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Genistein, an isoflavone included in soy, inhibits thrombotic vessel occlusion in the mouse femoral artery and in vitro platelet aggregation

Kazunao Kondo*, Yasuhiro Suzuki, Yasuhiko Ikeda, Kazuo Umemura

Department of Pharmacology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu 431-3192, Shizuoka, Japan

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Abstract

Diet can be the most important factor that influences risks for cardiovascular diseases. Genistein included in soy is one candidate that may benefit the cardiovascular system. Here, we investigated the inhibitory effects of genistein on thrombotic vessel occlusion in the mouse femoral artery using a photochemical reaction, and in vitro platelet aggregation in whole blood measured by single platelet counting. Genistein (10 mg/kg), intravenously administered 10 min before the rose bengal injection, significantly prolonged the thrombotic occlusion time from 6.1 ± 0.4 to 8.4 ± 0.8 min ($P<0.05$). Genistein at doses higher than 30 μM significantly ($P<0.01$) inhibited in vitro platelet aggregation induced by collagen (1 and 3 μg/ml). When 10 mg/kg genistein was intravenously administered, ex vivo platelet aggregation induced by collagen (1 and 3 μg/ml) was significantly suppressed ($P<0.01$). In conclusion, genistein prevented in vivo thrombogenesis and suppressed in vitro platelet aggregation. These results suggest that dietary supplementation of soy may prevent the progression of thrombosis and atherosclerosis.

Keywords: Genistein; Thrombosis; Soy; Isoflavone; Platelet aggregation

1. Introduction

Diet can be the most important factor that influences risks for cardiovascular diseases (Keys et al., 1986). Saturated fatty acid is one example that correlates with death rate, and on the contrary, unsaturated fatty acid or soy food attenuates the risk (Potter, 1998). The large consumption of soy food in Asian countries is noteworthy as the basis for the lower incidence of cardiovascular disease in this area (Kromhout et al., 1989; Golbitz, 1995). The prevention of cardiovascular diseases using soy or soy products is, if possible, a promising approach because it is easier to take daily, as meals, than medication.

Among the active components of soy, isoflavones seem to play some important role in preventing cardiovascular diseases. Genistein is one of the isoflavones included in soy, and is known as a tyrosine kinase inhibitor (Akiyama and Ogawara, 1991). It has been reported that genistein is the most abundant phytoestrogen in soy (Setchell et al., 1987), and its plasma concentration is apparently higher in Japanese who eat a lot of soy products than in Finnish who eat a western diet (Adlercreutz et al., 1993). Thus, the inhibitory effects of genistein on the progression of thrombosis or atherosclerosis need to be clarified when considering the dietary benefit of soy food for preventing cardiovascular diseases.

Therefore, in the present study, we have investigated the inhibitory effects of genistein on thrombotic vessel occlusion in the mouse femoral artery, and in vitro platelet aggregation in whole blood by the single platelet counting method. In the present study, the anti-thrombotic effect of genistein was investigated using a photothrombotic model, which has been widely used in our institute. This model employs a nonmechanical approach to achieve vessel wall injury. This model demonstrates the intravascular events following endothelial injury by singlet oxygen molecules produced by a photochemical reaction between a rose bengal injection and green light irradiation, such as platelet adhesion to the damaged vessel wall, thrombus growth by platelet aggregation, thrombotic vessel occlusion and blood flow cessation (Hirata et al., 1993; Saniabadi et al., 1995; Suzuki et al., 2001).

* Corresponding author. Tel.: +81-53-435-2271; fax: +81-53-435-2270.

E-mail address: k17kondo@hama-med.ac.jp (K. Kondo).

