—Regular Articles—

Z-ligustilide Extracted from *Radix Angelica Sinensis* Decreased Platelet Aggregation Induced by ADP *Ex Vivo* and Arterio-venous Shunt Thrombosis *In Vivo* in Rats

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Antithrombotic therapy has become an important goal for the treatment of ischemic disorders such as cerebral ischemia. Our recent studies found that Z-ligustilide (LIG), a characterized 3-n-alkylphthalide constituent of *Radix Angelica sinensis* essential oil, exerted significant neuroprotection against cerebral ischemic damage in several animal models. The present study evaluated the antithrombotic activity of LIG and its effect on platelet aggregation and coagulation time. LIG (10 or 40 mg/kg) was intragastrically administered to rats once daily for 3 days. Our results showed that LIG significantly and dose-dependently reduced arterial thrombus weight in an arteriovenous shunt thrombosis in rats and platelet aggregation induced by adenosine diphosphate in rats *ex vivo*. Meanwhile, LIG at 10 or 40 mg/kg had no significant effect on coagulation time, including activated partial thromboplastin time and prothrombin time, in rats *ex vivo*. The present study demonstrated for the first time that LIG may exert efficient antithrombotic activity through inhibition of platelet aggregation, without effecting coagulation time of peripheral blood. These data, together with the previously reported neuroprotective effects of LIG on cerebral ischemia, suggest that the antithrombotic activity of LIG may contribute to its potential for the treatment of ischemic diseases, including ischemic stroke.

Key words——*Radix Angelica sinensis*; Z-ligustilide; thrombosis; platelet aggregation; coagulation time

INTRODUCTION

Numerous studies indicate that thrombosis is the main source of thromboembolic complications of ischemic disorders, and antithrombotic therapy has become an important strategy for the treatment of cerebral ischemia.[1,2] Two pharmacological strategies for antithrombotic treatment of ischemic stroke have been previously developed. One approach aims to reestablish blood flow to the ischemic site by dissolving the intraarterial clot using thrombolytic agents (*e.g.*, tissue plasminogen activator or urokinase). However, the narrow time window and the risk of cerebral hemorrhage associated with the use of thrombolytic agents greatly restrict the beneficial effects for ischemic stroke patients.[3] Another strategy is to prevent the formation and extension of intravascular clots using antithrombotic agents (*e.g.*, antiplatelet drugs or anticoagulation agents). Considering the importance of thrombosis in ischemic disorders, the search for better antithrombotic agents has continued.

Radix Angelica sinensis (RAS), also known as Danggui in Chinese, is a popular traditional Chinese medicine. In addition to being clinically used to treat female menstrual disorders *via* nourishing the blood and invigorating blood circulation, RAS has been long included in a number of traditional Sino-Japanese herbal prescriptions for the treatment of ischemic diseases.[4,5] Phytochemical studies show that the effective constituents of RAS are classified into essential oil and its water-soluble part. Z-ligustilide (LIG) (Fig. 1), a characterized 3-n-alkylphthalide derivative, has long been regarded as the main effective constituent of RAS essential oil.[6–10] We recently found that LIG at the doses of 20 or 80 mg/kg (p.o.) exerted dose-dependent and significant neuroprotection in permanent cerebral ischemic model in rats *via* a variety of pharmacological properties, including an-

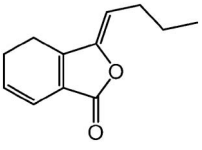

Fig. 1. Chemical Structure of Z-ligustilide (LIG)

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tioxidative and antiapoptotic effects.[11–14] In addition, sodium ferulate, the water-soluble constituent of RAS, was reported to significantly inhibit platelet aggregation at the doses of 100 or 200 mg/kg.[15] However, no report has investigated the effects of LIG on platelet function or thrombopoiesis, which is significantly related to the outcome of ischemic disorders including ischemic stroke. To further explore the potential hemodynamic basis of LIG in the treatment of ischemic diseases, the effects of LIG on thrombopoiesis and its potential modes of actions were investigated.

MATERIALS AND METHODS

Animals SPF male Sprague-Dawley rats (7–8 weeks old, 280–320 g) were obtained from the Institute of Experimental Animals, Sichuan Academy of Medical Sciences. The animals were acclimated for 5 days at 22±1°C with a 12 h light/dark cycle and allowed free access to a commercial pellet diet obtained from the institute and drinking water before experiments. All animal experiments were performed in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China (November 14, 1988).

