Astragaloside Alleviates Hepatic Fibrosis Function via PAR2 Signaling Pathway in Diabetic Rats

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Key Words
Hepatic fibrosis • Diabetes • PAR2 • PAK • PKC • Astragaloside

Abstract
Background/Aims: Astragaloside (AGS) extracted from radix astragalin (Huangqi) has been considered to be beneficial to liver diseases. In this study, we examined the role played by AGS in alleviating hepatic fibrosis function via protease-activated receptor-2 (PAR2) mechanisms. We hypothesized that AGS affects PAR2 signaling pathway thereby improving hepatic function in rats with hepatic fibrosis induced by carbon tetrachloride (CCl₄). We further hypothesized that AGS attenuates impaired hepatic function evoked by CCl₄ to a greater degree in diabetic animals.

Methods: ELISA and Western Blot analysis were used to examine PAR2 signaling pathway in diabetic CCl₄-rats and non-diabetic CCl₄-rats.

Results: AGS inhibited the protein expression of PAR2 and its downstream pathway PKA and PKCε in CCl₄-rats. Notably, the effects of AGS were greater in CCl₄-rats with diabetes. AGS also significantly attenuated the CCl₄-induced upregulations of pro-inflammatory cytokines, namely interleukin-1β, interleukin-6 and tumor necrosis factor-α accompanied with decreases of collagenic parameters such as hexadecenoic acid, laminin and hydroxyproline. Additionally, AGS improved the CCl₄-induced exaggerations of liver index and functions including alanine aminotransferase, aspartate aminotransferase. Moreover, TGF-β1, a marker of hepatic fibrosis, was increased in CCl₄-rats and AGS inhibited increases in TGF-β1 induced by CCl₄.

Conclusions: AGS alleviates hepatic fibrosis by inhibiting PAR2 signaling expression and its effects are largely enhanced in diabetic animals. Targeting one or more of these signaling molecules may present new opportunities for treatment and management of hepatic fibrosis; and results of our study are likely to shed light on strategies for application of AGS because it has potentially greater therapeutic effectiveness for hepatic fibrosis in diabetes.
Introduction

Hepatic fibrosis is featured by the over production and deposition of extracellular matrix, and excessive build up connective tissue in the liver. This process leads to liver structure disruption and loss of liver function [1]. Although promising progresses have been made in animal models [2], the current treatment of hepatic fibrosis is inadequate, partly due to our poor understanding of the underlying mechanisms responsible for pathophysiological process of the disease and lacking of satisfactory drugs.

Activated hepatic stellate cells, portal fibroblasts, and myofibroblasts of bone marrow origin have been identified as major collagen-producing cells in the injured liver; among the cells, hepatic stellate cells (HSCs) are more important in the development of hepatic fibrosis [1, 2]. A recent study suggests that the activation of the hepatic stellate cell causes accumulation of extracellular matrix and formation of scar, leading to deterioration in the hepatic functions [3]. The hepatic stellate cells are also called Ito cells, which store fat, produce excessive amounts of abnormal matrix including collagen, other glycoproteins and glycans, and matricellular proteins [4]. Thus, stellate cell activation results in abnormal extracellular matrix, both in quantity and composition [4]. Nonetheless, the regulation of hepatic stellate cell activities in hepatic fibrosis remains to be determined.

Protease-activated receptors (PARs) are a family member of G-protein-coupled receptors and are activated by a proteolytic mechanism [5]. Among the four members of PARs, PAR2 is largely distributed in various tissues, including gastrointestinal, liver, cardiovascular, and respiratory systems [5]. Extracellular serine proteases, such as trypsin, tryptase, tissue factor VIIa, and kallikreins, activate PAR2, and thus the receptors are N-terminally truncated by proteases to enable coupling and activation of intracellular G-protein signaling cascades [6]. Although serine proteases activate PAR2, several members of the serine protease family, including neutrophil elastase, cathepsin G, and proteinase 3, have been shown to inactivate PAR2 by cleaving downstream of the tethered ligand [7, 8]. PAR2 activation is generally pro-inflammatory both in vitro and in vivo [6]. The PAR2-activating tissue factor VIIa can also stimulate adipose tissue macrophages, and cause insulin resistance and metabolic dysfunction in mice [9], but little is known about other extracellular signals or dietary ligands that may influence expression or activation of PAR2.

