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The protective action of scutellarin against immunological liver injury induced by concanavalin A and its effect on pro-inflammatory cytokines in mice

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Abstract

Scutellarin is a natural compound from a Chinese herb. The purpose of this paper was to study the protective effect of scutellarin on concanavalin A (Con A)-induced immunological liver injury and its effect on liver nuclear factor κB (NF-κB), tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), and inducible nitric oxide synthase (iNOS) expression in mice. Mouse liver injury was produced by injection of Con A 25 mg kg^{-1} via the tail vein. Scutellarin 50 or 100 mg kg^{-1} was peritoneally administered to mice 9 or 1 h before injection of Con A. The levels of serum alanine aminotransferase (ALT) and asparatate aminotransferase (AST), NO_2^-/NO_3^- and TNF-α were determined with biochemical kits, and ELISA using Quantikine Mouse TNF-α kit according the manufacturer's instructions. Liver lesions were examined by light microscope. The expression of TNF-α, IFN-γ, iNOS and Fas mRNA in the livers was detected by RT-PCR; and the expression of c-Fos, c-Jun, iNOS and IκB proteins was measured by Western Blotting. As a result, pretreatment with scutellarin 100 mg kg^{-1} significantly decreased the serum ALT, AST, NO_2^-/NO_3^- and TNF-α levels, and also reduced liver lesions induced by Con A. Scutellarin 100 mg kg^{-1} down-regulated expression of TNF-α and iNOS mRNA, and c-Fos, c-Jun and iNOS protein, while scutellarin enhanced the degradation of IκBα in the livers of mice injected with Con A. The results suggest that scutellarin has a protective action against Con A-induced liver injury in mice, and its active mechanism may be related to the inhibition of the NF-κB–TNF-α–iNOS transduction pathway.

Introduction

Scutellarin, 4′,5,6-trihydroxylflavone-7-*O*-glucuronoside (Figure 1), is an active principle from *Erigeron breviscapine (vant) Hand Mass*. An injection containing scutellarin has been used in the treatment of cardio-cerebral vascular diseases in China (Xiao & Cheng 1987). Scutellarin was shown to have scavenging activity on hydroxyl radicals, superoxide anion radicals and hydrogen peroxide in-vitro (Liu et al 2002), and reduce liver injury elicited secondary due to brain ischaemia/reperfusion in rats (Yang et al 2003). Scutellarin was also shown to be hepatoprotective in an experimental model of liver toxicity of selenium in rats (Eltayeb et al 2004); however, there is no report about the effect of scutellarin on immune-mediated liver injury in the literature. It was reported that the injection of concanavalin A (Con A) could induce liver injury through immune mechanism (Tiegs et al 1992), and some cytokines such as tumour necrosis factor α (TNF-α), interferon γ (IFN-γ) and Fas/FasL were directly involved in the process of hepatocyte damage induced by Con A (Mizuhara et al 1994; Gantner et al 1995; Tagawa et al 1997). Nuclear factor κB (NF-κB) plays a pivotal role in regulating the immune system, inflammation and cell apoptosis (Ghosh & Karin 2002). It is interesting to study whether scutellarin has a protective effect on Con A-induced liver injury and effects on the expression of several pro-inflammatory cytokines and NF-κB activation in mice. This paper reports the results of our study on this question.

Materials and Methods

Reagents

Scutellarin (purity > 95%) was purchased from Kunming Longjin Pharmaceutical Co. Ltd (Kunming, China). Con A was obtained from Sigma Chemical Co. (St Louis, MO). TRIZOL

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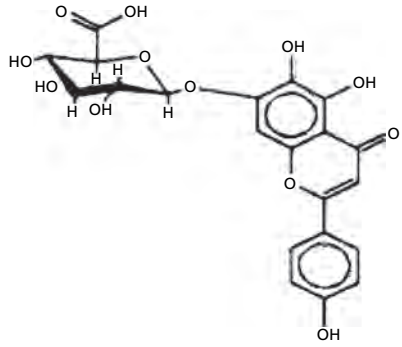

Figure 1 Chemical structure of scutellarin.

reagent was a product of Invitrogen; Access RT-PCR System was obtained from Promega. Quantikine Mouse TNF-α kit was purchased from R&D Systems Inc. (Minneapolis, MN). Alanine aminotransferase (ALT) and asparatate aminotransferase (AST) kits were products of Beijing BHKT Clinical Reagent Co. Ltd (Beijing, China). NO_2^-/NO_3^- kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animal treatment

Male ICR mice, 22–24 g, were obtained from Beijing Weitong Lihua animal company. The mice were housed 5 per cage in a thermo-regulated environment (23 ± 1°C, 50 ± 5% humidity) with free access to food and water, under a 12-h light–dark cycle. All animal experiments were performed in accordance with the instructions of the Ethical Committee for Care and Welfare of Laboratory Animals from Beijing municipal government.

