The Effect of Dietary *Bacillus Natto* Productive Protein on *in vivo* Endogenous Thrombolysis

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Key Words

Natto · Nattokinase · Fibrinolysis · Thrombolysis

Abstract

The influence of dietary *Bacillus natto* productive protein (BNPP) on endogenous thrombolysis was investigated in the rat. Animals were given a standard feed for 14 weeks, to which 0.2 or 1% BNPP was added. Thrombolysis was evaluated using an He-Ne laser-induced thrombosis model in mesenteric microvessels. Changes in thrombus volume, reflecting thrombolysis, decreased to 82% of the initial value in the control group. In contrast, the thrombus volume decreased to 67% in the animals fed 0.2% BNPP, and decreased to 51% in the group given 1% BNPP. The extent of thrombolysis in the 1% BNPP group was equivalent to that seen in animals treated with a bolus intravenous infusion of 0.2 mg/kg tissue plasminogen activator. The results demonstrated that the dietary administration of BNPP enhanced endogenous thrombolysis in a dose-dependent manner. Argatroban (2 mg/kg/h) enhanced endogenous fibrinolysis only in control animals, but not in the BNPP groups. The results support the suggestion that dietary supplementation with BNPP may

provide a simple means to promote fibrinolysis not only in the treatment of thromboembolism but also in the prevention of venous occlusion.

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Introduction

Intravenous fibrinolytic agents such as streptokinase, urokinase and tissue plasminogen activator (t-PA) have been widely used in clinical practice for thrombolytic therapy since the 1960s, 1970s and 1980s, respectively. However, the biological activity of these substances is short lived in the circulation after intravenous administration and there is a significant risk of hemorrhagic complications with a pronounced activation of fibrinolytic activity in whole blood in some circumstances [1–3]. Sumi and his colleagues [4, 5] were the first to demonstrate fibrinolytic activity *in vivo* after oral administration of the fibrinolytic enzyme, urokinase. The enzyme was shown to cross the intestinal tract and stimulate systemic fibrinolytic activity. Nevertheless, the therapeutic efficacy of this approach remains to be established.

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The prevention of life style-related diseases such as cardiovascular disease and stroke is a major priority in developed and developing countries at present. It is clear beyond doubt that principle risk factors for these disorders are associated with diet. Thus, apart from controlling the intake of macronutrients such as fat, the use of dietary supplements to maintain or induce fibrinolytic activity in vivo could provide a very attractive means to reduce the risk of atherothrombotic conditions. Sumi et al. [6, 7] demonstrated that extracts of fermented soybeans (natto) that form part of the traditional Japanese diet promoted fibrinolytic activity in the circulation in a similar manner to oral urokinase. Others have shown that these extracts moderate a number of different physiological mechanisms [8–11]. Originally, natural natto was produced by fermentation using a mixture of bacteria, and was known to contain various substances including nattokinase, which has been shown to have potent fibrinolytic activity, greater than that of plasmin [11, 12]. More recently, however, specific bacteria may be used and various other nattokinases may be produced depending on the particular *Bacillus* species employed in the fermentation process. *Bacillus natto* productive protein (BNPP) is one of these enzymes. Hence, the active components of different natto extracts may not be the same in each product and the modes of action may not be uniform. Nevertheless, it has been demonstrated that the active component(s) can be absorbed through the intestinal tract and express their action in the circulation. Nattokinase may induce prourokinase release from liver and thus activate plasminogen in the circulation as suggested for urokinase [5]. In addition, Urano et al. [13] demonstrated that their specific nattokinases, subtilisin NAT, inactivated plasminogen activator inhibitor (PAI-1) resulting in enhanced fibrinolysis in vitro. However, the absorption of nattokinase may not be sufficient to overcome the abundant fibrinolytic inhibitors present in the circulation and the role of this mechanism in vivo is unclear. The aim of the present study was to clarify the mode of action of dietary BNPP with special reference to the endogenous fibrinolytic mechanism, using an in vivo, laser-induced thrombosis model in the rat.