A relationship between PAR2 and stellate cell activities has recently been recognized [10]. Results of this prior study indicate that PAR2 activation augments TGF-β production and promotes hepatic fibrosis in mice and thereby induces a profibrogenic phenotype in human hepatic stellate cells [10].

Radix astragali (Huangqi) is one of main components in a decoction of traditional Chinese medicine usually used to improve chronic liver diseases. Astragaloside (AGS) extracted from radix astragali has been considered to play a central role [11-15]. Thus, in this study we examined the effects of AGS on PAR2 signal pathway in rats with hepatic fibrosis induced by carbon tetrachloride (CCl4). We hypothesized that AGS inhibits PAR2 signal expression and thereby improves hepatic function during development of hepatic fibrosis. It should be noted that chronic liver diseases and diabetes are often present in the same patient [16, 17]. Clinical studies have shown that diabetic patients have a higher risk to have hepatic fibrosis [16]. Notably, numerous cellular and molecular mechanisms are involved in hepatic fibrosis as well as in diabetes [17, 18]. Thus, in this study we further hypothesized that the effects of AGS appear greater in CCl4-rats with diabetes in improving hepatic fibrosis.

Materials and Methods

Animals

All experimental procedures were in accordance with the guidelines of the International Association for the Study of Pain and were approved by the Animal Research Committee of our medical institution. Male Sprague-Dawley rats weighing 200-250 g were used in this study. Streptozotocin (STZ) was freshly
dissolved in 0.9% sterile saline and diabetes was induced by a single injection of STZ (60 mg/kg, Sigma Co.). Diabetes was confirmed by measurements of blood glucose concentrations in samples obtained from the tail vein six weeks after injection of STZ. Note that rats with blood glucose concentration > 350 mg/dl were included in the study. Age- and body weight-matched rats with saline injection were used as controls. Hepatic fibrosis was induced by subcutaneous injection of carbon tetrachloride (CCl₄) 1:1 in olive oil, 3 ml/kg twice a week for consecutive 6 weeks. The rats in the control group were injected with an equal volume of olive oil without CCl₄. In a subgroup, the rats intragastrically received 120 mg/kg of AGS (dissolved in saline) daily for consecutive 6 weeks. The body weights of all rats were examined once a week. At the end of the experimental period, the rats were weighed and euthanized with 120 mg/kg of sodium pentobarbital. The livers were immediately harvested and weighed after blood was collected from the abdominal aorta. Accordingly, the rats were divided into six groups: control rats (n=12); control + STZ (n=12); CCl₄ rats (n=15); CCl₄ rats + STZ (n=15); CCl₄ rats + AGS (n=20); and CCl₄ rats + STZ + AGS (n=20).

Western Blot analysis
Total protein in tissue was extracted by homogenizing sample in ice-cold immunoprecipitation assay buffer with protease inhibitor cocktail kit (Roche). The lysates were centrifuged and the supernatants were collected for measurements of protein concentrations using a bicinchoninic acid assay reagent kit. Fifty microgram of total protein was loaded to run 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the protein was transferred to nitrocellulose membranes (GE Healthcare Biosci). Then, the membranes were incubated for 30 minutes in 5% low-fat milk prepared with Tris-buffered saline containing 0.05% Tween-20 (TTBS). Subsequently, membranes were incubated with the rabbit anti-PAR2 primary antibody (1:500, Neumorics) and goat anti-rabbit secondary antibody (1:200). After being fully washed, the membrane was incubated with horseradish peroxidase-linked goat anti-rabbit secondary antibody (1:250) and visualized for immunoreactivity. The membrane was also processed to detect β-actin for equal loading. Likewise, the protein expression of PKCζ, PKA and TGF-β1 was determined. Rabbit anti-PKCζ (1:500), anti-PKA (1:500) and anti-TGF-β1 (1:250) primary antibodies (all obtained from Cayman Chemical Co., USA), and goat anti-rabbit secondary antibody (1:250) were applied. The bands recognized by the primary antibody were visualized and the optical densities of protein bands were analyzed using the ImageJ software.

