Involvement of Inducible Nitric Oxide Synthase in Blood Flow Decrease in Vein Induced by Hen-Egg White Lysozyme

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Our in vivo assay system developed to search for allergy-preventive substances, assesses the blood flow decrease in tail vein microcirculation of mice subjected to sensitization with hen-egg white lysozyme (HEL). The blood flow decrease appears to be regulated by various factors such as nitric oxide (NO), thromboxane (TX) A₂, prostacyclin (PGI₂) and endothelin (ET)-1 together with cyclooxygenase (COX)-1, COX-2, inducible nitric oxide synthase (iNOS), and constitutive nitric oxide synthase (cNOS). In this study, we examined in detail the roles of iNOS in this assay system using an iNOS knockout (KO) mouse. We found that the blood flow decrease in the HEL-sensitized INOS KO mice was slightly weaker than that in their wild type (WT) mice. This blood flow decrease was not affected by a selective COX-1 inhibitor, a selective COX-2 inhibitor and a PGI₂ agonist unlike the case of the WT mice. However, it was inhibited by a nonselective NOS inhibitor, a specific TXA₂ synthase inhibitor and a specific ET-1 receptor blocker as in the case of the WT mice. The present results indicate that the blood flow decrease occurs via two pathways; one is an iNOS-independent response involving TXA₂ and ET-1, and the other is an iNOS-dependent response involving COX-1, COX-2 and PGI₂. cNOS appears to play some roles in the blood flow decrease and iNOS acts as an exacerbation factor. Our method using HEL-sensitized should be useful for searching for agents that can prevent allergy via new mechanisms.

Key words blood flow; inducible nitric oxide synthase (iNOS); allergy preventive medicine; nitric oxide (NO); hen-egg white lysozyme (HEL)

Our in vivo assay system was developed to quantitatively estimate mouse hen-egg white lysozyme (HEL)-anaphylaxis, including fatal shock.1) This assay system monitors the decrease in blood pressure2) or blood flow3) as anaphylaxis response and makes possible investigation of the dynamics of the anaphylactic response in the same individual animals without killing them. We have also discovered a distinctive phenomenon in which the blood flow of vein microcirculation is markedly decreased by HEL-sensitization alone without the HEL-challenge.4) The blood flow of the HEL-sensitized mice reproducibly decreases to about 65—75% of that of normal mice. Thus, the afferent (promotion) stage of allergy caused by xenobiotics can be dynamically and easily measured by using blood flow monitoring. This blood flow decrease is considered to be due to contraction of peripheral blood vessels and increase in blood viscosity, because no relationship with blood pressure was observed. In this assay system, mice are sensitized with a mixture of HEL and adjuvant. When only HEL or only adjuvant was used, the degree of the blood flow decrease was very small compared with the case of the mixture. Although anti-HEL IgE antibody significantly increased after HEL-sensitization, there was no significant increase in the number of leukocytes. Thus, the decrease of blood flows reflects the promoter process of an allergic reaction by the cooperation action of HEL and adjuvant. Using this blood flow decrease as a guide, we developed an in vivo assay method to search for substances, which can prevent allergies.5) Various factors are involved in blood flow decrease, including nitric oxide (NO), cyclooxygenase (COX)-1, COX-2, thromboxane (TX) A₂, endothelin (ET)-1, prostacyclin (PGI₂) and granulocytic elastase (GE) from vascular endothelial cells. We also found that the plasma levels of nitrite/nitrate (NO₃), metabolites of NO, together with expression of iNOS protein in the thoracoabdominal aorta synchronously increase with the blood flow decrease in HEL-sensitized mice.4)

In the present study, we examined how iNOS is involved in the blood flow decrease, using an iNOS KO mouse. The relationships among iNOS and NO, COX-1, COX-2, TXA₂ or ET-1 were also examined.