Materials and Methods

Bacillus Natto Productive Protein

BNPP was kindly supplied by Daiwa Pharmacology, Japan. It was purified from *Bacillus natto*-cultured filtrate and had a molecular weight of 20,000–30,000 (257-amino acid residues). The volume of BNPP administered was decided according to the amount of

Table 1. Composition of the diets (total calories: 360 kcal/100 g)

Component	Control group, g	BNPP group, g
Moisture content	7.7	7.7
Crude protein	23.6	23.6
Crude fat	5.3	5.3
Crude ash content	6.1	6.1
Crude fiber	2.9	2.9
Soluble nonnitrogenous matter	54.4	54.4
BNPP	0	0.2 or 1.0
Total	100	100.2 or 101

intake as natto (traditional Japanese food). 1.0 g of BNPP roughly equates the usual intake of natto.

Preparation of Feed

BNPP was added to the standard feed (MF, Oriental Yeast) for rat. The composition of the feed is shown in table 1.

Animals

Male Wistar/ST rats weighing 250–330 g were obtained from Japan SLC, Hamamatsu, Japan. They were maintained in compliance with the ‘Guiding Principles for the Care and Use of Animal in the Field of Physiological Sciences’, published by the Physiological Society of Japan. Feed and water intake was recorded, and a change in weight was used to confirm adequate compliance. In order to circumvent the possibility that short-term dietary changes might mediate enhanced fibrinolysis through stress-related mechanisms and be misleading, thrombolysis experiments were performed after 14 weeks of continuously feeding BNPP. Rats maintained over the same period of time in a similar manner but only on standard feed were used as controls.

Recombinant Tissue Plasminogen Activator

Recombinant t-PA (rt-PA; E-6056, Monteplase) was kindly donated by EIZAI, Japan. The rt-PA was dissolved in saline (2,750,000 IU/vial) and stored at –80 °C until use.

Argatroban

{(2R, 4R)-4-methyl-1-[N2-[(3-methyl-1,2,3 and 4-tetrahydro-8-quinoline sulfonyl)-L-arginyl]-2-piperidine carboxylic acid monohydrate]} was purchased from Daiichi Pharmaceutical Co.

Experimental Thrombolysis Models

Animals were anesthetized with sodium pentobarbital (60 mg/kg i.m.) and femoral blood vessels exposed by a median incision. Canulae (PE 50, Becton Dickinson) were introduced into a femoral vein and artery for the administration of the study agents and the monitoring blood pressure, respectively. A loop of intestine was extracted through the midline abdominal incision, spread over a Perspex plate and an O-ring placed around the tissue to stop vessel movement during peristalsis. The Perspex plate was attached to the stage of a microscope (Olympus BX51) and the preparation observed using a long

Fig. 1. Evaluation of thrombus size using Image Pro Plus software. Upper images represent time 0, lower images were made after 60 min. Thrombus size = IOD_n / IOD_o . IOD_n = IOD at various time intervals during thrombolysis; IOD_o = IOD immediately after the stabilizing thrombus.

working distance objective. Venules (30–35 μ m in diameter) were selected for irradiation. An He-Ne laser beam was introduced into the microscope using a dichroic mirror and focused on the center of the selected vessels. The diameter of the laser spot on the focal plane was 15 μ m with a power of 25 mW. Evans blue (1.6 mg/kg, E. Merck, Germany) was injected through the femoral vein cannula. Irradiation for 5 s was repeated every 30 s until a mural thrombus occluding 90% of the vessel lumen was formed. After stabilization for 10 min, saline or argatroban (0.5 mg/kg, B.I. + 2.0 mg/kg/h) or rt-PA (0.4, 0.2, 0.1 mg/kg) was administered through the cannula as a single bolus dose. Saline was administered in the same manner to control animals.

Analysis of Thrombus Size

Changes in thrombus volume were analyzed using the image analysis software, Image-Pro Plus (Media Cybernetics, USA). In brief, two-dimensional images of thrombi were captured in situ on computer at 5-min intervals. Subsequently, three-dimensional images were constructed by establishing optical density values relative to that of an area of the blood vessel lumen not involved in thrombus formation (fig. 1). Integrative optical density (IOD) values were computed corresponding to the thrombus size. Changes in thrombus size were calculated according to the following formula: thrombus size = $IOD_n \div IOD_o$ (IOD_n = IOD at various time intervals during thrombolysis; IOD_o = IOD immediately after the stabilizing thrombus). The extent of thrombolysis was expressed as a percentage of the initial thrombus volume.