Mice were injected with 25 mg kg^{-1} Con A or a corresponding volume of normal saline via the tail vein, respectively. Scutellarin dissolved in normal saline at 50 mg kg^{-1} and 100 mg kg^{-1} was administered intraperitoneally at two times, 9 and 1 h before Con A injection. A control group received normal saline instead of scutellarin. The mice were killed 2, 8 and 16 h after treatment with Con A, respectively, for different measurements.

Measurement of serum ALT and AST levels

Sixteen hours after Con A injection, the mouse serum ALT and AST levels were measured using the biochemical kits according to the manufacturer's instructions.

Measurement of serum NO_2^-/NO_3^- level

Eight hours after the injection of Con A, the mouse serum NO_2^-/NO_3^- was measured using the biochemical kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute).

TNF-α assay

Two hours after Con A injection, the serum TNF-α level was measured by ELISA using Quantikine Mouse TNF-α kit according to the manufacturer's instructions (R&D Systems, USA).

Detection of TNF-α, IFN-γ, iNOS, Fas and FasL mRNA by semi-quantitative RT-PCR

Two hours after the injection of Con A, mouse liver was removed and placed in liquid nitrogen for detection of TNF-α, IFN-γ, inducible nitric oxide synthase (iNOS) and Fas mRNA by RT-PCR. The total RNA of the liver was extracted using Trizol reagent according to the manufacturer's instructions (Gibco USA). The primers used were as follows: 5′-TGC ACA GAA GGG AAG GAG TA-3′ and 5′-ATG GTT TCA CGA CTG GAG GT-3′(395bp) for Fas; 5′-GAC AGC AGT GCC ACT TCA TC-3′ and 5′-TTA AGG CTT TGG TTG GTG AA-3′(317bp) for FasL; 5′-GGC GGT GCC TAT GTC TCA G-3′ and 5′-GGG CAG CCT TGT CCC TTG A-3′(364bp)for TNF-α; 5′-CTC AAG TGG CAT AGA TGT GG-3′ and 5′-ACT CCT TTT CCG CTT CCT GA-3′(346bp) for IFN-γ; 5′-GCC TCA TGC CAT TGA GTT CAT CAA CC-3′ and 5′-GAG CTG TGA ATT CCA GAG GCC TGA AG-3′(372bp) for iNOS; and 5′-CTC CTA CCA CAC CCA TTC TCA TCC-3′ and 5′-GCA ATG CCT GGG TAC ATG GTG G-3′(492bp) for β-actin. The β-actin gene was amplified as an internal control. PCR conditions were 35 cycles of denaturizing at 94°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 45 s. PCR products were separated by electrophoresis through 2% agarose containing 0.5 μg mL^{-1} ethidium bromide. The DNA bands were imaged using a Kodak digital imaging system (Kodak DC120, Digital Science 1D system, USA). The relative quantity of Fas, TNF-α, IFN-γ and iNOS mRNA are presented as the ratio of the intensity of each band relative to the intensity of the house-keeping gene β-actin.

Detection of the expression of IκBα, c-Jun, c-Fos and iNOS by Western blotting

Two hours after the injection of Con A, the livers were placed in liquid nitrogen for measurement of the expression of IκBα, c-Jun, c-Fos and iNOS. The liver tissues were homogenized in RIPA lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% TritonX-100, 0.5% Nonidet P40, 1 μg mL^{-1} aprotinin, 1 μg mL^{-1} leupeptin, 1 mM PMSF) at 4°C, and centrifuged at 10 000 *g* at 4°C for 30 min. The supernatants were mixed in Laemmli loading buffer, boiled for 4 min, and then subjected to SDS-PAGE. After electrophoresis, the proteins were transferred onto nitrocellulose membranes and blotted against primary antibodies for 2 h. The membranes were washed with TBST (20 mM Tris-base, 150 mM NaCl, 0.05%Tween-20) and incubated with a 1:300 dilution of HRP-conjugated secondary antibodies for 2 h. The protein bands were visualized by DAB system and were scanned.

Liver histology examination by light microscope

Sixteen hours after the Con A injection, the livers from mice of the normal control, Con A control and scutellarin 100 mg kg^{-1} groups were removed. Liver sections, 5 μm thick, were stained with hematoxylin–eosin, and submitted to a pathologist who was blinded, to observe liver lesions. Each section was examined

under a microscope. The histological degree of liver injury was classified according to the numbers of coagulative necrosis foci localized in the intermediate zone of the lobule (zone 2 of the liver lobule) in Con A hepatitis (grade 0, no necrosis per view; grade 1, 1–3 small coagulating necrosis foci; grade 2, 4–7 coagulating necrosis foci; grade 3, > 8 coagulating necrosis focusi, or a large scale of coagulating necrosis).

