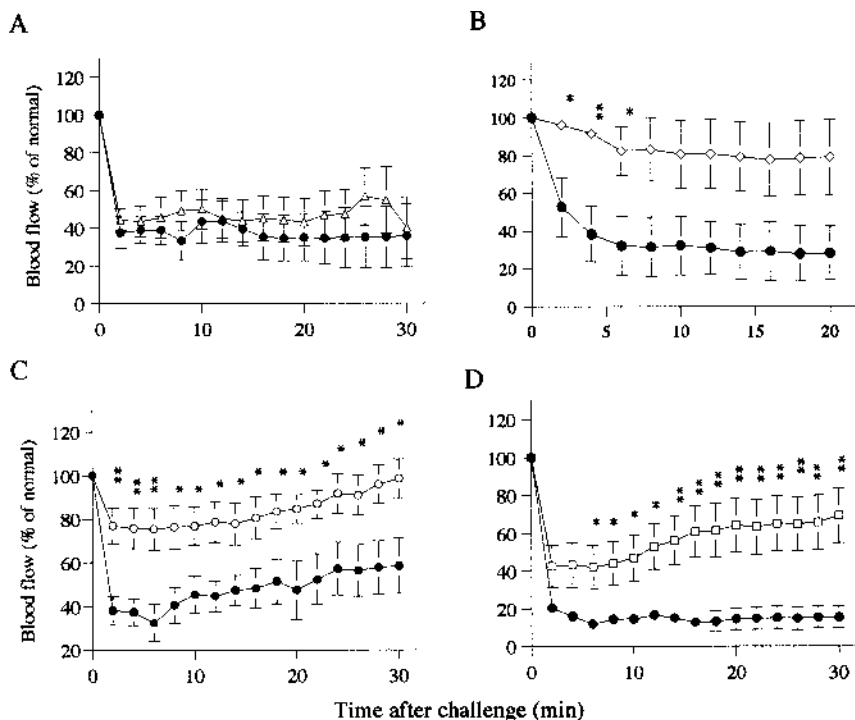


Fig. 2. Effect of DPH, Ketanserin, DSCG and CV-3988 on the HEL-Induced Decrease of Blood Flow

Blood flow was measured after HEL challenge in HEL-sensitized mice. ●: control; △: pretreatment with DPH(A); ◇: pretreatment with ketanserin tartrate (B); ○: pretreatment with DSCG (C); □: pretreatment with CV-3988 (D) at 1 h before challenge (10 mg/kg, i.v., each). Points represent the mean $\pm$ S.E. for 5 or 7 mice. * $p<0.05$, ** $p<0.01$ compared with control by Student's *t*-test.

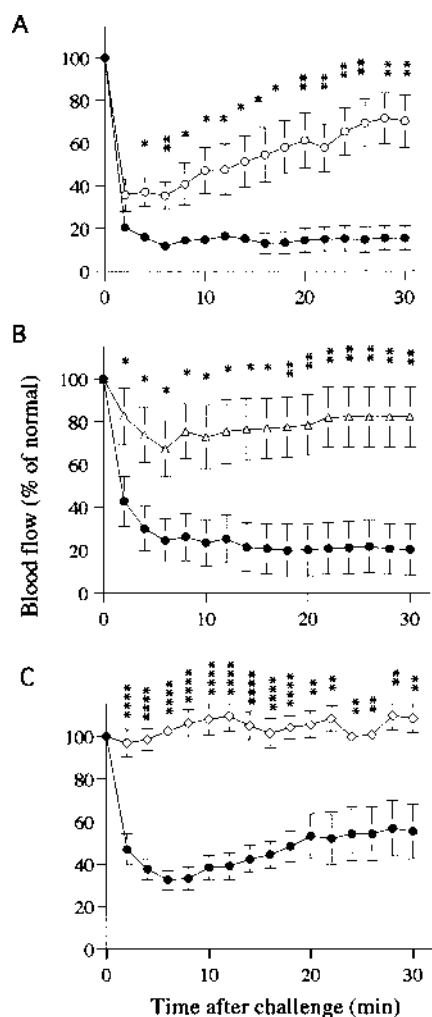


Fig. 3. Anti-anaphylactic Effect of IB, Kaempferol 3-rutinoside (**1**) and Lawsone (**2**) on the HEL-Induced Decrease of Blood Flow

Blood flow was measured after HEL challenge in HEL-sensitized mice. ●: control; ○: pretreatment with IB (100 mg/kg, i.v.) (A); △: pretreatment with **1** (10 mg/kg, *p.o.*) (B); ◇: pretreatment with **2** (10 mg/kg, *p.o.*) (C) at 1 h before challenge. Points represent the mean ± S.E. for 7 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ compared with control by Student's *t*-test.

caused a significant difference ($p < 0.01$ – 0.05) compared with the control group (Fig. 2B). DSCG (Fig. 2C) and CV-3988 (Fig. 2D) also showed significant differences ($p < 0.01$ – 0.05) compared with the control group.

Anti-anaphylactic Effect of IB as Reflected by Blood Flow HEL-sensitized mice were injected i.v. with 100 mg/kg of IB on the 9th day just 1 h before the challenge with HEL. Figure 3A shows that the decrease in blood flow in HEL-specific anaphylaxis was significantly blocked by a single IB pretreatment in the monitoring period, and returned to normal at about 30 min (—○—). As a control, normal mice were monitored for their blood flow from 1 h after i.v. IB injection. IB itself did not cause an increase in the blood flow (data not shown), but a slight decrease, although it was not significant. Other groups of normal unsensitized mice were injected similarly with IB and 1 h later challenged with HEL. This combination of IB with HEL also did not result in an increase in the blood flow (data not shown).

