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Inhibitory effect of polysaccharides isolated from *Angelica sinensis* on hepcidin expression

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ABSTRACT

Ethnopharmacological relevance: Angelica sinensis polysaccharide is an important bioactive component of *Angelica sinensis* (Oliv.) Diels that has been used in traditional Chinese medicine for treating gynecological disorders and anemia.
Aim of the study: Previous study indicated that *Angelica sinensis* polysaccharide (ASP) may promote plasma iron levels by suppressing the expression of hepcidin, a negative regulator of body iron metabolism, in the liver. The present study aims to clarify the inhibitory effect of ASP on hepcidin expression as well as the involved mechanisms.
Materials and Methods: ASP (1 g/kg) or vehicle (normal saline) was intragastrically administrated to rats everyday for 14 d. Intraperitoneal injections of recombinant human erythropoietin (rhEPO, 2000 U/kg) were given to positive control group. Erythropoietin and hepcidin levels in serum at different time points were determined by enzyme-linked immunosorbent assay. Western blot was used to investigate the expression of 6 pertinent signal proteins in liver.
Results: ASP significantly reduced hepcidin expression by inhibiting the expression of signal transducer and activator of transcription 3/5 (STAT3/5) and mothers against decapentaplegic protein 4 (SMAD4) in liver and stimulating the secretion of erythropoietin, which further down-regulated hepcidin by repressing CCAAT/enhancer-binding protein α (C/EBPα), SMAD4, and the phosphorylation process of STAT3/5.
Conclusions: ASP can suppress the expression of hepcidin in normal rats, and may be used in the treatments of hepcidin-induced diseases.

1. Introduction

Angelica sinensis (Oliv.) Diels (family Umbelliferae, Chinese name Danggui) is a well-known oriental herb used for the treatment of anemia, uterine bleeding, abnormal or painful menstruation, and other diseases affecting women (Raman et al., 1996). Many customary applications of the root of *Angelica sinensis* are based on empirical allegations of its effects on blood tonification and promotion of the blood circulation (Zhang and Sun, 1990). As one of the active components, polysaccharide isolated from *Angelica sinensis* was found to be effective in enhancing hematopoiesis by increasing the secretion of some hematopoietic growth factors such as erythropoietin via stimulating hematopoietic cells and muscle tissues (Sarker and Nahar, 2004). In our previous research, we found that *Angelica sinensis* polysaccharide (ASP) could promote the amount of iron in serum and hypothesized that ASP could participate in the regulation of iron homeostasis (Wang et al., 2007).

Hepcidin, a 25 amino-acid antimicrobial peptide produced by hepatocytes, is the central regulator of body iron metabolism (Kemna et al., 2008). Hepcidin binds to ferroportin, the channel for cellular iron efflux, leading to internalization and degradation of ferroportin. The binding diminishes duodenal transfer of iron into the circulation and promotes iron retention within macrophages (Du et al., 2008; Theurl et al., 2008). In this manner, hepcidin lowers plasma iron levels. Chronic elevation of hepcidin levels causes systemic iron deficiency. Therefore, hepcidin has become an attractive therapeutic target for the treatment of iron deficiency diseases. Recently, erythropoietin administration has been used to treat hepcidin-induced anemia since erythropoietin was demonstrated to be effective in suppressing hepcidin expression by enhancing body erythropoiesis (Handelman and Levin, 2008).

Accordingly, it was proposed that ASP which can stimulate erythropoietin secretion may affect iron homeostasis in vivo by regulating hepcidin expression. The present study was undertaken

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to evaluate the hepcidin-suppressing efficacy of ASP. Furthermore, to clarify the underlying mechanism in the hepcidin suppression caused by ASP, we monitored the serum concentration of erythropoietin and the liver expressions of 6 proteins belonging to 3 pathways which were previously demonstrated important for hepcidin regulation (Viatte and Vaulont, 2009). These proteins include: STAT3/phospho-STAT3 and STAT5/phospho-STAT5 in the Janus-kinase/signal transducer and activators of transcription proteins (JAK/STAT) signal transduction pathway, SMAD4 in the bone morphogenetic protein-mothers against decapentaplegic protein (BMP-SMAD) pathway, and CCAAT/enhancer-binding protein α (C/EBPα) in the erythropoietic pathway.