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2. Materials and methods

2.1. Thrombotic vessel occlusion model

The experimental protocol was reviewed and approved by the Animal Experiments Committee of Hamamatsu University School of Medicine. Thrombotic vessel occlusion was produced in the femoral arteries of mice using the photochemical thrombosis model as described previously (Hirata et al., 1993; Saniabadi et al., 1995; Suzuki et al., 2001). Briefly, 33 male Institute of Cancer Research mice (ICR: 5 weeks old, 27–36 g) were anesthetized with sodium pentobarbital (80 mg/kg, i.p.). A cannula was inserted into the jugular vein of each animal for injection of rose bengal, and then the right femoral artery was exposed and a pulsed Doppler flow probe (PDV-20; Crystal Biotech, Hopkinson, MA, USA) was placed on it for monitoring the blood flow. Transillumination with green light (wavelength: 540 nm) was achieved using a xenon light with both a heat-absorbing filter and a green filter (model 4887; Hamamatsu Photonics, Hamamatsu, Japan). Irradiation was directed via an optical fiber positioned 3 mm away from a segment of the intact femoral artery proximal to the flow probe. Irradiation at an intensity of 0.9 W/cm^2 was started when the baseline blood flow was stable and then rose bengal (15 mg/kg) was injected for 5 min. Irradiation was continued until the blood flow stopped. The femoral artery was considered to occlude when the blood flow stopped for more than 1 min. The occlusion time was taken from the start of rose bengal infusion to the cessation of blood flow.

Genistein at doses of 3 and 10 mg/kg dissolved in dimethylsulfoxide (DMSO) was intravenously administered at a volume of 0.02 ml/10 g body weight at 10 min before the start of the rose bengal infusion in six different mice. As a control group for genistein, DMSO was administered in the same manner to six mice. In five different animals, acetylsalicylic acid (aspirin) at doses of 3 and 10 mg/kg, dissolved in 0.1 M sodium bicarbonate ($NaHCO_3$), was administered in the same manner. As a control group for acetylsalicylic acid, 0.1 M $NaHCO_3$ was administered in the same manner to five mice.

2.2. In vitro platelet aggregation in whole blood

Platelet aggregation in whole blood was measured using the single platelet counting method as previously described (Saniabadi et al., 1998), with a slight modification. Briefly, 28 male Institute of Cancer Research mice (5 weeks old) were anesthetized with sodium pentobarbital (80 mg/kg, i.p.). Blood was collected from the inferior vena cava and drawn into a prewarmed (37 °C) syringe containing one-tenth the volume of 3.8% sodium citrate. The blood obtained from one animal was diluted with the same volume of prewarmed saline, and divided into a series of five plastic tubes of 280-μl aliquots. In a series of tubes, 10-μl aliquots of each concentration of genistein or vehicles (0.1% DMSO as the final concentration) were added and incubated for 5–10 min at 37 °C. In a different series of tubes, acetylsalicylic acid or vehicle (0.1 M $NaHCO_3$) was added in the same manner. Platelet aggregation was induced by adding 10-μl aliquots of collagen (1 or 3 μg/ml as the final concentration) into the tubes, followed by 5-min constant agitation at 140 shakes/min using an automatic shaker (Taiyo Incubator Personal, Taitec, Tokyo, Japan). Then, 300 μl of 1% formaldehyde was added to stop further aggregation and disaggregation. The number of single platelets in each test tube was determined using a whole-blood cell counter (Celltac, Nihon Kohden, Tokyo, Japan). Eight and six different mice were used to evaluate the effect of genistein and acetylsalicylic acid, respectively, for each platelet aggregation induced by 1 and 3 μg/ml of collagen.

2.3. Ex vivo platelet aggregation in whole blood

Genistein, 10 mg/kg, or vehicle (DMSO) was intravenously administered to male Institute of Cancer Research mice (5 weeks old) via a cannula inserted into the jugular vein. Under anesthesia with sodium pentobarbital (80 mg/kg, i.p.), blood was collected from the inferior vena cava. Platelet aggregation in the whole blood induced by 1 or 3 μg/ml of collagen was measured using the single platelet counting method. Blood was collected from five different mice treated with genistein at 5 and 60 min after administration. In the control group, blood was collected from five mice that received the vehicle 5 min after administration.