LIG Preparation and Administration LIG was isolated from the essential oil of RAS by silica-gel column chromatography and identified by nuclear magnetic resonance as described previously.[16] The purity of LIG was examined by high-performance liquid chromatography. LIG with >97.6% purity was used, prepared daily in 3% (v/v) Tween-80 (AR, Tianjin Chemical Company, China), and orally administered at doses of 10 or 40 mg/kg once daily for 3 days. Rats in the control group received volume-matched vehicle. Aspirin (Xi'an Bodyguard Pharmaceutical Co. Ltd., China) or warfarin sodium (Shanghai Pharmaceutical Co. Ltd., China) was used as a positive control in the appropriate experiments.

***In Vivo* Antithrombotic Activity** The antithrombotic activity of LIG was determined in a rat arteriovenous shunt thrombosis model described previously with minor modifications.[17] Rats were orally given LIG, aspirin (40 mg/kg), or vehicle once daily for 3 days. One hour after administration, two 8 cm polyethylene tubes (0.6 mm inner diameter, 1.0 mm outer diameter) were placed between the right carotid artery and the left jugular vein of chloral hydrate-anesthetized rats (300 mg/kg, i.p.). The tubes were linked to a central part (6 cm long, 1.0 mm inner diameter) containing a 5 cm cotton thread (0.25 mm diameter) and filled with a heparin saline solution (25 IU/ml). Extracorporeal circulation was maintained for 15 min, during which time a thrombus formed *via* adhesion to the cotton thread. The shunt was then removed, and the thread with its associated thrombus was removed and immediately weighed. The thrombus wet weight was determined by subtracting from the value obtained from the weight of the dry 5 cm cotton thread determined previously.

***Ex Vivo* Antiplatelet Aggregation Activity** Rats received oral administration of LIG, aspirin, or vehicle once daily for 3 days. One hour after the final administration, 5 ml of blood samples was taken from the carotid artery and collected using 3.8% trisodium citrate as the anticoagulant (9/1, v/v) in chloral hydrate-anesthetized rats (300 mg/kg, i.p.). Platelet-rich plasma (PRP) was obtained by centrifuging the blood sample at 100 g for 10 min. Platelet-poor plasma (PPP) was obtained by centrifuging at 1200 g for 10 min continuously. PRP was adjusted to 3×10^8 platelets/ml with PPP. Platelet aggregation induced by the addition of adenosine diphosphate (ADP; final concentration, 5 μM) was determined according to the method of Born[18] with a TYXN-96 multifunctional intelligent aggregometer (Shanghai General Machine Electricity Technological Research Institute, Shanghai, China). The light transmission of PRP is 0%, and that of PPP is 100%. The extent of platelet aggregation was estimated quantitatively by measuring ADP-induced maximal aggregation.

***Ex Vivo* Anticoagulation Activity** Anticoagulation activity was evaluated by measuring plasma clotting times. Rats received LIG, warfarin (1 mg/kg, p.o.), or vehicle once daily for 3 days. One hour after the final administration, citrated blood was collected and centrifuged to obtain PPP as above. The plasma clotting times, activated partial thromboplastin time (APTT), and prothrombin time (PT) were measured by an optical method on a STA-R Coagulometer with PT and APTT reagent kits (Diagnostica Stago, Inc., France). PT was measured by incubation of 50 μl of PPP for 4 min, followed by the addition of 100 μl of prewarmed PT agent. APTT was measured by incubation of 50 μl of PPP with 50 μl of APTT agent for 4 min, followed by the addition of 50 μl of 25 mM

$CaCl_2$.

Statistical Analysis All data are expressed as mean±S.D. SPSS software (version 11.5) was used for all statistical calculations. The difference between the treated groups and control groups was analyzed by one-way analysis of variance (ANOVA). When appropriate, *post hoc* comparisons were made using the Least Significant Difference (LSD) test (equal variances assumed) or Dunnett's T3 test (equal variances not assumed). Values of $p<0.05$ were considered significantly significant.