2. Materials and methods

2.1. Plant material and preparation of raw polysaccharide (ASP)

The dry roots of *Angelica sinensis* (Oliv.) were purchased from Union Hospital and were identified according to the identification standard of Pharmacopoeia of People's Republic of China. 200 g of powdered material was extracted twice with boiled distilled water (1000 mL) for 0.5 h. The filtered extract was combined and concentrated under reduced pressure at 50 °C. After removing acidity protein and alkaline protein by modulating pH, the aqueous extract was precipitated by ethanol (final concentration 75% (v/v)). The resulting precipitate was suspended in distilled water and lyophilized. The raw polysaccharide (ASP) was obtained (carbohydrate content: 75.4%, yield: 17.2%). Phytochemical screening showed the presence of two subtypes of ASP:ASP I and II (7.41:1) with average molecular weight of 8000 Da and 76,000 Da, respectively. Both of APS I and II consist of arabinose, galactose, and glucose.

2.2. Experimental animals and treatment

36 male Sprague–Dawley (SD) rats weighing 220–250 g were housed in temperature – (22 ± 2 °C) and humidity – (55–65%) controlled rooms with a 12/12 h off/on light cycle. All animals had free access to food and water. Rats were randomly divided into 3 groups. ASP treatment group consisted of 12 rats intragastrically administered with ASP (1 g/kg) each day for 14 days. RhEPO treatment group was made up of 12 rats given recombinant human erythropoietin (rhEPO, Sunshine Pharmaceutical Co., Shenyang, China) by intraperitoneal injection at a dose of 2000 U/kg each day for 3 days. 12 rats in negative control group were intragastrically administered with 1 mL normal saline each day for 14 days. Dosages and time were determined according to preliminary experiments. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Huazhong University of Science and Technology (Wuhan, China).

2.2.1. Blood collection (animal treatment for investigation of hepcidin-suppressing effect of ASP and rhEPO)

7 rats in each group were used to investigate the suppression of hepcidin levels by ASP and rhEPO. Animals were anesthetized (ketamine 70 mg/kg and acepromazine 3 mg/kg, intraperitoneally) and blood was taken from the retro-orbital sinus before administration and 0 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h, 72 h after the last administration.

2.2.2. Tissue collection (animal treatment for mechanism study on hepcidin suppression by ASP and rhEPO)

The other 5 rats of each group were used to investigate the expression of 6 related signal proteins in liver. Tissues were taken 8 h (rhEPO group) or 24 h (ASP and control group) after the last administration. In order to remove the great amount of blood in liver, liver perfusion (perfusion media: normal saline with 200 U/mL heparin) was performed in situ under light ether anesthesia as previously described (Seglen, 1976). After perfusion, livers were cut off and kept at −80 °C.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Blood samples were centrifuged at 5000 rpm/min for 10 min after coagulation. Supernatant serum was stored at −20 °C. Erythropoietin and hepcidin in serum were determined by ELISA according to the manufacturer's instructions (USCN Life Co., USA).

2.4. Western blot analysis

Liver tissue lysates were prepared as follows: minced liver samples were lysed in a pH 7.5 buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 5 mM EGTA, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Diagnostics, Meylan, France). Protein concentrations were determined by Bio-Rad protein assay. Total cellular proteins (100 μg/lane) were resolved with SDS–PAGE and transferred to nitrocellulose membrane (Amersham Biosciences, USA). Membranes were blocked in 5% non-fat milk in TBS, incubated in primary antibody (illustrated below), washed and incubated with peroxidase-conjugated secondary antibody, washed, and then incubated with chemiluminescence substrate (ECL Plus). The signal was visualized using X-ray film in dark room. The relative and normalized protein expression was calculated by a ratio of density of each estimated protein to density of β-actin.