2.4. Reagents

Genistein and acetylsalicylic acid were purchased from Sigma (St. Louis, MO, USA). Rose bengal was purchased from Wako, Osaka, Japan. Collagen was obtained from Nycomed Pharma, Munich, Germany.

2.5. Statistical analysis

Results were expressed as means ± S.E.M. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonnferroni's multiple comparison test. P values of <0.05 were considered to be statistically significant.

3. Results

3.1. Inhibitory effect of genistein on thrombotic vessel occlusion

In the control group for genistein, the femoral artery was occluded by 6.1 ± 0.4 min (Fig. 1A). Treatment with genistein at doses of 3 and 10 mg/kg prolonged the occlusion time to 7.4 ± 0.5 and 8.4 ± 0.8 min, respectively. The inhibitory effect of genistein at the 10 mg/kg dose reached

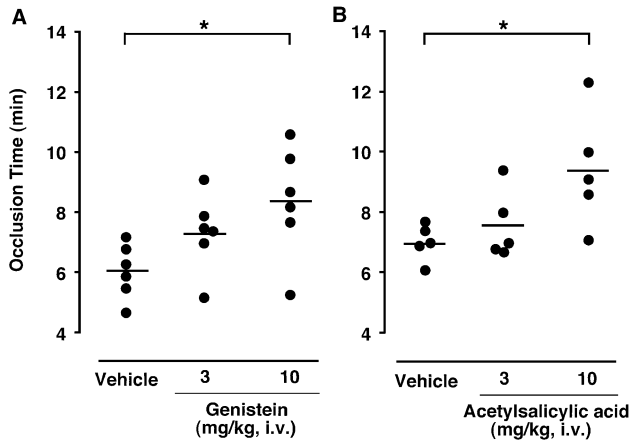

Fig. 1. Effects of (A) genistein and (B) acetylsalicylic acid on the thrombotic vessel occlusion induced by the photochemical reaction. Drugs were intravenously administered (A) 5 or (B) 10 min before rose bengal administration (15 mg/kg, i.v.). Dots indicate individual data and bars represent the average values of each group. *$P<0.05$ versus the vehicle control by Bonnferroni's multiple comparison test.

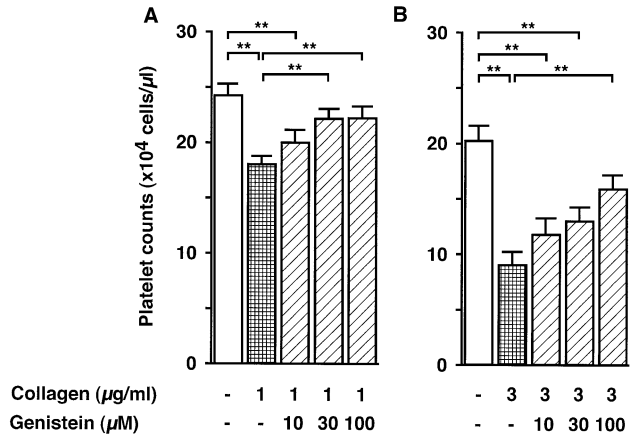

Fig. 2. Effect of genistein on platelet aggregation in whole blood, measured by the platelet counting method. Each drug was added to the blood sample 5 to 10 min before collagen stimulation at (A) 1 or (B) 3 μg/ml. Results are presented as means ± S.E.M. ($n=8$). **$P<0.01$ versus the collagen-stimulated control (meshed bars) by Bonnferroni's multiple comparison test.

statistical significance compared with the control group ($P=0.012$).

Acetylsalicylic acid had a similar effect to genistein on the inhibition of thrombotic vessel occlusion (Fig. 1B). The occlusion times were 7.0 ± 0.3, 7.6 ± 0.5 and 9.4 ± 0.9 min for vehicle, and acetylsalicylic acid at doses of 3 and 10 mg/kg, respectively. The occlusion time in mice treated with 10 mg/kg aspirin was significantly longer than that of the control group ($P=0.015$). There was no significant difference in the occlusion time between animals treated with DMSO or $NaHCO_3$ as vehicles.