RESULTS

Effects of LIG on Arteriovenous Shunt Thrombosis in Rats The antithrombotic effect of LIG is demonstrated in a rat arteriovenous shunt model (Fig. 2). Pretreatment with LIG for 3 days led to a dose-related reduction in thrombus formation. The wet weight of the thrombus in response to 10 and 40 mg/kg of LIG was 19.5±6.4 mg and 13.6±3.2 mg, respectively, and was significantly lower than control (46.4±8.5 mg; $p<0.01$).

***Ex Vivo* Antiplatelet Aggregation of LIG** We investigated the effects of LIG pretreatment on maximal platelet aggregation induced by ADP. As shown in Fig. 3, the control rats treated with vehicle had platelet aggregation of 44.6±4.6%. LIG pretreatment for 3 days dose-dependently reduced ADP-induced platelet aggregation in rats *ex vivo*. The maximal platelet aggregation was significantly lower in the 10 mg/kg LIG group (6.8±2.5%) and 40 mg/kg LIG group (2.0±1.0%) compared with controls ($p<0.01$). Aspirin at a dose of 40 mg/kg decreased the maximal platelet aggregation to 21.1±3.8% ($p<0.01$ *vs* control group).

***Ex Vivo* Effects of LIG on Coagulation Time** The effects of LIG on coagulation times were evaluated by APTT and PT assays in rats *ex vivo*. As shown in Fig. 4, oral administration of LIG (10 or 40 mg/kg) for 3 days did not affect PT or APTT compared with controls ($p>0.05$), whereas 1.0 mg/kg warfarin significantly prolonged coagulation times compared with controls and the LIG-treated groups ($p<0.01$). These data indicate that LIG at these doses did not affect the coagulation system.

DISCUSSION

Antithrombotic therapy has been proven to be effective for the treatment of ischemic stroke by preventing the recurrence of thrombus and thus preserving cerebral blood flow.[2] The present study examined the effects of LIG on arterial-type thrombus formation in a rat arteriovenous shunt model in which developing thrombi contain large platelet aggregation, originating from the thrombogenic silk thread, surrounded by erythrocytes and fibrin.[19] Oral administration of LIG at doses of 10 and 40 mg/kg once daily for 3 consecutive days significantly reduced

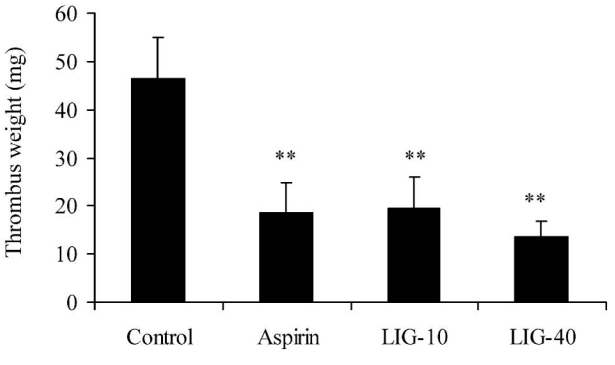

Fig. 2. Effects of Z-ligustilide on Thrombus Formation in Rat Arteriovenous Shunt Thrombosis

Rats were pretreated with aspirin (40 mg/kg, p.o.), Z-ligustilide (LIG; 10 or 40 mg/kg, p.o.), or the same volume of vehicle (control) once daily for 3 days. One hour after the final administration of the tested agents, the A-V bypass thrombus was allowed to form on the thread segment during 15 min of extracorporeal circulation and immediately measured. Data are expressed as mean±S.D. ($n=8$). $^{**}p<0.01$, compared with control.

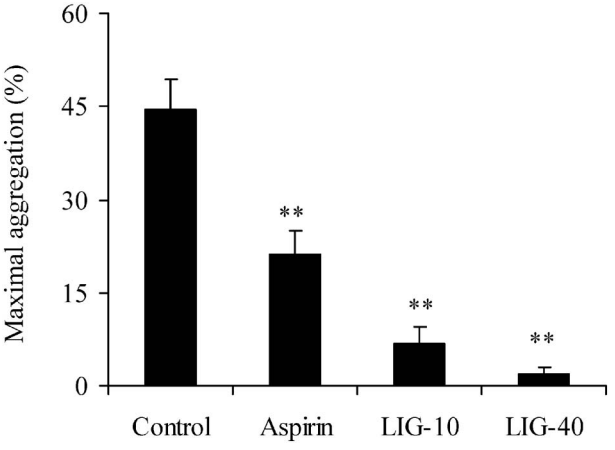

Fig. 3. Effects of Z-ligustilide on Adenosine Diphosphate (ADP)-induced Platelet Aggregation in Rats