The antibody used in western blot assay were: rabbit polyclonal directed against pY^{705}-STAT3 (1/1000; No. 44380G; Invitrogen, USA), mouse monoclonal directed against STAT3 (1/1000; No. 9139; Cell Signaling Technology, USA), rabbit monoclonal directed against pT^{694}-STAT5 (1/1000; No. 9359; Cell Signaling Technology, USA), rabbit polyclonal directed against STAT5 (1/1000; No. 9363; Cell Signaling Technology, USA), mouse monoclonal directed against Smad4 (1/50; No. 39-1200; Invitrogen, USA), rabbit monoclonal directed against C/EBPα (1/1000; No. ab40761; Abcom, UK), mouse monoclonal directed against β-actin (1/2000; Sigma, USA), peroxidase-conjugated goat anti-rabbit immunoglobulins (1/4000; No. KC133423; Thermo, USA), peroxidase-conjugated goat anti-mouse immunoglobulins (1/4000; No. LA142254; Thermo, USA).

2.5. Statistical analysis

Results were expressed as mean ± S.D. Student's *t* test was used for estimation of statistical significance ($p < 0.05$was considered as significant).

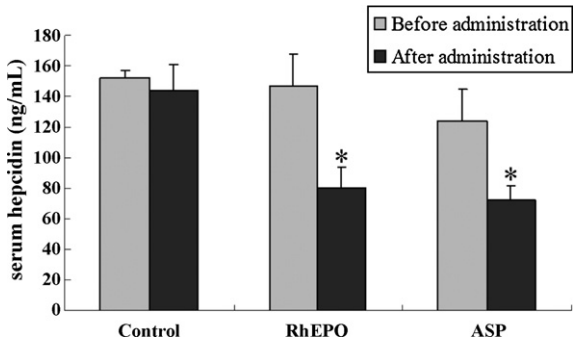

Fig. 1. Changes of hepcidin levels in rats of different treatment groups. Control, rats ($n = 7$) administrated normal saline (1 mL) for 14 days; RhEPO, rats ($n = 7$) administrated rhEPO (2000 U/kg) for 3 days; ASP, rats ($n = 7$) administrated ASP (1 g/kg) for 14 days. *$p < 0.05$ as compared with those before administration.

3. Results

3.1. Erythropoietin promotion and hepcidin suppression

After 3-day treatment of rhEPO and 14-day treatment of ASP, hepcidin levels were significantly reduced by 45.6% and 25.8%, respectively (Fig. 1). However, hardly any changes in erythropoietin levels were observed (data not shown).

It was demonstrated that hepcidin expression decreases following repeated erythropoietin administration (Nicolas et al., 2002). To investigate the correlation between hepcidin expression and erythropoietin levels in body, blood was taken at different time points after the last administration. In both ASP and rhEPO groups, erythropoietin levels in serum were dramatically promoted, and hepcidin levels began to decline 6–8 h after that (Fig. 2).

After the last administration of ASP, serum erythropoietin reached the maximum level at 4 h and went back to the initial level at 72 h; serum hepcidin began to reduce at 12 h and reached the minimum level at 48 h (Fig. 2B). However, in rhEPO group (Fig. 2A), the changing of both erythropoietin and hepcidin lev-

