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Short Communication

Effects of a diet containing Brazilian propolis on lipopolysaccharide-induced increases in plasma plasminogen activator inhibitor-1 levels in mice

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ABSTRACT

Background: Brazilian propolis has many biological activities including the ability to help prevent thrombotic diseases, but this particular effect has not been proven. Plasma levels of plasminogen activator inhibitor-1 (PAI-1), an inhibitor of fibrinolysis, increase under inflammatory conditions such as infection, obesity and atherosclerosis and such elevated levels predispose individuals to a risk of developing thrombotic diseases. **Aim:** This study aimed to determine the effects of a diet containing Brazilian propolis on lipopolysaccharide (LPS)-induced increases in plasma PAI-1 levels. **Materials and Methods:** Mice were fed with a diet containing 0.5% (w/w) Brazilian propolis for 8 weeks. Thereafter, the mice were subcutaneously injected with saline containing 0.015 mg/kg of LPS and sacrificed 4 h later. **Results:** Orally administered Brazilian propolis significantly suppressed the LPS-induced increase in PAI-1 antigen and its activity in mouse plasma. **Conclusion:** This study indicated that Brazilian propolis contains natural products that can decrease thrombotic tendencies in mice.

KEY WORDS: Dietary supplement, mouse, plasminogen activator inhibitor-1, thrombosis

INTRODUCTION

Prothrombotic factors play a key role in the development of thrombotic diseases [1]. For instance, plasminogen activator inhibitor-1 (PAI-1) is the main physiological inhibitor of the fibrinolytic system, where it plays an important role in disease prevention by removing thrombi from the vascular system [2]. It is also the primary physiological inhibitor of tissue-type plasminogen activator, a key protease of the fibrinolytic system [2], through forming irreversible 1:1 complexes. An increase in plasma PAI-1 levels suppresses the normal fibrinolytic system and leads to prothrombotic states. Many factors such

as blood lipids, glucose, and insulin influence habitual PAI-1 levels. Therefore, elevated PAI-1 levels are intimately related to prothrombotic states such as hypertension, obesity, insulin resistance, and diabetes as well as aging [3-6]. Increased levels of plasma PAI-1 comprise a risk factor for thrombotic diseases and serve as a marker of the onset of such diseases [2]. Maintaining physiological plasma levels and activities of PAI-1 might thus represent a promising intervention for treating and preventing thrombotic diseases [7].

Many compounds that affect coagulation and platelet function are derived from natural sources such as garlic, ginger, gingko, and mushrooms [8,9]. Furthermore, the reports indicate that some products derived from natural sources inhibit PAI-1 production *in vitro* and in experimental animals *in vivo*. For example, Liu *et al*. reported that green tea polyphenols inhibit PAI-1 expression and secretion in endothelial cells [10], and Zhou *et al*. reported that salvianolic acid B attenuates PAI-1 production in human umbilical vein endothelial cells (HUVEC) incubated with tumor necrosis factor α (TNF-α) [11]. A citrus extract containing flavones represses PAI-1 expression in human colon fibroblasts [12] and xanthoangelol isolated from *Angelica keiskei* inhibits PAI-1 increases in mice plasma induced by lipopolysaccharide (LPS) and in culture media of HUVEC induced by TNF-α [13].

Propolis is a hive product comprising resinous materials collected by honey bees from plants and it includes over 300 chemical compounds [14]. Propolis is a feature of folk medicines and health supplements worldwide, and various biological activities have been indicated [14,15]. The composition and biological activities of propolis greatly depend on the location of the honey bees and the plant source from which it is derived [16]. Brazilian propolis contains various biologically active organic compounds in abundance such as artepillin C [17]. The effect of Brazilian propolis on various pathological conditions such as tumors, inflammation, diabetes, and immunocompromised patients have mainly been investigated *in vitro* and in experimental animals [18-22], inflammatory conditions alter the coagulation and fibrinolytic system, frequently leading to a procoagulant state [23]. Proinflammatory cytokines and endotoxins play a central role in the effects on the coagulation and fibrinolysis pathways [24]. Brazilian propolis inhibits increases in PAI-1 antigen induced by TNF-α in culture media of HUVEC [25]. Anti-inflammatory properties of Brazilian propolis have been demonstrated in mouse models of inflammation and in cultured activated macrophages [20,26,27]. This study aimed to determine the effects of a diet containing Brazilian propolis on LPS-induced plasma PAI-1 increases in model mice.

