Comparative Study of the High Molecular Mass Fraction and Low Molecular Mass Fraction of Sho-saiko-to in a Murine Immunologically Induced Liver Injury Model

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We compared the pharmacological actions of the high and low molecular mass fractions of Sho-saiko-to using a murine immunologically induced liver injury model to estimate the roles of these fractions in the expression of the pharmacological action. In a Bacillus Calmette–Guérin (BCG)/lipopolysaccharide (LPS)-induced liver injury model, Sho-saiko-to and both of its fractions significantly reduced the increases in the aminotransferase levels in serum. They also reduced the increase in the nitric oxide (NOx) level in serum. On the other hand, Sho-saiko-to and its high molecular mass fraction suppressed the increase in plasma NOx level in an LPS-induced endotoxin shock model but its low molecular mass fraction did not. These results suggest the possibility that both fractions act hepatoprotectively in a different manner. We believe that these results can help to elucidate the mechanism of action of ingredients in Sho-saiko-to.

Key words Sho-saiko-to; high molecular mass fraction; low molecular mass fraction; liver injury

Many researchers have reported on the efficacies of Japanese and Chinese herbal remedies, so-called kampo-hozai. Since kampo-hozai are mixtures of plant and animal materials and contain numerous ingredients, most researchers have regarded each kampo-hozai as one drug for the investigation of the pharmacological actions using in vivo and in vitro systems. Alternatively, there have been many reports concerning the isolation and structure elucidation of major ingredients and the pharmacological and biochemical actions of crude drugs composing kampo-hozai. However, few reports described the relationship between the mixture, kampo-hozai, and their active ingredients to clarify how their ingredients worked in kampo-hozai function.

Sho-saiko-to has been extensively investigated and found to have diverse pharmacological activities. Sho-saiko-to has been used for the treatment of various infectious diseases such as chronic viral hepatitis, and it has been shown that Sho-saiko-to has immunomodulating activity, antiinflammatory effects, and antipneumococcal activity. Previously, we demonstrated that the immunomodulating actions of Sho-saiko-to, such as augmentation of macrophage function and mitogenic activity, were mainly due to its polysaccharide fractions in vivo. Yamaoka et al. also reported the augmentation of natural killer (NK) cells by Sho-saiko-to was mainly due to its acidic polysaccharide fraction and that the polysaccharide fraction of Zizyphi Fructus was involved in this activity. Furthermore, the group of Yamada has done much work in this area and isolated 22 types of immunomodulating polysaccharides, which were classified into pectins, pectic arabinogalactans, and pectic heteroglycans, from Juze-nihai-to. These results suggest that one of the pharmacological roles of the polysaccharide fraction in kampo-hozai is as an immunomodulator. However, the pharmacological roles of the low molecular mass fraction and the interaction of both high and low molecular mass fractions in specific pharmacological action of Sho-saiko-to are still unclear, even though the pharmacological properties of each ingredient composing Sho-saiko-to, such as glycyrrhizin, baicalin, ginsenosides, and saikosaponins, are well characterized.

In the present study, we attempt to compare the pharmacological activities of the high molecular mass fraction and the low molecular mass fraction of Sho-saiko-to using an immunologically induced liver injury model to estimate the role of these fractions in the expression of the pharmacological action of Sho-saiko-to.

MATERIALS AND METHODS

Animals Male ICR mice, 5–6 weeks old, were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). They were housed in standard plastic cages in an air-conditioned room (24 °C) and given a commercial diet (CE-2, Clea Co., Tokyo, Japan) and water ad libitum. The animals were kept for at least 7 d after their arrival.

Chemicals Lipopolysaccharide (LPS, Escherichia coli 0111:B4) was purchased from Difco laboratories (Detroit, MI, U.S.A.) and Bacillus Calmette–Guérin (BCG) was from Nippon BCG Co. (Tokyo, Japan). All other chemicals used were of special grade.

Preparation of Sho-saiko-to and Its High and Low Molecular Fractions Sho-saiko-to was prepared using the authentic method. Briefly, a mixture of Bupleuri Radix (7 g), Pinelliae Tuber (5 g), Scutellariae Radix (3 g), Ginseng Radix (3 g), Zingiberis Rhizoma (1 g), Zizyphi Fructus (3 g), and Glycyrrhizae Radix (3 g) were extracted with 600 ml of water at 100 °C for 1 h. The decoction was filtered and then lyophilized to obtain the dry extract powder (8.29 ± 0.04 g). The powdered Sho-saiko-to extract was dissolved in water and then the addition of 3 volumes of ethanol gave the precipitate and the ethanol-soluble supernatant. The precipitate was washed thoroughly with 75% ethanol and 100% ethanol, suspended in the appropriate volume of water, and then lyophilized to give a powder (ES fraction, 1.87 ± 0.01 g). The ethanol-soluble supernatant was concentrated and then lyophilized to produce a powder (ES fraction, 6.35 ± 0.11 g).