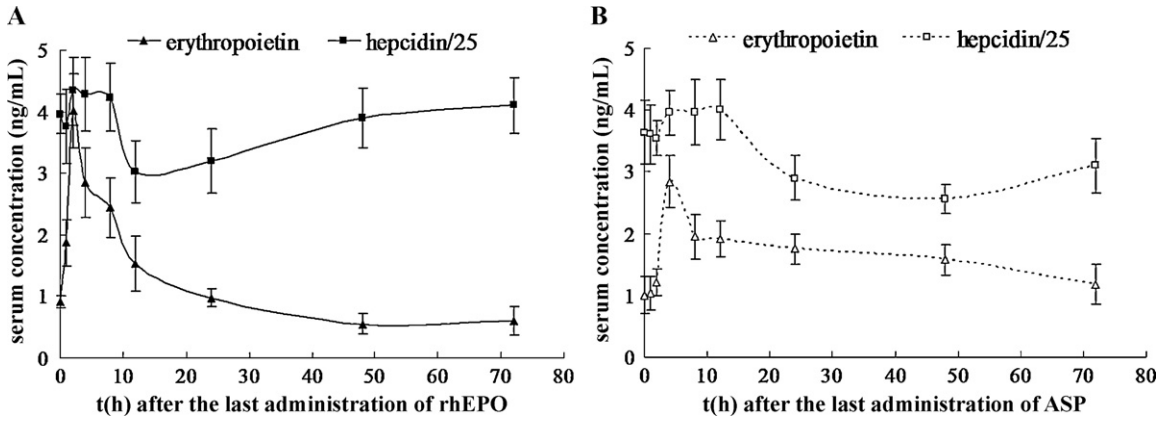

Fig. 2. Erythropoietin promotion and hepcidin suppression induced by rhEPO and ASP. (A) Intraperitoneal injection of rhEPO (2000 U/kg) increased erythropoietin levels in serum immediately; serum hepcidin reduced to the minimum level after 12 h of the injection and returned to the initial level 36 h later. (B) Intragastrically administration of ASP (1 g/kg) stimulated endogenous erythropoietin secretion and kept serum erythropoietin at a high level till 72 h after administration; serum hepcidin declined to the minimum level at 48 h, and was kept at a low level for 72 h after administration.