MATERIALS AND METHODS

Materials

Brazilian propolis (Institute for Bee products and Health Science, Yamada Bee Company Inc., Okayama, Japan) is an ethanol extract comprising 55% (w/w) solids. Samples of propolis were collected from colonies of Scaptotrigona bees between February 2007 and December 2008 in Minas Gerais, Brazil. Insoluble matter was removed by passage through diatomaceous earth and filter paper. LPSs from *Escherichia coli* 0111:B4 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other materials were commercial products of the highest grade available.

HPLC Analysis of Extract

The ethanol extract of Brazilian was analyzed by HPLC [28] using a Cosmosil 5C18-ARII column (Nacalai Tesque, Kyoto, Japan) and a gradient CH_3CN in 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min. Compounds were detected at 260 nm.

Animal Experiments

About 7-week-old male kwl ICR mice (Tokyo Laboratory Animals Science Co. Ltd., Tokyo, Japan) were housed at 24 ± 2°C and provided with water and the MF diet (Oriental yeast Co. Ltd., Tokyo, Japan) *ad libitum*. The mice were subcutaneously injected with saline containing 0.015 mg/kg of LPS, sacrificed at indicated times, and then, blood was collected into plastic tubes containing 3.2% sodium citrate. The effect of propolis was evaluated by providing the mice with water and the MF diet (Oriental yeast Co. Ltd., Tokyo, Japan) with or without 0.5% (w/w) of propolis ethanol extract for 2, 4 and 8 weeks *ad libitum*. The mice were then subcutaneously injected with saline containing 0.015 mg/kg of LPS. Blood specimens collected from the inferior vena cava using a plastic syringe and needle under pentobarbital (40 mg/kg i.p.) anesthesia 4 h later was mixed with 0.2 volumes of 3.2% sodium citrate. Platelet-poor plasma prepared by centrifugation at 7000 rpm (3800 ×g) for 10 min at 4°C in an MX-100 micro-centrifuge (TOMY, Tokyo, Japan) was stored at −80°C. All animal experiments proceeded in accordance with the Guide for the Care and Use of Laboratory Animal at Teikyo University and were approved by the Animal Care and Use Committee at Teikyo University (Permission No. 12-013).

Measurement of Plasma PAI-1 Levels and Activity

Levels of total PAI-1 and PAI-1 activity in mouse plasma and corresponding active PAI-1 antigen levels were measured using relevant ELISA kits (Molecular Innovations Inc., Southfield, MI, USA) according to the manufacturer's instructions.

Statistics

Data are expressed as means ± standard deviation. Statistical significance was determined using Mann–Whitney U tests. $P < 0.05$ was considered to represent significance.

RESULTS

The effects of subcutaneous injection of LPS on plasma PAI-1 levels in mice were observed. The mice were subcutaneously injected with 0.015 mg/kg LPS, blood was collected at the indicated times, and then total PAI-I antigen in plasma

was determined. Figure 1 shows the time course of PAI-1 antigen levels in plasma after LPS injection. The LPS caused a significant increase in plasma PAI-1 levels that peaked 4 h after injection. The LPS-induced PAI-1 increase in plasma was statistically significant at 3 and 4 h compared with control mice (3 h control [means = 1.06, (95% confidence interval [CI], 0.43-1.69), $n = 4$] vs. 3 h LPS [means = 12.43, (95% CI, 7.87-17.00), $n = 3$], $P = 0.034$; 4 h control [means = 1.34, (95% CI, −0.26-2.94), $n = 4$] vs. 4 h LPS [means = 12.98, (95% CI, 8.12-17.84), $n = 3$], $P = 0.034$).

The inhibitory effects of dietary propolis on LPS-induced PAI-1 production were assessed in mice fed with a diet containing 0.5% (w/w) Brazilian propolis for 8 weeks. Thereafter, the mice were subcutaneously injected with saline containing 0.015 mg/kg of LPS and sacrificed 4 h later because PAI-1 levels peaked at this point [Figure 1]. Stimulation with LPS (LPS [+]) significantly increased levels of PAI-1 antigen in plasma compared with control LPS (−) mice (LPS [−] control [means = 1.49 (95% CI, 0.78-2, 21), $n = 8$] vs. LPS (+) control [means = 14.87 (95% CI, 12.36-17.38, $n = 11$], $P < 0.001$). Orally administered propolis significantly suppressed the LPS-induced increase of PAI-1 antigen in mouse plasma (LPS (+) control vs. LPS (+) propolis, [means = 7.74 (95% CI, 4.66-10.81, $n = 12$], $P = 0.002$) [Figure 2].