HPLC Analysis of Glycyrrhizin and Baicalin in Sho-
saiko-to and Its Fractions  Each 5 mg of Sho-saiko-to or its fractions was dissolved in water and filtered through a 0.45 μm membrane filter (Tosoh, Tokyo, Japan), and then made up to 5 ml. Ten microliters of each sample was subjected to HPLC analysis. A Shimadzu model LC-6A chromatograph with a stainless steel column (250 mm×4.6 mm i.d.), packed with reverse-phase Hypersil ODS (5 μm, Eruma Optical Works, Tokyo, Japan) for baicalin or TSK-gel ODS-120T (5 μm, 150 mm×4.6 mm i.d., Tosoh) for glycyrrhizin, was used. The mobile phase was methanol–water–sulfuric acid (43 : 57 : 0.03) for baicalin or methanol–water–tetraethylammonium hydrogen sulfate (54 : 46 : 0.03) for glycyrrhizin. The column temperature was 50 °C, the flow rate was 1.0 ml/min, and detection wavelength was 280 nm for baicalin or 254 nm for glycyrrhizin.

Chemical Analysis of the EP Fraction  The total carbohydrates and uronic acid contents were determined by the phenol–sulfuric acid20 and the sulfuric acid–carbazole21 methods, respectively, using glucose and galacturonic acid as the standards. Protein was assayed by the Bradford method22 with bovine serum albumin as the standard.

Effects of Sho-saiko-to and Its Fractions on BCG/LPS-Induced Liver Injury in Mice  Male ICR mice were intravenously primed with BCG 1 mg suspended in pyrogen-free saline and 7 d later injected with LPS 10 μg intravenously. Sho-saiko-to and its EP and ES fractions were orally administered during the experimental period at a dose of 0.70, 0.21, and 0.53 g/kg/d, respectively. These dosages were equivalent to five times the human dosage per day. Eight hours after injection of LPS, the mice were anesthetized with ether and blood samples were collected by exsanguination from the inferior vein. Serum was separated by centrifugation, and the NOx concentration was determined with Griess reagent as described above.

Statistical Analysis  Results are given as mean±S.E.M.; mean values are compared using the two-tailed Student's t-test.

RESULTS

Fractionation of Sho-saiko-to and Chemical Analysis of Its Fractions  Ethanol precipitation fractionated Sho-saiko-to into a high molecular mass fraction (EP fraction) and a low molecular mass fraction (ES fraction). The chemical analysis showed the EP fraction contained 78% total sugar as glucose, 14.8% uronic acid as galacturonic acid, and 2.1% protein. These data suggest that the EP fraction consisted mainly of polysaccharide. The occurrence of low molecular mass compounds such as glycyrrhizin and baicalin in the ES fraction was confirmed by HPLC. For example, the representative contents of glycyrrhizin in Sho-saiko-to and its ES fraction were 121 mg and 117 mg per human daily dose and those of baicalin were 223 and 215 mg, respectively. The EP fraction did not contain those compounds.

Effects of Sho-saiko-to and Its Fractions on BCG/LPS-induced Liver Injury in Mice  Male ICR mice were primed with BCG and 7 d thereafter nonlethal dose of LPS was injected to cause liver injury. In this model, severe liver injury was observed 8—12 h after LPS injection. A preliminary experiment had revealed that oral administration of Sho-saiko-to for 7 d significantly reduced the increase in AST and ALT levels at a dose of 0.7 g/kg/d. This dosage was equivalent to five times the human dosage. We therefore chose this dosage for this study. As shown in Fig. 1, Sho-saiko-to significantly reduced the serum levels of AST and ALT. The EP fraction and ES fraction also reduced the increases in these aminotransferases.

To determine the hepatoprotective mechanism of Sho-saiko-to and its fractions, the serum level of NOx, one of the mediators in this model, was measured. As shown in Fig. 2, the intravenous injection of LPS markedly increased the serum NOx level in BCG-primed mice. Sho-saiko-to and its fractions reduced this increase in serum NOx level and the EP fraction was particularly effective.

Effects of Sho-saiko-to and Its Fractions on the LPS-Induced Endotoxin Shock Model  It is well known that the
intranavenous injection of LPS alone causes an increase in the NOx level in serum and plasma. We studied the effects of Sho-saiko-to and its fractions on the increase in plasma NOx level induced by LPS alone to determine how they suppressed the increase compared with that in the case of BCG/LPS. When various doses of LPS were injected intravenously into untreated mice, an increase in plasma NOx level was observed at a dose of more than 0.1 mg/kg. We therefore used this dosage in this experiment.