Anti-anaphylactic Effect of Kaempferol 3-Rutinoside (1**) and Lawsone (**2**) as Reflected by Blood Flow** As these compounds were insoluble in the amount of saline (10 μ l

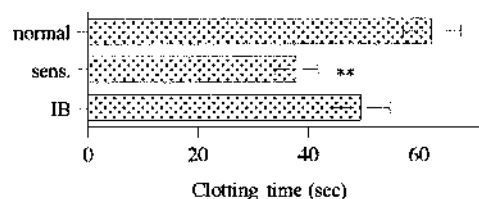


Fig. 4. Blood Clotting Time in HEL-Sensitization

Normal: without treatment; Sens.: sensitization with HEL; IB: IB pretreatment (100 mg/kg, *p.o.*) 24 h before sensitization. Results are the means ± S.E. for 7 mice. ** $p < 0.01$ compared with normal by Student's *t*-test.

saline/10 g body weight) used for an i.v. injection, they were administered orally (*p.o.*) at 10 mg/kg (100 μ l saline/10 g body weight) to HEL-sensitized mice on the 9th day just 1 h before the challenge with HEL. Figure 3B shows the inhibitory effects of **1** against the reduction of blood flow by anaphylaxis. Compound **1** showed a significant difference ($p < 0.05$ – 0.01). Figure 3C shows the inhibitory effects of **2**. Compound **2** also showed a highly significant difference ($p < 0.01$ – 0.0001).

Changes of Natural Clotting Time by Sensitization

The influence on blood flow by sensitization was considered to be reflected by the blood clotting time. Measurement of the natural blood clotting time of sensitized mice showed a significantly ($p < 0.01$) shortened clotting time compared with normal mice, as shown in Fig 4.

The sensitized mice pretreated by IB showed slight, but not significant, prolongation of clotting time as compared with the sensitized mice.

DISCUSSION

The anaphylaxis-induced model we developed causes not only a decrease in blood pressure based on the vasodilation of NO,²⁾ but also shows a remarkable decrease in the blood flow in the tail. In the anaphylaxis provocation, the blood flow of the heart muscle may be maintained by sacrificing other blood flows.¹³⁾ It is considered that the reduction of both blood flow and blood pressure causes serious anaphylactic shock in this model. Therefore, it would be worthwhile to try to prevent the reduction of both blood pressure and blood flow to the point of serious anaphylaxis.

Fluctuations of blood flow in parts of the body have been reported in anaphylaxis provocation. However, there has been no detailed report concerning the assay method. We have found that the blood flow of the venous microcirculation of the tail hypodermic of mice in HEL-anaphylaxis significantly decreased as compared to normal blood flow. Measurement of the reduction of blood flow was developed as a time-dependent quantitative method for anaphylaxis.

We previously reported that histamine and PAF are involved as initiators of a blood pressure decrease, whereas leukotrienes, PG and serotonin sustain anaphylaxis. DSCG continually inhibited the decrease of blood flow, suggesting the involvement of chemical mediators which exist in granules, since DSCG is an anti-degranulate agent. However, the typical mediator, histamine, would not be involved in the decrease of blood flow, since DPH, an anti-histaminic agent, did not show an inhibitory effect. Histamine liberates blood vessel relaxation factors, such as NO and PGI₂ which expand the blood vessels or improve blood flow from endothelial

cells. Therefore, the present method cannot be used to search for histamine antagonists, as shown by our DPH results. On the other hand, the participation of serotonin was suggested as a principal mediator, because Ketanserin inhibited the decrease of the blood flow. Ketanserin, an antagonist of serotonin 5HT_{2A} receptor, is involved in platelet aggregation or vasoconstriction.

It has also been suggested that PAF is not an initiator of the decrease in blood flow, since CV-3988 did not suppress this decrease just after the provocation of anaphylaxis. In a preliminary experiment, a transient reduction of blood flow was observed in an exogenous PAF single administration (data not shown), and the blood flow recovered faster than with the provocation of anaphylaxis. Therefore, PAF sensitivity in mice appears to increase by the sensitization. Since the natural clotting time of blood was shortened with sensitization (Fig. 4), blood clotting may be the cause of the blood flow decrease. Thus, PAF may participate in blood clotting.

In our previous study, several flavonoids and 1,4-naphthoquinones isolated from IB showed significant inhibitory effects on the mortality¹⁾ and hypotension⁵⁾ caused by anaphylaxis, on the heterologous PCA reaction, PAF-induced hypotension,⁷⁾ chronic pruritus and the development of dermatitis using NC mice, a model of atopic dermatitis.¹⁴⁾ Compounds isolated from IB contain large quantities of kaempferol, kaempferol 3-rutinoside (**1**), 2-methoxy-1,4-naphthoquinone and lawsone (**2**).⁴⁾ Among these compounds, **1** and **2**, which have different molecular skeletons, showed strong activity in all of the above-mentioned biological studies. They were thus chosen for the present study and found to significantly inhibited the decrease in blood flow.

Therefore, the remarkable anti-anaphylactic effects of *I.*

balsamina are ascribed to the suppression of both blood pressure and blood flow.

These findings indicate that the present method of measuring blood flow can be used as an assay method for the anti-anaphylactic effect of natural plant extracts or isolated chemical compounds. In addition, the method can be used to search for inhibitors of serious anaphylaxis by simultaneously measuring blood pressure. This new evaluation system for anaphylaxis should play an important role in studies on serious anaphylaxis.

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