The plasma levels of PAI-1 activity were then measured using an ELISA that detects active PAI-1 antigen. Since the active form spontaneously converts to the latent form, understanding levels of the active form are important for evaluating plasma PAI-1 activity. Figure 3 shows that LPS also increased the level of active PAI-1 (PAI-1 activity) in plasma. Dietary propolis for 8 weeks significantly decreased the LPS-induced increase in PAI-1 activity (LPS [+] control [means = 13.07 (95% CI, 5.40-20.75), $n = 6$] vs. LPS (+) propolis, [means = 4.47 (95% CI, −0.09-6.40, $n = 6$], $P = 0.014$) [Figure 3]. The oral administration of propolis for 8 weeks did not affect plasma PAI-1 antigen levels in non-stimulated mice (LPS [−] control vs. LPS [−] propolis, [means = 1.16 (95% CI, 0.93-1.39), $n = 9$], $P = 0.643$) [Figure 2]. These results show that oral propolis administered for 8 weeks does not affect spontaneous plasma PAI-1 levels.

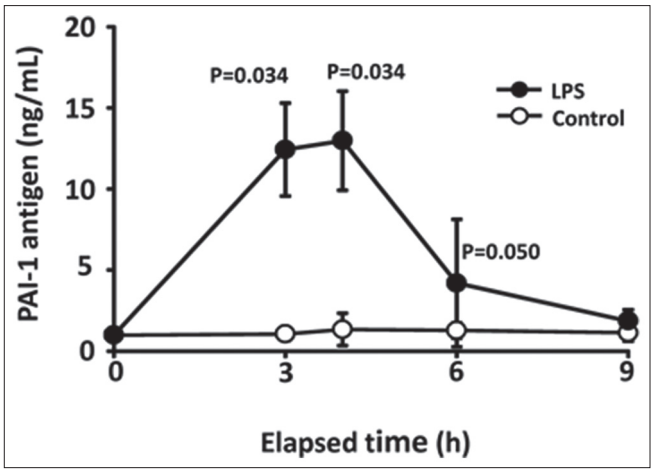

Figure 1: Time course of plasminogen activator inhibitor-1 (PAI-1) antigen levels in plasma after lipopolysaccharide (LPS) injection. Mice were subcutaneously injected with 0.015 mg/kg LPS, blood was collected at indicated times and then total PAI-1 antigen in plasma was determined using ELISA kits. Data are expressed as means ± standard deviation (LPS, $n = 3$; control $n = 4$)

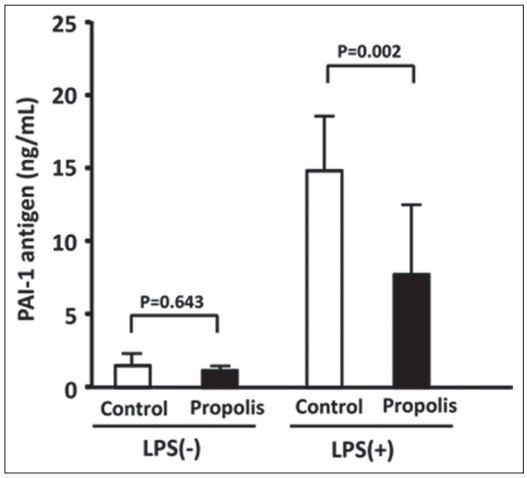

Figure 2: Effects of dietary propolis on lipopolysaccharide (LPS)-induced increase in plasminogen activator inhibitor-1 (PAI-1) antigen in mouse plasma. Mice were fed with a diet containing 0.5% (w/w) Brazilian propolis for 8 weeks. Thereafter, the mice were subcutaneously injected with saline containing 0.015 mg/kg of LPS and sacrificed 4 h later. Total PAI-1 antigen in plasma was determined using ELISA kits. Data are expressed as means ± standard deviation ($n = 8$~12)

DISCUSSION

PAI-1 inhibits the normal fibrinolytic system and the increase in plasma are involved in the onset of thrombotic diseases. PAI-1 is a powerful acute-phase reactant, reflected by rapid, large increases in plasma levels during the acute-phase response [29]. Thus, the suppression of increased PAI-1 expression during the acute phase would improve prothrombotic conditions. This study aimed to determine the effects of diets containing Brazilian propolis on LPS-induced plasma PAI-1 increases in model mice. The amount of LPS used was considerably less than that applied in the LPS-induced disseminated intravascular coagulation model [30]. These findings confirmed that the present mouse model has a slight thrombotic tendency without clot formation, despite having increased plasma PAI-1 levels.