As shown in Fig. 3, Sho-saiko-to and its EP fraction significantly suppressed the increase in plasma NOx level 4 h after the injection of LPS, but the ES fraction did not. At 8 h after the injection, the plasma NOx level reached the maximum and the tendency of the effects of Sho-saiko-to and the EP fraction was similar to that at 4 h.

**DISCUSSION**

Numerous reports have shown that Sho-saiko-to has protective effects against various forms of acute and chronic experimental liver injury, such as carbon tetrachloride-induced, N-galactosamine-induced, alcohol-induced, and immunologically induced (allergic) liver injury. As described in the Results section, we chose BCG/LPS liver injury model as an immunologically induced liver injury model and demonstrated that Sho-saiko-to had a protective effect in this model as well as in others. Both high and low molecular mass fractions of Sho-saiko-to also reduced the serum level of aminotransferases in this model. Animals treated with microorganisms, such as BCG and Corynebacterium, are sensitive to LPS, which causes lethal toxicity with fulminant liver injury. Tumor necrosis factor (TNF) is thought to be one of the mediators of LPS-induced lethal toxicity, because passive immunization against TNF partially protected mice. It has been reported that Sho-saiko-to protected the recombinant human TNF (rhTNF)-induced lethality in galactosamine-hypersensitized mice and against the decrease in rectal temperature after rhTNF in normal mice. Thus we should investigate the effects of Sho-saiko-to and its fractions on serum TNF levels in this model.

Alternatively, it is known that reactive oxygen species and NO are also important mediators in this model. We measured the serum NOx concentration and showed that Sho-saiko-to and its fractions, especially the EP fraction, reduced the serum NOx concentration. Sho-saiko-to and its EP fraction suppressed the increase in plasma NOx level in the LPS-induced endotoxin shock model but the ES fraction did not. These results suggest the possibility that both fractions have hepatoprotective action with different mechanisms, consistent with the absorption and metabolism of the high and low molecular mass fractions.

In a previous study, we demonstrated that the immunomodulating action of Sho-saiko-to, such as augmentation of macrophage function, was mainly due to its polysaccharide fractions. Oral administration of the EP fraction enhanced phagocytosis of casein-induced murine peritoneal macrophages and NO production of thioglycollate broth-induced peritoneal macrophages to the same degree as Sho-saiko-to. We assume that Sho-saiko-to and its EP fraction could up-regulate the host defense system through the augmentation of macrophage function. It has been reported that Sho-saiko-to improved the function of hepatic macrophages blocked by gum arabic when activated after partial heptectomy. Thus hepatic macrophages such as Kupffer cells may be one of the target cells affected by Sho-saiko-to and its EP fraction. Sho-saiko-to and its EP fraction may influence macrophage function such as NO production bi-directionally. That is, they may up-regulate that in the physiological state. We cannot explain the reason for such bi-directional actions like at present. Further study is needed to clarify how Sho-saiko-to and the EP fraction influence sensitivity to LPS in peritoneal and hepatic macrophages. The immunomodulating effect of the EP fraction may be involved in the hepatoprotective actions of Sho-saiko-to and the EP fraction.

With respect to the ES fraction, it contained well-known hepatoprotective compounds such as saikosaponins and gly-
cytchaloside. Unfortunately, the amounts of saikosaponins are too low to explain the hepatoprotective action. For example, a few milligrams of saikosaponin a are present in the usual daily dose of Sho-saiko-to but saikosaponin d, the most potent compound among the saikosaponins, is not contained in Sho-saiko-to. 34) Because of its acid lability, saikosaponin d is easily converted to saikosaponin b2 by coexisting organic acids during the preparation of the decoction. On the other hand, Sho-saiko-to contains about 1.5% glycyrrhizin and 0.05% glycyrrhetinic acid. During the preparation of the decoction, on the other hand, Sho-saiko-to contains about 1.5% glycyrrhizin and thus we assumed glycyrrhizin could be involved in the hepatoprotective effect of the ES fraction.

In this study, we found that both the high and low molecular mass fractions protected against BCG/LPS-induced liver damage and that their protective mechanism might be different. We speculate that the EP fraction may act as an immunomodulating agent, while the ES fraction may act as nonimmunomodulating agent, such as the radical scavenger against NO and other reactive oxygen intermediates or as a membrane-protective agent. Further comparative study, for example, using a nonimmunologically induced liver injury model such as carbon tetrachloride-induced liver injury, is needed. We believe that these results help us to understand how the ingredients work in the pharmacological actions of Sho-saiko-to.

REFERENCES

19) Yamada H., Kiketissos, 1994, 7—16.