ELISA
The levels of IL-1β, IL-6 and TNF-α were determined using an ELISA assay kit (Promega Corp. Madison, WI) according to the provided description and modification. Briefly, polystyrene 96-well microtiter immunoplates were coated with respective affinity-purified rabbit anti-IL-1β, anti-IL-6 and anti-TNF-α antibodies. Parallel wells were coated with purified rabbit IgG for evaluation of nonspecific signal. After overnight incubation at room temperature and 2 hours of incubation with the coating buffer containing 50 mM carbonate buffer (pH 9.5) in 2% BSA, plate were washed with 50 mM Tris-HCl. After extensive washing, the diluted samples and IL-1β, IL-6 and TNF-α standard solution were distributed in each plate, respectively, and left at room temperature overnight. The plates were then washed and incubated with their respective anti-galactosidase per well. Then, the plates were washed and incubated with substrate solution. After an incubation of 2 hours at 37°C, the optical density was measured using an ELISA reader.

Radioimmunoassay (RIA)
In order to assess collagenic parameters, serum concentrations of hexadecenoic acid (HA), laminin (LN) and concentrations of hydroxyproline (HYP) in liver tissues were measured using commercial RIA kits (Beifang Biotech, Beijing, China) according to the manufacturer’s protocols and as described previously [19]. Also, serum concentrations of liver functional enzymes ALT and AST were determined using commercial kits (Beifang Biotech, Beijing, China) according to the manufacturer’s manual.

Statistical analysis
All data were analyzed using a two-way repeated-measures analysis of variance. Values were presented as means ± standard error of mean (SEM). For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed by using SPSS for Windows version 17.0 (SPSS, USA).
Results

General measurements

First, we examined body weight of rats during experimental interventions. Figure 1A illustrates a decrease in body weight in those animals with increases of the concentrations of blood glucose after injection of STZ. This figure also shows that the body weight of rats in the CCl\textsubscript{4} group was significantly reduced as compared with those in the respective control group. Loss of the body weight was worsened in diabetic CCl\textsubscript{4}-animals. Note that with the treatment with AGS, the CCl\textsubscript{4}-rats gained weight faster than that those without treatment. Previous studies have reported that the levels of blood glucose begin to increase 1 day after systemic injection of STZ and remain at a higher level for >10 weeks [20, 21]. Figure 1B shows that hyperglycemia largely developed in animals during six weeks after STZ (P<0.05 vs. control rats) as compared with saline control rats. The levels of blood glucose were not observed to be changed significantly after CCl\textsubscript{4} per se.

Expression of PAR2 pathway

Figure 2A shows that the protein expression of PAR2 and its downstream pathways, namely PKA and PKCe was significantly increased in the liver tissues of non-diabetic CCl\textsubscript{4}-rats and diabetic CCl\textsubscript{4}-rats as compared with their respective controls (P<0.05 vs. controls). Treatment with AGS attenuated the exaggerated PAR2 pathway expression induced by CCl\textsubscript{4} (P<0.05 vs. CCl\textsubscript{4}-rats with no AGS) and the effects appeared to be a greater degree in diabetic rats compared with non-diabetic rats. The percentage inhibitory effects of AGS on PAR2, PKA and PKCe were 27%, 25%, and 30% in non-diabetic CCl\textsubscript{4}-rats; and 49%, 43% and 48% in diabetic CCl\textsubscript{4}-rats, respectively (P<0.05, non-diabetic CCl\textsubscript{4}-rats vs. diabetic CCl\textsubscript{4}-rats).