Determination of Plasma Coagulation and Fibrinolysis Factors

Blood was taken using a 21-gauge needle, with 3.14% sodium citrate, from the abdominal aorta of rats after 14 weeks of oral intake of 1% BNPP. All samples (citrated plasma) were stored at -80°C until assay.

The plasma fibrinogen level was determined with the thrombin time method (Sysmex). The fibrin degradation product (FDP) was determined with the latex agglutination method (Teikoku Hormone Manufacturing) with antifibrinogen antibody. The plasminogen activity was measured with the chromogenic method (S-2251: H-D-Val-Leu-Lys-pNA, Chromogenix). Rat plasminogen was activated with urokinase (Mitsubishi Pharma).

Statistical Analysis

Data were analyzed by repeated-measure ANOVA with reference to each saline control and to the thrombus size prior to drug administration. The differences between means were considered significant at $p < 0.05$ with the Duncan post hoc test with unequal sample size. Results are expressed as mean $\pm$ SEM.

Results

Intake of Feed and Weight Gain

The feed intake and changes in weight during the experimental period are shown in figure 2. No statistically significant differences were recognized between the control group and the BNPP-fed groups.

Thrombolysis by t-PA

The process of the thrombolysis induced by t-PA in this model is illustrated in figure 3. The thrombi induced by laser irradiation demonstrated low levels of spontaneous thrombolysis and maintained 80% of their original volume for 60 min after irradiation. The relative throm-

Fig. 2. Changes in body weight during feeding the experimental diets. **a** Feed intake. **b** Body weight. ○ = Control group; △ = 0.2% BNPP group; ▲ = 1% BNPP group (n = 8 animals in each group).

Fig. 3. Time course of thrombolysis induced by bolus injections of t-PA. ○ = Placebo; ● = t-PA 0.1 mg i.v./kg; △ = t-PA 0.2 mg i.v./kg; ▲ = t-PA 0.4 mg i.v./kg (n = 8 animals in each group; * p < 0.05).

Fig. 4. Effect of dietary BNPP on spontaneous thrombolysis. ○ = Control; ● = 0.2% BNPP; △ = 1% BNPP (n = 8 animals in each group; * p < 0.05).

bus size decreased significantly after bolus infusions of t-PA at doses of 0.4, 0.2 and 0.1 mg/kg, and the rate of thrombolysis was dependent on the dose of t-PA.

Effect of Dietary BNPP on Thrombolysis

The effects of dietary BNPP on spontaneous thrombolysis are shown in figure 4. The relative thrombus volume at 60 min decreased to $67.4 \pm 10.2\%$ of the original volume in the group given 0.2% BNPP and decreased to $51.4 \pm 7.7\%$ in those fed 1% BNPP ($p < 0.05$). Both 0.2% BNPP and 1% BNPP enhanced thrombolysis compared with the control group. This enhancement effect was not recognized at less than 0.2% BNPP. The enhancement of

thrombolysis mediated by dietary 1% BNPP was equivalent to that of 0.2 mg/kg rt-PA administered by bolus injection.

Effect of Argatroban on BNPP-Enhanced Thrombolysis

The effects of the concomitant administration of argatroban are shown in figure 5. The relative thrombus volume at 60 min decreased to $58 \pm 7.3\%$ of the original volume in the group infused only argatroban. Argatroban alone significantly enhanced spontaneous thrombolysis. But in combination with dietary 1% BNPP, the relative thrombus volume at 60 min was $38 \pm 5.6\%$ of the original

Fig. 5. Comparison between 1% BNPP and argatroban in spontaneous thrombolysis. ○ = MF; ● = MF + argatroban (5 mg i.v./kg/h); △ = MF + 1% BNPP; ▲ = MF + 1% BNPP + argatroban (5 mg i.v./kg/h) (n = 8 animals in each group; * p < 0.01).

volume in the 1% BNPP-argatroban-treated group in comparison with $51.4 \pm 7.7\%$ in the 1% BNPP group not given argatroban. Argatroban did not show an additive effect on thrombolysis induced by 1% BNPP ($p = 0.27$; fig. 5).