3.2. Inhibitory effect of genistein on in vitro platelet aggregation in whole blood

The platelet counts prior to stimulation by collagen were the same for the genistein- and acetylsalicylic acid-treated groups. The collagen treatment reduced the platelet counts, which indicated the induction of platelet aggregation. The platelet aggregation induced by 1 μg/ml collagen in the vehicle (DMSO) was significantly weaker compared with that induced by 1 μg/ml collagen in $NaHCO_3$ ($P<0.05$: meshed columns in Fig. 2A vs. Fig. 3A), whereas there was no difference between the platelet aggregation induced by 3 μg/ml collagen in either DMSO or $NaHCO_3$ (meshed columns in Fig. 2B vs. Fig. 3B).

Genistein inhibited the collagen-induced platelet aggregation in a concentration-dependent manner (Fig. 2). The inhibitory effects of genistein at doses of 30 to 100 μM or 100 μM were significant ($P<0.01$) for platelet aggregation induced by 1 or 3 μg/ml collagen, respectively. The inhibitory effect of acetylsalicylic acid on platelet aggregation is shown in Fig. 3. Acetylsalicylic acid at 100 to 1000 μM or 300 to 1000 μM significantly ($P<0.01$) inhibited 1 or 3 μg/ml collagen-induced platelet aggregation. The inhibitory effects of acetylsalicylic acid and genistein on collagen-induced platelet aggregation were similar.

3.3. Inhibitory effect of genistein on ex vivo platelet aggregation

The inhibitory effect of genistein on platelet aggregation was investigated when genistein, at the dose which prolonged thrombotic occlusion time, was intravenously administered. Stimulation by collagen (1 and 3 μg/ml) reduced the platelet counts in DMSO-treated animals, and genistein administration restored the reduction of platelet counts to the original levels (Table 1). The inhibitory effect of genistein on 1 μg/ml collagen-induced platelet aggregation was maintained up to 60 min after genistein administration ($P<0.01$). The inhibitory effect of genistein on 3 μg/ml collagen-induced aggre-

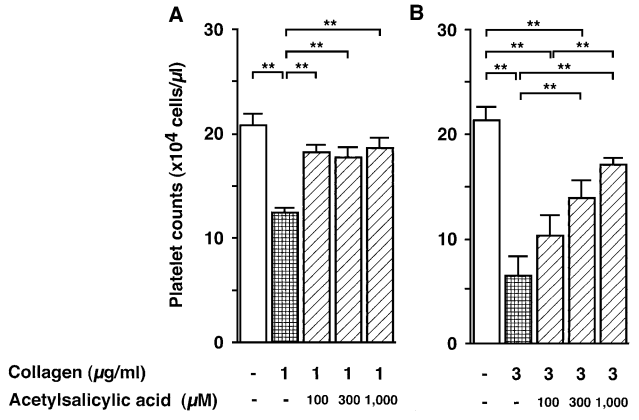

Fig. 3. Effect of acetylsalicylic acid on platelet aggregation in whole blood, measured by the platelet counting method. Each drug was added to the blood sample 5 to 10 min before collagen stimulation at (A) 1 or (B) 3 μg/ml. Results are presented as means ± S.E.M. ($n=6$). **$P<0.01$ versus the collagen-stimulated control (meshed bars) by Bonnferroni's multiple comparison test.