Likewise, Figure 2B further shows that TGF-β1 was increased in the liver tissues of non-diabetic CCl\textsubscript{4}-rats and diabetic CCl\textsubscript{4}-rats. AGS also inhibited increases in TGF-β1 induced by CCl\textsubscript{4} and the inhibitory effects of AGS on TGF-β1 were greater in diabetic CCl\textsubscript{4}-rats than those in non-diabetic CCl\textsubscript{4}-rats (21% in non-diabetic CCl\textsubscript{4}-rats vs. 53% in diabetic CCl\textsubscript{4}-rats; P<0.05 between two groups).

Fig. 1. The changes of body weight and blood glucose in six groups of rats during interventions. (A) *P<0.05 (4-6 weeks after AGS treatment shown by grey bars), indicated control rats vs. control rats + STZ, CCl\textsubscript{4}-rats (without AGS), and CCl\textsubscript{4}-rats + STZ (without AGS). *P<0.05 also indicated CCl\textsubscript{4}-rats vs. respective CCl\textsubscript{4}-rats with AGS. i.e., CCl\textsubscript{4}-rats vs. CCl\textsubscript{4}-rats with AGS, and CCl\textsubscript{4}-rats + STZ vs. CCl\textsubscript{4}-rats + STZ with AGS. There was no significant difference observed in body weight among control rats, CCl\textsubscript{4}-rats and CCl\textsubscript{4}-rats + STZ 4-6 weeks after AGS treatment. (B) *P<0.05, STZ rats vs. respective non-STZ rats over a six week intervention time (indicated by grey bars). i.e., control rats + STZ vs. control rats; CCl\textsubscript{4}-rats + STZ vs. CCl\textsubscript{4}-rats; and CCl\textsubscript{4}-rats + STZ with AGS vs. CCl\textsubscript{4}-rats with AGS. No significant difference in blood glucose was observed among three groups of rats with injection of STZ (control rats + STZ, CCl\textsubscript{4}-rats + STZ and CCl\textsubscript{4}-rats + STZ with AGS).
Fig. 2. Effects of AGS on expression of PAR2 pathways and TGFβ. A&B: Top panel and bottom panel represent typical bands and averaged data. Expression of PAR2 and its downstream pathway PKA and PKCε was significantly increased in the liver tissues of non-diabetic CCl₄-rats and diabetic CCl₄-rats as compared with their respective controls. Likewise, TGF-β1 was also increased in the liver tissues of non-diabetic CCl₄-rats and diabetic CCl₄-rats. Treatment with AGS attenuated the exaggerated PAR2 pathway signals induced by CCl₄. AGS also inhibited increases in TGF-β1 induced by CCl₄. *P<0.05 vs. control rats and CCl₄-rats with AGS. The percentage inhibitory effects of AGS on PAR2, PKA and PKCε as well as TGF-β1 were greater in diabetic rats as compared with non-diabetic rats.

Levels of PICs
Figure 3 demonstrates the levels of IL-1β, IL-6 and TNF-α in six groups of rats. Administration of CCl₄ significantly elevated these PIC levels in liver (P<0.05 vs. respective control rats) in both non-diabetic rats and diabetic rats. Moreover, treatment with AGS significantly attenuated increases of IL-1β, IL-6 and TNF-α evoked by injection of CCl₄. Nonetheless, the effects of AGS appeared to be greater in diabetic rats.

Collagenic parameters
The levels of HA, LN, and HYP were examined to evaluate the collagen contents. Figure 4A&B shows that the productions of HA and LN were significantly increased in CCl₄-rats in comparison to the control group rats (P<0.05). Administration of AGS attenuated upregulation of HA and LN, which appeared to be greater in diabetic CCl₄-rats than those in non-diabetic CCl₄-rats. Figure 4C further shows that the levels of HYP in CCl₄-rats were higher than those in control rats (P<0.05). By contrast, the levels of HYP in CCl₄-rats were significantly inhibited (P<0.05) following treatment of AGS. The inhibitory effects of AGS appeared to be greater in diabetic CCl₄-rats.
Liver index and functions
In addition, Figure 5A demonstrates that the liver indices of CCl\(_4\)-rats largely increased as compared to those of the control rats. Treatment with AGS lessened the amplified liver indices. STZ injection failed to significantly affect the liver indices per se.