This study showed that orally administered Brazilian propolis significantly suppressed LPS-induced increases of PAI-1 antigen and activity in mice. PAI-1 production was significantly suppressed in mice that consumed Brazilian propolis for 8 weeks. This time frame is appropriate to explore anti-thrombotic and anti-inflammatory effects in mice [13,31,32].

Brazilian propolis contains many natural components [33,34] and Table 1 lists some of the components of the Brazilian propolis used in the present study. Among many natural compounds such as cinnamic acid derivatives (drupanin, artepillin C, and baccarin), benzoic acid derivatives (caffeic acid and coumaric acid), chlorogenic acid, and flavonoids (chrysin,

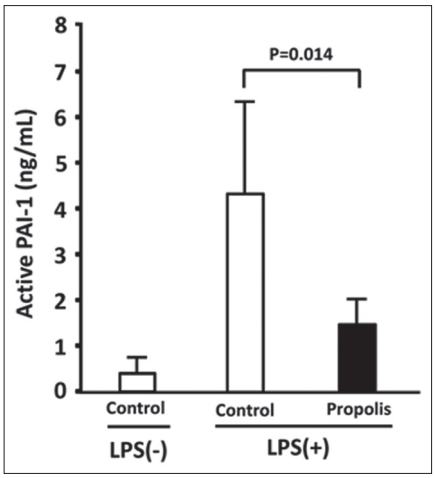

Figure 3: Effect of dietary propolis on lipopolysaccharide (LPS)-induced increase in plasminogen activator inhibitor-1 (PAI-1) activity in mouse plasma. Mice were fed with a diet containing 0.5% (w/w) Brazilian propolis for 8 weeks. Thereafter, the mice were subcutaneously injected with saline containing 0.015 mg/kg of LPS and sacrificed 4 h later. PAI-1 activity in plasma was determined using active PAI-1 ELISA kits. Data are expressed as means ± standard deviation (n = 5~6)

Table 1: Contents of cinnamic acid derivatives, benzoic acid derivatives, chlorogenic acid and flavonoids in ethanol extract of Brazilian propolis

Compound	Contents of ethanol extract of solid Brazilian propolis (mg/100 g)
Chlorogenic acid	120
Coumaric acid	1200
Caffeic acid	140
Drupanin	1400
Artepillin C	9500
Baccharin	3500
Chrysin	2.9
Naringenin	1.9
Pinocembrin	37
Kaempferide	1700
Kaempferol	110

naringenin, pinocembrin, kaempferide, and kaempferol) [Table 1]; cinnamic acid derivatives are likely to have anti-thrombotic activities. The commercially-produced cinnamic acid derivative, artepillin C, the major cinnamic acid derivative in Brazilian propolis, tended to inhibit PAI-1 production in a previous study [25]. Konishi *et al*. have described the absorption and bioavailability of artepillin C in rats after oral administration [35]. The other cinnamic acid derivatives, drupanin and baccharin, are abundant in Brazilian propolis [Table 1] and have various biological activities [18,36-39]. Thus, one or more cinnamic acid derivatives in Brazilian propolis might contribute to the inhibition of PAI-1 production. Chrysin inhibits TNF-α -induced increases in PAI-1 antigen in culture media of human HUVEC, and LPS induces increases in PAI-1 antigen in mouse plasma [25]. Chrysin is abundant in European propolis [40], but the Brazilian propolis used in the present study contains only trace amounts (2.9 mg/100 g propolis extract). Thus, the contribution of chrysin in Brazilian propolis to inhibiting PAI-1 production might be minimal. Detailed information is needed about specific molecules in Brazilian propolis that suppress increases in PAI-1.

CONCLUSION

The present findings indicated that Brazilian propolis contains some natural products that can decrease thrombotic tendencies in mice by suppressing increased plasma PAI-1 levels.

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