Table 1
Effect of intravenously administered genistein on ex vivo platelet aggregation in whole blood

Genistein	Time after Administration (min)	Collagen concentration (μg/ml)	
		1	3
−		8.3 ± 2.0	6.1 ± 1.9
+	5	16.6 ± 0.6[a]	16.6 ± 0.5[b]
+	60	18.6 ± 1.6[a]	11.7 ± 1.1[c]

Data represent means ± S.E.M. of platelet counts (× 10^4 cells/μl, $n=5$). Animals had vehicle (DMSO: −) or genistein 10 mg/kg (+) intravenously administered. In control animals, the vehicle was administered and blood was collected after 5 min. The platelet count in the whole blood sample without collagen stimulation was 17.0 ± 0.8 ($n=15$), and there was no difference between different sampling times or drugs.

[a] $P<0.01$ versus the vehicle-treated blood stimulated with 1 μg/ml collagen, by Bonnferroni's multiple comparison test.

[b] $P<0.05$ versus the vehicle-treated blood stimulated with 3 μg/ml collagen, by Bonnferroni's multiple comparison test.

[c] $P<0.01$ versus the vehicle-treated blood stimulated with 3 μg/ml collagen, by Bonnferroni's multiple comparison test.

gation was partially reversed at 60 min after administration, but still significant ($P<0.05$).

4. Discussion

In the present study, genistein prolonged thrombotic vessel occlusion time and suppressed in vitro platelet aggregation induced by collagen. The inhibitory effects of genistein on thrombogenesis and platelet aggregation were similar to those of acetylsalicylic acid, which has been reported to be effective in the prevention of cardiovascular diseases and strokes (Antiplatelet Trialists' Collaboration, 1994). The dose of genistein used in the present study was chosen according to previous studies which demonstrated the effects of genistein in experimental animals (Hughes et al., 1991; Honore et al., 1997). In those studies, intravenous administration of genistein (140 mg per body) significantly dilated the coronary artery in rhesus monkeys, and intravenous administration of genistein at a dose of 8 mg/kg blocked luteinizing hormone secretion in ovariectomized rats. Based on our observations, the genistein included in soy and soy products is expected to be effective for the prevention of cardiovascular diseases and strokes. Furthermore, we have previously demonstrated that natto-kinase, one of the active compounds in soy products, suppressed vessel intimal thickening following endothelial injury of the rat femoral artery (Suzuki et al., 2002). It has been reported that genistein has an anti-proliferative effect by suppressing phosphorylation of growth factor receptors including those for epidermal growth factor, insulin or platelet-derived growth factor (Linassier et al., 1990; Pan et al., 2001). These findings suggest that soy and soy products would inhibit the progression of thrombogenesis and atherosclerosis.

Genistein is a tyrosine kinase inhibitor and it has been reported that tyrosine kinase activation plays a key role in the platelet aggregation induced by collagen (Fujii et al., 1994; Briddon and Watson, 1999). Thus, in the present study, genistein may suppress the platelet aggregation induced by collagen by inhibition of the tyrosine kinase activation. In our photothrombotic model, thrombotic vessel occlusion consists of platelet- and fibrin-rich thrombi (Saniabadi et al., 1995). These findings suggest that genistein may prolong the thrombotic vessel occlusion time by suppression of platelet aggregation. We have also demonstrated that anti-platelet agents prolonged thrombotic vessel occlusion time (Hirata et al., 1993). Platelet aggregation therefore plays a key role in thrombogenesis in our photothrombotic model. Endothelial functions and vessel constriction also contribute to the progression of thrombi. It has been reported that flavonoids prevent vessel constriction induced by agonists (Duarte et al., 2001; Kubota et al., 2001; Trigueiro et al., 2000) and improve endothelial function (Koga and Meydani, 2001; Stein et al., 1999). Furthermore, a flavonoid has been reported to inhibit platelet deposition on damaged vessel walls by suppression of platelet–endothelial adhesion (Mruk et al., 2000). These data suggest that flavonoids, including genistein, have multiple actions for the prevention of thrombogenesis.

In conclusion, the genistein included in soy prevented in vivo thrombogenesis and in vitro platelet aggregation. Based on our observations, soy and soy products taken as foods can be expected to prevent cardiovascular diseases and strokes.

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