Serum ALT and AST activities were measured to assess the effects of AGS on liver function. Figure 5B &C reveal that the levels of ALT and AST were significantly elevated in non-diabetic CCl\(_4\)-rats and diabetic CCl\(_4\)-rats as compared with those in control groups (\(P<0.05\)). However, rats treated with AGS showed a significant reduction of ALT and AST activities (\(P<0.05\)). These data demonstrate that treatment with AGS effectively suppressed the activities of impaired liver functional enzymes in rats after application of CCl\(_4\).

Discussion
AGS is the major active component extracted from *radix Astragali*, the root of *Astragalus membranaceus* Bunge [13], known as herbal remedy Huangqi in China. It has been reported that AGS plays a beneficial role without evident toxic or side effects such as the anti-
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Inflammatory, anti-aging, anti-oxidative and anti-myocardial injury [11, 12, 14, 15, 22-24]. In the current study, we demonstrated that AGS significantly inhibits the progression of hepatic fibrosis induced by CCl₄ and the effects of AGS appear to be greater in diabetic animals.

Considerable efforts have been made to create appropriate animal models of hepatic fibrosis for the study of the underlying molecular mechanisms in vivo and to evaluate anti-fibrotic drugs [25]. Among the hepatic fibrosis models, CCl₄-induced rat hepatic fibrosis has been widely used to study human disease [25]. Exposure to CCl₄ leads to hepatic injury, which causes the permeability alteration of the plasma and mitochondrial membranes [26]. As a result, cytoplasmic enzymes, including ALT and AST, discharge into circulation [27]. Both ALT and AST reflect the degree of liver cell injury and serve as a reliable diagnostic indicator of liver damage [28]. In our current study, the CCl₄-injury rats gained less weight with significantly higher ALT and AST levels than the control rats, while the rats treated with AGS showed significant reductions in these enzyme levels. This result indicates that AGS is effective on liver functions. Consistent with this result, we further demonstrated that treatment with AGS reduced the severity of liver injury since it significantly decreased the amplified liver indices of CCl₄-rats. Interestingly, STZ injection failed to affect the liver indices per se; however, the improving effects of AGS on liver functions and index were observed to be a greater degree in diabetic rats.

Prior studies suggest the critical role of inflammatory mechanisms in modulating hepatic fibrosis [29]. The inhibition of IL-1β, IL-6 and TNF-α ameliorates the hepatic aggravation during fibrogenesis [30]. It has also been reported that in the pathophysiological process cell loss in sensitive tissue of diabetic rats is observed at least 4 weeks after development of hyperglycemia [31]. Also, increases of IL-6 and TNF-α in diabetic sensitive tissue were observed from 4 weeks following STZ injection [32, 33]. Therefore, in this study, we examined PIC responses and the role played by AGS at 6 weeks of time points following STZ injection. We found that AGS attenuated the upregulation of IL-1β, IL-6 and TNF-α to a greater degree in diabetic CCl₄-rats than that in non-diabetic CCl₄-rats.

Continuous accumulation of extracellular matrix (ECM) is responsible for hepatic fibrosis. As the major component of ECM, type I collagen is composed of HYP [34]. Additionally,
HA and LN are important indices to evaluate the collagen extent of hepatic fibrosis [35]. Our data further showed that treatment with AGS predominantly suppressed the levels of HYP, HA and LN in CCl₄-induced rats, indicating that AGS likely plays an inhibitory role in the hepatofibrosis progression.