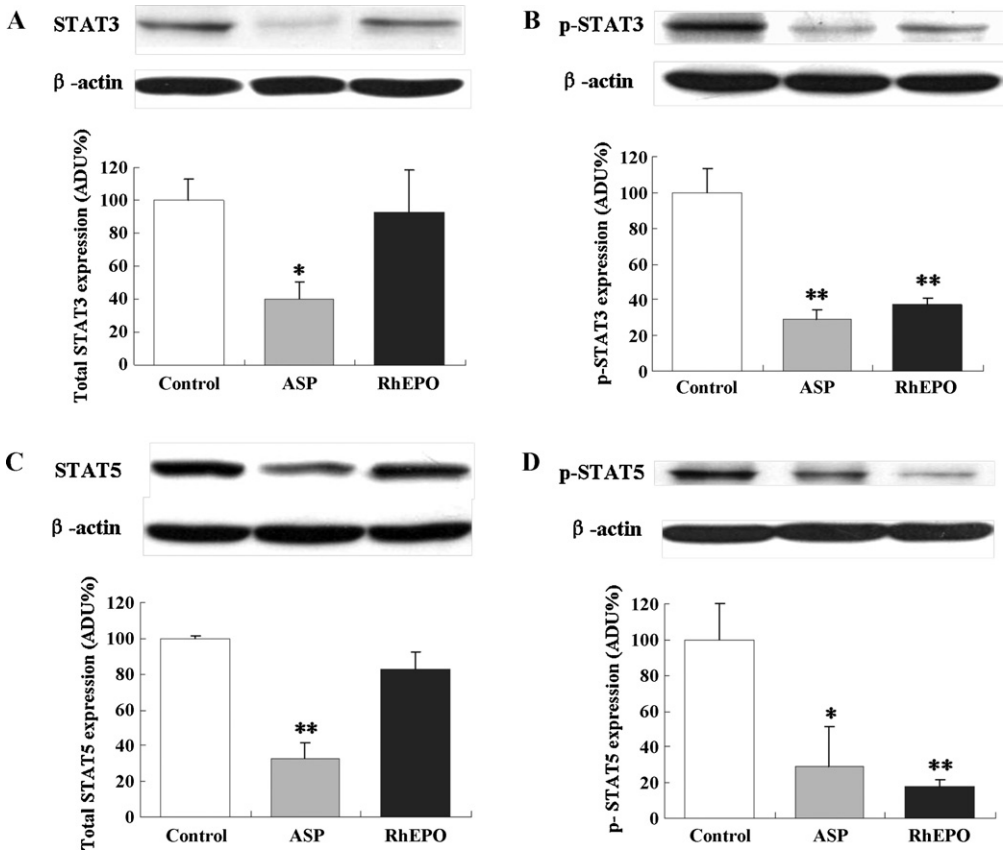

Fig. 3. Suppression of JAK/STAT pathway by ASP and rhEPO. (A) Total STAT3 expression, (B) phospho-STAT3 expression, (C) total STAT5 expression, and (D) phospho-STAT5 expression. Bars represent mean (±S.D.) of arbitrary densitometric units (ADUs) following Western blot analysis. Values are expressed as percentages relative to negative control group. Control, rats ($n=5$) administrated with normal saline (1 mL) for 14 days; ASP, rats ($n=5$) administrated with ASP (1 g/kg) for 14 days; RhEPO, rats ($n=5$) administrated with rhEPO (2000 U/kg) for 3 days. $^{*}p<0.05$; $^{**}p<0.01$.

els progressed more quickly than those in ASP group. It could be inferred that the maintenance of the high level of erythropoietin after ASP administration might result in the low maintenance of hepcidin.

3.2. Inhibition of signal proteins involved in hepcidin regulation

3.2.1. STAT3/phospho-STAT3 and STAT5/phospho-STAT5 expression

ASP administration reduced the mean levels of hepatic STAT3 and STAT5 by 60.1% and 67.2%, respectively (Fig. 3). Similar Western blot analysis of phospho-STAT3 and phospho-STAT5 revealed approximate 70.8% and 70.6% reduction in rats of ASP-treatment group. There were no apparent effects on the ratio of STAT3/5 and phospho-STAT3/5, suggesting that the phosphorylation process was not affected by ASP.

In rhEPO group, the decreases in STAT3 and STAT5 were not statistically significant. However, the mean levels of phospho-STAT3 and phospho-STAT5 were reduced by 62.8% and 81.8%, respectively, indicating that erythropoietin inhibited the phosphorylation processes of STAT3/5 rather than the proteins themselves.

3.2.2. SMAD4 expression

Previous reports showed that SMAD4 is the key protein in BMP-SMAD pathway and is essential for hepcidin production in response to several stimulants (Wang et al., 2005). Western blot analysis of SMAD4 indicated that ASP administration and rhEPO injection reduced liver expression of SMAD4 by 43.1% and 35.8%, respectively (Fig. 4).

3.2.3. C/EBPα expression

C/EBPα was the first transcription activator of the hepcidin gene identified. Its importance was supported by the fact that liver-specific deletion of C/EBPα gene in mice prevented hepcidin expression and induced a severe iron overload (Courselaud et al., 2002). In this study, C/EBPα levels were reduced by 46.8% and 68.9%, respectively after ASP and rhEPO treatment (Fig. 5).

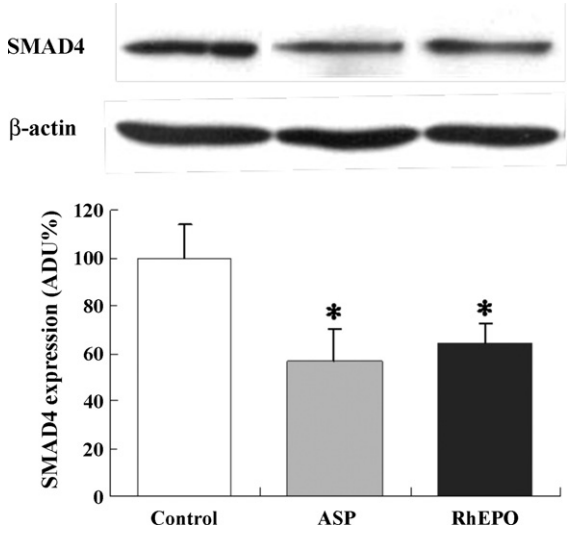

Fig. 4. Suppression of SMAD4 by ASP and rhEPO. Bars represent mean (±S.D.) of arbitrary densitometric units (ADUs) following Western blot analysis. Values are expressed as percentages relative to negative control group. Control, rats ($n = 5$) administrated normal saline (1 mL) for 14 days; ASP, rats ($n = 5$) administrated ASP (1 g/kg) for 14 days; RhEPO, rats ($n = 5$) administrated rhEPO (2000 U/kg) for 3 days. $^{*}p < 0.05$.