MATERIALS AND METHODS

Materials N-[3-(Aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride (1400W),6) 2-fluoro-α-methyl-4-biphenylacetic acid (Flurbiprofen)7) and N-[2-(cyclohexyl-oxoxy)-4-nitrophenoxy]-methanesulfonylamine (NS-398)8) were putchased from Cayman Co., Ltd.; N²-nitro-L-arginine-methyl-ester (l-NAME) and cyclo (d-α-aspartyl-l-prolyl-d-valyl-l-leucyl-l-tryptophyl) sodium salt (BQ-123)9) from Sigma Co., Ltd.; the stable analog of PGI₁ (Beraprost sodium)10) was from Yamanouchi Pharmaceutical Co., Ltd.; and 3-[4-(1H-imidazol-1-ylmethyl)phenyl] 2-propenoic acid hydrochloride monohydrate (Ozagrel)11) was from Ono Pharmaceutical Co., Ltd. These agents were dissolved in physiological saline or water. The saline or water solution was administered to mice at a volume of 10 μl/10 g body weight in the case of injection and 100 μl/10 g body weight in the case of oral use.

Animals Female iNOS KO mice with a C57BL/6 background (SPF grade), 7—8 week old, were obtained from Jackson Laboratory (Bar Harbor, Maine, U.S.A.). Male ddY mice of 5 weeks (SPF grade) and female WT C57BL/6 mice of 8 weeks old (SPF grade) were obtained from Japan Shizuoka Laboratory Animal Center (Shizuoka, Japan). The mice were housed at 24±2°C and 60±5% relative humidity.
Food (CE-2, Clea Japan, Inc.) and water were available ad libitum. All animal experiments were performed in accordance with the Guidelines for Animal Experiments of Mukogawa Women’s University.

**Hen-Egg White Lysozyme (HEL) Sensitization** Immunization with HEL was performed as previously described.\(^1\) The mice were subcutaneously sensitized on day 0 with 50 μg of HEL (Sigma Chemical Co., St. Louis, MO, U.S.A.) emulsified in complete Freund’s adjuvant (CFA; DIFCO).

**Blood Flow Measurement** The blood flow of venous microcirculation in the tail hypodermic of the unanesthetized mice was monitored using a laser Doppler blood flow meter of the contact type (FLO-C1, Neuroscience, Japan) as previously reported.\(^5\) Each mouse was pre-warmed for 10 min at 37 °C, prior to the experiment and was placed on a holder in a measuring chamber kept at 37 °C throughout the measurement. The normal blood flow was measured for 10 min at 1 d before the experiment. The blood flow of the sensitized mice was measured for 10 min every day for 9 d from the sensitization (0 d). The results were expressed as a relative percent change to the value before sensitization. Five animals per each group were used in all experiments.

**Expression and Amounts of iNOS** The expressions of iNOS protein of HEL-sensitized and -nonsensitized mice in the thoracoabdominal aorta were measured using the Western blot method at 9 d after sensitization. The X-ray film was scanned into an Adobe PhotoShop program (version 3.0) with an Epson scanner (GT-6600U) and transferred to the Macintosh NIH Image program (version 1.61). The density of the bands was measured using NIH Image gel macros. The iNOS protein signals were normalized to the signals of α-actin, a specific smooth muscle cell marker. The protein signals obtained were expressed as iNOS/α-actin ratios.

**Effects of Various Reagents on Blood Flow Decrease in the Tail Vein of HEL-Sensitized Mice** Reagents such as 1400W, flurbiprofen, NS-398, l-NAME, BQ-123, beraprost sodium and ozagrel were administered at 0 d (the sensitizing day), 3, 6 and 9 d after the sensitization. To focus on the relationship between iNOS and other factors, all reagents were used at effective doses that led to complete recovery of blood flow decrease as described previously.\(^5\) None of the reagents alone affected the blood flow. The statistical calculations are shown in comparison with the HEL-sensitized group (the control group).

**Statistical Analysis** Two-way analysis of variance (ANOVA) was used to test for statistical differences. When significant differences \(p<0.05\) were identified, the data were further analyzed by Dunnett’s multiple range test or the Tukey–Kramer test coupled with Bonferroni inequality for significant differences between each test group and the control group. For the Bonferroni test, 5 points were used after day 4 of the HEL-sensitization, because a significant difference was observed between the blood flows of non-treated and sensitized mice after day 4.