Comparison with Coagulation and Fibrinolysis Factors

Plasminogen, fibrinogen, and FDP were assayed after 14 weeks' oral intake of 1% BNPP. The plasma fibrinogen level was 206.9 ± 3.4 mg/dl in the 1% BNPP group and 375.6 ± 3.4 mg/dl in the control group (mean $\pm$ SE, n = 8, $p < 0.0001$). The plasma fibrinogen level in the 1% BNPP group decreased in comparison with the control group. FDP was less than 10 μ g/ml (negative) in both groups. Plasminogen activity was $133 \pm 10.5\%$ in the 1% BNPP group and $117 \pm 8.5\%$ in the control group (mean $\pm$ SE, n = 8). Plasminogen activity and FDP were not different between the BNPP group and the control group.

Discussion

The effects of the dietary administration of BNPP for 14 weeks were investigated in vivo in an experimental model of spontaneous thrombolysis in rat mesenteric microvessels. We adapted a modern, computerized image analysis system combined with a previously validated

laser-induced thrombosis model [14, 15] to continuously monitor and quantify the degree of thrombolysis. rt-PA induced thrombolysis in a dose-dependent manner in this model.

In this study, we confirmed that the dietary administration of BNPP enhanced spontaneous thrombolysis and reduction of plasma fibrinogen level without decreasing plasminogen activity. Furthermore, we demonstrated that the effects of 1% dietary BNPP were equivalent to 0.2–0.4 mg/kg intravenous t-PA. These results were partially in keeping with those of Fujita et al. [16]. These workers indicated that nattokinase was more effective than plasmin or elastase in restoring blood flow in a chemically induced thrombosis model in the rat. However, in our previous study, intravenous administration of BNPP does not show significant enhancement of thrombolysis compared with t-PA (data not shown). This might be because BNPP has poor affinity to fibrin compared with t-PA.

In vivo thrombolysis, the affinity to fibrin is one of the most important characteristics of a thrombolytic inducer. The biochemical characteristics of the fibrinolytic enzymes derived from natto have been investigated in several studies. Fujita et al. [12] first described the amino acid sequence of an extract from natural natto and demonstrated similarities between this purified nattokinase and subtilisin-like serine proteases. Others have utilized products derived from the supernatants of bacterial cultures and have similarly identified subtilisin activity [13, 17–19]. The subtilisins constitute, however, a superfamily of carbonyl hydrolases and are produced by various *Bacillus subtilis* strains and other *Bacillus* species. They exhibit high specific activity on proteinaceous substrates, function optimally at moderate temperatures, and are stable under alkaline conditions. It is likely, therefore, that naturally fermented soybeans produce several types of nattokinase and the functional properties of the different variants have not been clarified. Nevertheless, it is evident that in general, the nattokinase cleave cross-linked fibrin directly [17] and in addition might proteolyse and inactivate PAI-1 [13]. PAI-1 and thrombin-activatable fibrinolysis inhibitor (TAFI) are factors of paramount importance in the regulation of thrombolysis. In particular, PAI-1 is the major inhibitor of endogenous thrombolysis, thereby promoting thrombosis [20]. Furthermore, spontaneous thrombolysis appears to be largely governed by thrombin-related mechanisms involving TAFI [21]. We have previously demonstrated that the specific thrombin inhibitor, argatroban, and the synthetic factor Xa inhibitor, DX-9065a, significantly promoted spontaneous thrombolysis [14, 15, 22].

In the current studies we confirmed that argatroban alone enhanced spontaneous thrombolysis. However, we also demonstrated that the thrombin inhibitor did not have an additive effect on thrombolysis induced by BNPP. The results were consistent with the concept that the thrombolytic potential of BNPP was independent of thrombin activity, and that the endogenous thrombolysis mechanisms might be regulated by a thrombin-related and a non-thrombin-related mechanism. Therefore, it could be speculated that dietary intake of BNPP enhanced spontaneous thrombolysis through a non-thrombin-related mechanism. So, BNPP would mainly affect PAI-1, but not TAFI.

From these observations, it was suggested that dietary administration of BNPP leads to an enhancement of spontaneous thrombolysis by indirect activation of t-PA and a reduction of fibrinogen as a structural factor of thrombus. From the amount of ingestion and the body weight, the volume of the dietary intake of BNPP in this experiment is equal to more than 10 times the usual dietary intake.

It is known that nattokinase is absorbed from the intestinal tract [23]. Therefore, further studies are necessary to determine if thrombolytic activity is similarly affected in vivo at sites distant from the mesenteric vasculature. Nevertheless, our data support the view that BNPP could provide the basis for simple and effective methods for the prophylactic enhancement of thrombolytic activity.

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