Rats were pretreated with aspirin (40 mg/kg, p.o.), Z-ligustilide (LIG; 10 or 40 mg/kg, p.o.), or the same volume of vehicle (control) once daily for 3 days. One hour after the final administration, rat blood samples were collected using 3.8% trisodium citrate solution (9/1, v/v), and platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared. The extent of platelet aggregation induced by ADP (5 μM) was estimated quantitatively by measuring the maximal aggregation with a TYXN-96 multifunctional aggregometer. Data are expressed as mean±S.D. ($n=6$). $^{**}p<0.01$, compared with control.

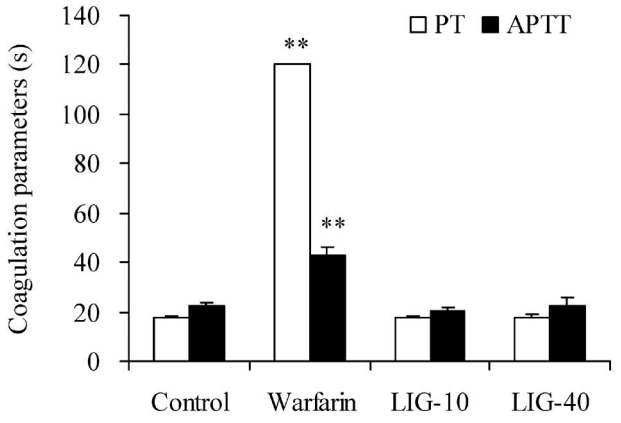

Fig. 4. Effects of Z-ligustilide on Coagulation Parameters in Rats

Rats were pretreated with warfarin (1 mg/kg, p.o.), Z-ligustilide (LIG; 10 or 40 mg/kg, p.o.), or the same volume of vehicle (control) once daily for 3 days. One hour after the final administration, rat blood samples were collected into a 3.8% trisodium citrate solution (9/1, v/v), and platelet-poor plasma (PPP) was prepared. Activated partial thromboplastin time (APTT) and prothrombin time (PT) were measured by an optical method on a STA-R Coagulometer. Data are expressed as mean±S.D. (n=6). **p <0.01, compared with control.

thrombus formation on the silk thread in rat arteriovenous shunt thrombosis. The antithrombotic activity of LIG at these doses was similar to aspirin at 40 mg/kg.

Arterial thrombi are well known to be largely composed of platelet aggregation. Platelets play a vital role in both the initiation and growth of thrombi. Thus, inhibition of platelet function represents a promising approach for the prevention of thrombotic disorders. In the present study, the effect of LIG on platelet function was assessed with regard to ADP-induced platelet aggregation. After oral administration for 3 days, LIG (10 or 40 mg/kg) significantly prevented platelet aggregation induced by ADP. The inhibitory potency of LIG at these doses was greater than that of aspirin at 40 mg/kg, suggesting that the antithrombotic activity of LIG might result from antiplatelet aggregation.

To investigate the interactions of LIG with coagulation factors, the effects of LIG on coagulation time were evaluated by APTT and PT assays using rat PPP *ex vivo*. After oral administration for 3 days, LIG (10 or 40 mg/kg) did not affect either coagulation parameter, although warfarin (1 mg/kg) significantly prolonged APTT and PT as reported previously,[20] indicating that the antithrombotic activity of LIG may not be associated with the extrinsic or intrinsic coagulation system.

Considering the traditional uses of RAS in traditional Chinese medicine, most of which have been employed to nourish the blood and to improve blood circulation for the treatment of ischemic diseases, the antithrombotic effect of LIG demonstrated in the present study might provide new evidence for the use of RAS. Further studies investigating the exact mechanisms of action of the antithrombotic activity of LIG are ongoing.

LIG may be a novel potent antithrombotic agent, and the modes of antithrombotic action are likely associated with antiplatelet aggregation rather than anticoagulation. The present data suggest that antithrombotic action might contribute to the neuroprotective effect of LIG against cerebral ischemia damage.

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