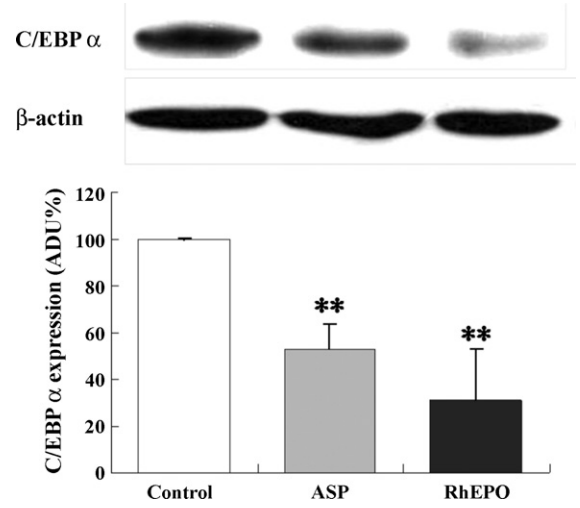

Fig. 5. Suppression of C/EBPα by ASP and rhEPO. Bars represent mean (±S.D.) of arbitrary densitometric units (ADUs) following Western blot analysis. Values are expressed as percentages relative to negative control group. Control, rats ($n = 5$) administrated normal saline (1 mL) for 14 days; ASP, rats ($n = 5$) administrated ASP (1 g/kg) for 14 days; RhEPO, rats ($n = 5$) administrated rhEPO (2000 U/kg) for 3 days. $^{**}p < 0.01$.

4. Discussion and conclusion

In this study, we investigated the effect of ASP on hepcidin suppression as well as its related mechanism. The results showed that ASP could promote erythropoietin secretion and reduce hepcidin levels in rats. Consistent with earlier reports (Nicolas et al., 2002; Huang et al., 2009), the suppression effect of rhEPO administration on hepcidin expression was demonstrated in our study. Interestingly, no variation in hepcidin levels was observed when erythropoietin levels reached its maximum; however, hepcidin concentration began declining when erythropoietin levels fell back. In other words, the inhibition of hepcidin only appears in the duration of high erythropoietin level. This phenomenon demonstrated that the directly inhibiting factor of hepcidin expression was not erythropoietin, but the downstream molecules of erythropoietin. This speculation was consistent with literatures which support the concept that the decrease of hepcidin following repeated administration of erythropoietin is secondary to the effect of erythropoietin on erythropoiesis (Pak et al., 2006; Huang et al., 2009). Comparing the duration of hepcidin suppression in both two treatment groups, it could be noticed that the rats' hepcidin concentration stayed low for a longer time in ASP treatment group, implicating the prolonged action of ASP in hepcidin suppression.

Researches conducted during the last few years indicated that the regulation of hepcidin expression is a complex process (Muckenthaler, 2008). It is becoming increasingly clear that hepcidin gene transcription is influenced by three main pathways: the inflammatory pathway, which increases hepcidin transcription through JAK/STAT signal transduction in response to inflammatory mediators; the BMP-SMAD pathway, which mediates hepcidin up-regulation by iron and hypoxia; and the erythropoietic pathway, which decreases hepcidin mRNA levels following an increase in the rate of erythropoiesis (Viatte and Vaulont, 2009). Accordingly, inhibitory effects of erythropoietin and ASP on key proteins in these pathways were investigated to clarify the molecular mechanism involved in hepcidin suppression.

Western blot analysis showed that ASP can significantly down-regulate STAT3 and STAT5 with little more decreases in phospho-STAT3 and phospho-STAT5. Whereas, erythropoietin can inhibit the phosphorylation process without affecting STAT3 and