There are numerous mechanisms responsible for promoting collagen formation in regulation of hepatic fibrosis. TGF-β1 is considered a key activator of HSCs and the most potent fibrogenic factor. α-SMA is a typical index for the activated HSCs during fibrotic process [36]. The expressions of α-SMA and type I collagen increases in TGF-β1-induced HSC-T6 cells [37]. NF-κb p65 is a central signaling factor for hepatic fibrosis in control of the generation of PICs [38]. As the critical molecules, PI3K/Akt/ mTOR pathway mediate protein expression and proliferation through the inhibition of DNA synthesis in HSCs [39] and play the critical role in transmitting signaling in hepatic fibrosis [40]. The suppression of mTOR/p70S6K signaling results in the inhibition of type I collagen accumulation and HSC proliferation [41]. Nonetheless, a relationship between PAR2 and HSC activities has recently been recognized [10]. Results of this prior study indicate that PAR2 activation augments TGF-β1 production and promotes hepatic fibrosis in mice and thereby induces a profibrogenic phenotype in human HSCs [10]. In the present study, our data also demonstrated that TGF-β1 was increased in hepatic tissues of CCl₄-rat in addition to PAR2 activation. In addition, AGS inhibited increases in TGF-β1 induced by CCl₄ to a greater degree in diabetic rats. To the best of our knowledge, our results for the first time show that AGS plays a beneficial role via PAR2 mechanisms in diabetic animals.

A prior study showed that the primary effects of AGS are generally via reducing oxidative stress, i.e., decreasing reactive oxygen species (ROS) and increasing glutathione (GSH) [42]. This process thereby inhibits HSC activation and prevents fibrosis. PAR2 pathway is activated by extracellular serine proteases including trypsin, tryptase, tissue factor VIIa, and kallikreins. In the present study, inhibition of PAR2 pathway was observed after treatment with AGS. This is likely due to a consequence of the lack of HSC activation and consequently leading to decreases in production of proteases.

PAR2 is known to be coupled to Gq/11 and its activation leads to the activation of PLCβ and its downstream pathway PKC [5]. Also, PKA pathway is likely to be engaged in the role played by PAR2 [5]. Thus, in the current study we also examined these signaling pathways in CCl₄-rats after AGS treatment. Our data demonstrated that AGS inhibited PAR2 as well as PKCe and PKA pathways in CCl₄-rats, which was likely to result in the improving effects on liver functions and injuries. Overall, our data suggest that intracellular PKCe and PKA signaling is involved in the role played by AGS in attenuating PAR2 in CCl₄-induced hepatic fibrosis.

Moreover, hepatic fibrosis can be induced by common bile duct ligation (BDL). It is noted that the primary pathological lesions in hepatic fibrosis induced by BDL occur in the area surrounding the bile duct epithelium. This triggers compensatory proliferation and destruction of biliary epithelial cells, which promotes HSC activation leading to development of cholestatic hepatic fibrosis. AGS has been previously reported to significantly reduce the deposition of collagen and HYP content of liver tissue and inhibit activation of HSCs in BDL-induced hepatic fibrosis [43]. Results of this prior study further demonstrated that AGS significantly decreased the protein and mRNA expressions of TGF-β1 and α-SMA. This was accompanied with reduced biliary epithelial cell proliferation whereas AGS enhanced expression of the Smad 7 protein and decreased expression of Notch signal. Similarly, our present study also observed that AGS has inhibitory effects on amplified TGF-β1 and HYP content induced by CCl₄. Although there are differences in the pathological process of hepatic fibrosis by BDL and CCl₄, AGS can play a beneficial role in improving hepatic function in both models. It should be noted that our results specifically indicate greater effects of AGS on hepatic fibrosis in diabetes.

Finally, a limitation of the study needs to be acknowledged. The present study did not include histological examination of inflammation and fibrosis to exemplify the anti-fibrotic effects of AGS. Nevertheless, we observed that in CCl₄-rats AGS significantly inhibited
increases in TGF-β1, as a marker of hepatic fibrosis, and AGS also decreased the collagen content of hepatic fibrosis. It is speculated that beneficial effects of AGS would improve inflammatory and fibrotic hepatic tissues on histological aspect.

Conclusions

Our data indicate that AGS alleviates hepatic fibrosis function by inhibiting PAR2 signaling expression and its effects are enhanced in diabetes. Targeting one or more of these signaling molecules may present new opportunities for treatment and management of hepatic fibrosis; and results of our study are likely to shed light on strategies for application of AGS or radix astragali because it has potentially greater therapeutic effectiveness for hepatic fibrosis in diabetes.

Disclosure Statement

None.

References


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