### Results

#### Change in NO Production and iNOS Expression by HEL-Sensitization

The protein expression of iNOS markedly increased with the blood flow decrease from day 1 to day 9 after the sensitization, when it was measured in the thoracoabdominal aorta of ddY mice (Fig. 1). The serum NO\(_x\) level also increased from day 3 to day 9 as described previously.\(^5\) On the other hand, the level (12.5 ± 1.7 μM) of day 9 was not so large as that causing decrease of blood pressure such as in anaphylaxis shock (150—200 μM).

**Effect of iNOS in HEL-Induced Blood Flow Decrease** \(N\)-[3-(Aminomethyl) phenylmethyl]-ethanimidamide (1400W, 5 mg/kg, s.c.), a selective iNOS inhibitor,\(^9\) did not significantly inhibit the blood flow decrease in HEL-sensitized ddY mice (Fig. 2).

**HEL-Sensitization in iNOS KO Mice** iNOS KO mice and their wild type (WT) C57BL/6 mice were sensitized with HEL, and the changes in the blood flow of the tail vein were measured. The blood flow in both mice showed a significant decrease compared with each non-treated (normal) group (Fig. 3). The present results are consistent with the data of 1400W shown above. However, the proportion of the blood flow decrease in the iNOS knockout mice was smaller than that of the WT mice.

**Effects of Various Reagents on HEL-Induced Blood Flow Decrease in iNOS KO Mice** Vascular endothelial cells are deeply involved in the maintenance of smooth microcirculation and blood vessel tension, local inflammation
and blood coagulation. PGI2 and TXA2, produced by COX in vascular endothelial cells and platelets, respectively, show contradictory actions against thrombocytes and vascular smooth muscles, and thus, the homeostasis of platelets is balanced by these two mediators. ET-1 is a vasoconstrictor and blood coagulation factor, which is released from vascular endothelial cells by the stimulation of inflammatory cytokines. When vascular endothelial cells are injured via neutrophils resulting from various types of shock, invasion of xenobiotics or systemic organ failure, the production of NO and PGI2 is inhibited and thus the vasoconstrictor action by ET-1 become relatively predominant. Consequently, microcirculation failure would be induced. We therefore used the following reagents were used with HEL-sensitized iNOS KO mice to elucidate the mechanism of the blood flow decrease.

1. L-NAME and L-arginine: L-NAME, a nonselective NOS inhibitor, inhibited the blood flow decrease in both WT and KO mice (Fig. 4). When L-arginine (L-Arg), a biosynthetic precursor of NO, was administered together with L-NAME, the recovery rate of the blood flow decrease was less than that caused by L-NAME alone.

2. Flurbiprofen: Flurbiprofen is a selective COX-1 inhibitor. COX-1 works not only in the defense against a rapid physiological change in the circulatory organs, but also induces the production of TXA2, followed by platelet aggregation in hematoblasts. Flurbiprofen (10 mg/kg, p.o.) significantly inhibited the blood flow decrease in the WT mice after day 6 of the HEL-sensitization, but did not inhibit that of iNOS KO mice (Fig. 5A). Therefore, COX-1 does not seem to be involved in the blood flow decrease in iNOS KO mice i.e. in the absence of iNOS.

3. NS-398: NS-398 is a selective COX-2 inhibitor. NS-398 is generally induced in vascular endothelial cells by inflammatory stimulation, followed by the production of various PGs such as PGI2, which leads to venous vasodilation and improvement of the blood flow. NS-398 (3 mg/kg, i.p.) significantly inhibited the blood flow decrease in the WT mice after day 6 of the HEL-sensitization, but did not inhibit that of iNOS KO mice (Fig. 5B). Thus, COX-2 also does not seem to be involved in the blood flow decrease in the absence of iNOS.

4. Beraprost: Beraprost sodium (40 μg/kg, p.o.), a stable analog of PGI2, significantly inhibited the blood flow decrease in WT mice after day 5 of the HEL-sensitization (Fig. 5C). On the other hand, beraprost sodium did not inhibit the blood flow decrease in the KO mice. A large amount of PGI2 is known to be synthesized by COX-2. Thus, sensitivity for vasodilator action for PGI2 may be reduced in the iNOS KO mice.