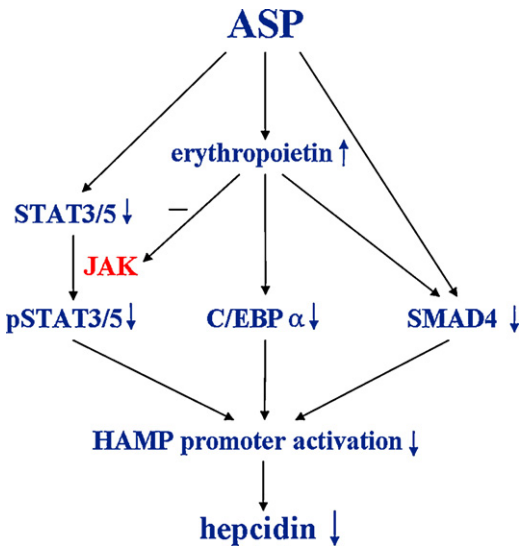

Fig. 6. Molecular mechanism speculated to be involved in hepcidin (HAMP) suppression by ASP. ASP down-regulates the transcription regulators STAT3/5 and SMAD4, and stimulates endogenous erythropoietin secretion which further decreases the signal proteins in liver. As a result, hepcidin expression was suppressed. Erythropoietin inhibits the phosphorylation of STAT3/5 and the expression of C/EBPα and SMAD4, which cause the reduction of activating factors and the suppression of hepcidin. Further researches are needed to demonstrate the inhibitory effect of erythropoietin on JAK which phosphorylate STATs.

STAT5, suggesting that erythropoietin may affect Janus-kinases (JAK) which phosphorylate STATs. Therefore, it could be speculated that the slight inhibitory activity of ASP in phosphorylation process was attributed to the effect of erythropoietin stimulated by ASP.

In hepcidin regulation, BMP signaling can enhance the phosphorylation of SMAD1/5/8 (Kautz et al., 2008). Then, SMAD4 is needed to bind these activated SMADs and the complex moves to the nucleus where it can stimulate hepcidin expression. As a further support for the role of SMAD4 in the regulation of hepcidin expression, ablation of SMAD4 specifically in the liver triggers an iron overload in multiple organs due to decreased levels of liver hepcidin (Wang et al., 2005). The results presented in this study suggest that both erythropoietin and ASP can suppress hepatic SMAD4 expression and may interrupt the entire BMP-SMAD pathway.

Pinto et al. recently proposed that erythropoietin could act directly to suppress hepcidin in hepatocytes through the regulation of the transcription factor C/EBPα. Similarly, we found erythropoietin can significantly inhibit hepatic C/EBPα in rats. Nevertheless, the suppression of C/EBPα by ASP administration was to a less extent than that by rhEPO administration. This may be due to the less levels of erythropoietin in blood promoted by ASP than that by rhEPO. These results also indicated that erythropoietin stimulated by ASP could take part in the hepcidin suppression of ASP.

Recent researches clarified that erythropoietin can inhibit hepcidin expression directly through the inhibition of C/EBPα in vitro (Pinto et al., 2008) and indirectly via the suppression of STAT3/phospho-STAT3 and SMAD4 signaling in vivo (Huang et al., 2009). Consistently, we confirmed that erythropoietin can interrupt these three signal transduction ways to suppress hepcidin expression in rats. In addition, the STAT5/phospho-STAT5 pathway was also affected by erythropoietin in our study. It is proposed that erythropoietin mediates hepcidin by inhibiting C/EBPα, SMAD4, and the phosphorylation process of STAT3/5 (Fig. 6).

Effects of Angelica polysaccharides on blood promotion and erythropoiesis activation have been already confirmed by many previous studies (Raman et al., 1996; Wang and Zhu, 1996). We further demonstrated its activity in the stimulation of endogenous erythropoietin secretion as well as the positive correlation between erythropoietin promotion and hepcidin repression. Therefore, it is speculated that the stimulation of erythropoietin as well as the inhibition of STAT3/5 and SAMD4 are involved in the suppression of hepcidin expression induced by ASP (Fig. 6).

In conclusion, results from present study showed that ASP can suppress hepcidin expression in vivo by stimulating erythropoietin secretion and interrupting other 2 main pathways of hepcidin regulation. This finding supported the previous speculation that ASP could participate in the regulation of iron homeostasis. As a hepcidin inhibitor, ASP could be applied in the treatments of diseases caused by hepcidin over-expression. Moreover, because of its safety, various bioactivities (Liu et al., 2010) and convenience in administration, ASP would be more widely used than rhEPO.

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