5. Ozagrel: Ozagrel is a specific synthase inhibitor of TXA2, which is an arachidonic acid metabolite, and causes platelet aggregation. Ozagrel (300 mg/kg, p.o.) significantly inhibited the blood flow decrease after day 7 of the HEL-sensitization in both WT and KO mice (Fig. 5D). Thus, the increase of TXA2 does not seem to be associated with the blood flow decrease induced by the HEL-sensitization, regardless of iNOS expression.

6. BQ-123: BQ-123, a specific receptor blocker of ET-1, is a vasoconstrictor released from vascular endothelial cells by calcium mobilization (calcikinesis) and activation of protein kinase and is induced by inflammatory cytokines and blood coagulation factors. BQ-123 (1 mg/kg, i.v.) significantly inhibited the blood flow decrease in both WT and KO mice after day 5 of the HEL-sensitization (Fig. 5E).
These results suggest that increased production of ET-1 is independent to iNOS expression in vascular endothelial cells.

**DISCUSSION**

We previously reported that the mechanism of the blood flow decrease in the vein caused by HEL-sensitization in ddY mice is very complicated and involves various factors such as NO, COX-1, COX-2, PGI2, TXA2, and/or ET-1.4) We assumed the following process for the mechanism of the blood flow decrease.4) After the sensitization, (i) cytokines induced by HEL activate nucleophils which release granulocytic elastase (GE). (ii) Injury of vascular endothelial cells by GE inhibits the production of NO and PGI2. As the result, the relatively predominant ET-1 induces microcirculation failure, followed by blood flow decrease. (iii) The resulting ischemia facilitates further production of cytokines, which induces the expression of COX-2 and iNOS.

In this study, we investigated the actual role of iNOS in the blood flow decrease caused by HEL-sensitized mice. In the HEL-sensitized ddY mice, the iNOS expression (Fig. 1) and the serum levels of NOx, metabolite of NO, markedly increased synchronously with the blood flow decrease. Therefore, the increased NOx level may result from iNOS expression. However, the blood flow decrease was inhibited partially by selective iNOS inhibitor 1400W (Fig. 2). Furthermore, blood flow decrease in the HEL-sensitized iNOS KO mice was slightly weaker than that in the WT mice (Fig. 3). These results indicate that the iNOS expression is not indispensable for the blood flow decrease and that perhaps NO generated by iNOS assists in amplifying of the serious decrease in blood flow. cNOS may play some roles in the HEL-induced blood flow decrease in the absence of iNOS, because l-NAME, an nonselective NOS inhibitor, inhibited the blood flow decrease in both WT and its KO mice (Fig. 4).

We also found that the characteristics of the blood flow decrease in sensitized WT C57BL/6 mice, which was used as the WT mice for iNOS KO mice, were similar to those in ddY mice.5) On the other hand, in the HEL-sensitized iNOS KO mice, the blood flow decrease was not affected by a selective COX-1 inhibitor (Fig. 5A), a selective COX-2 inhibitor (Fig. 5B), and PGI2 agonist (Fig. 5C), unlike in the case of the WT mice, whereas it was inhibited by a specific TXA2 synthase inhibitor (Fig. 5D) and a specific ET-1 receptor blocker (Fig. 5E) like in the case of the WT mice. These findings indicate that the HEL-induced blood flow decrease is caused by two pathways, an iNOS-independent response involving TXA2 and ET-1 and an iNOS-dependent response involving COX-1, COX-2 and PGI2.

This is the first report to clarify a causal relationship between iNOS expression and a decrease in blood flow of vein microcirculation in the promotion (afferent) stage of allergy.
Our animal model using HEL should be useful for the search for preventive agents of allergy with new mechanisms. The present research can also be applied to the development of preventive medicines against thrombosis and lifestyle-related diseases, which must possess multi-target inhibitory activities to be effective.

We are now using iNOS knockout mouse to examine how the blood coagulation system is related to the blood flow decrease. The results will be reported elsewhere together with the application of this assay method.

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