Statistical analysis

All the data are expressed as means ± s.d. The differences of the biomarkers between groups were analysed using one-way analysis of variance test, and then the individual differences between the groups were evaluated using Dunnett's test.

Results

Effect of scutellarin on the levels of serum ALT and AST, and liver lesions in Con A-injected mice

As shown in Table 1, the serum ALT and AST levels significantly increased 16 h after the injection of Con A. Pretreatment of mice with scutellarin 50 mg kg^{-1} and 100 mg kg^{-1} reduced the levels of ALT and AST significantly.

The histological examination of the livers from mice of the Con A group revealed widespread necrosis localized in the intermediate zone of the lobule (zone 2 of the liver lobule), along with a moderate infiltration in portal areas by mononuclear and polymorph nuclear cells. Pretreatment with scutellarin markedly reduced all the above liver lesions in Con A hepatitis mice (Table 1, Figure 2).

Effect of scutellarin on serum levels of TNF-α and NO_2^-/NO_3^- in Con A-injected mice

The serum level of TNF-α increased markedly at 2 h after the Con A injection. TNF-α level increased nearly 50-fold compared with the normal group. The serum TNF-α level of mice pretreated with 100 mg kg^{-1} scutellarin reduced by more than 50% in comparison with the Con A control group. The serum NO_2^-/NO_3^- levels also slight increased, but not statistically significantly, at 8 h after the injection of Con A. Prior administration of 100 mg kg^{-1} scutellarin markedly decreased the elevated serum NO_2^-/NO_3^- levels, and even reduced them to lower than those in saline-injected control mice (Table 2). The effect of 50 mg kg^{-1} scutellarin on serum TNF-α and NO_2^-/NO_3^- levels was not significant.

Effect of scutellarin on iNOS, TNF-α, IFN-γ and Fas mRNA in the livers of Con A-injected mice

Two hours after Con A injection, the expression of iNOS and IFN-γ mRNA in the liver increased significantly, while there was no significant alteration of liver Fas and TNF-α mRNA expression (Figure 3, Table 3). Pretreatment with 100 mg kg^{-1} scutellarin markedly down-regulated the expression of iNOS and TNF-α mRNA, but no regulating effect on the Fas and IFN-γ mRNA expression was observed. The 50 mg kg^{-1} scutellarin pretreatment was shown to have no effect the expression of iNOS, TNF-α, IFN-γ or Fas mRNA.

Effect of scutellarin on the expression of c-Fos, c-Jun, iNOS and IκBα protein in the livers of Con A-injected mice

From the visualized protein bands in Figure 4, it may be seen that the level of c-Fos, c-Jun and iNOS protein in the livers of normal group mice were low, whereas the protein bands of c-Fos, c-Jun and iNOS in the liver were more visualized at 2 h after Con A injection. Prior administration of 100 mg kg^{-1} scutellarin reduced the expression of c-Jun and iNOS protein in the liver stimulated by Con A injection, but it had no effect on c-Fos protein expression. The IκBα protein level in the livers of normal mice was higher. After the injection of Con A, the IκBα protein level decreased, indicating the increase of IκBα degradation from NF-κB. The liver IκBα protein in mice treated with 100 mg kg^{-1} scutellarin was near to that of normal mice. In the Western blotting detection, two livers from each group were measured, and every sample was repeated twice. Because there were only two samples per group, the protein bands were not measured quantitatively by densitometry.

Discussion

Concanavalin A is a lectin with high affinity for the hepatic sinus (Mizuhara et al 1994). The injection of Con A into mice induced liver injury. It was proved that TNF-α and IFN-γ are directly involved in hepatocyte damage, because anti-TNF-α and anti-IFN-γ antibodies protected against liver injury in the Con A hepatitis model, and Con A could not induce liver damage in IFN-γ and TNF-α(–/–) mice (Mizuhara et al 1994; Gantner et al 1995; Tagawa et al 1997). Both TNF-α and IFN-γ can activate liver iNOS expression (Taylor et al 1998a). The results clearly indicated that scutellarin protected against immune-mediated liver injury induced by Con A as shown by decreases of all the elevated serum ALT, AST,

Table 1 Effect of scutellarin on the serum ALT and AST levels and liver lesions in Con A-injected mice (determined at 16 h after Con A injection)

Group	No. mice	ALT (U L^{-1})	AST (U L^{-1})	Histological grade of liver injury
Control	10	27.9 ± 12.5***	59.1 ± 12.9***	0.0 ± 0.0***
Con A	13	550.0 ± 77.0	448.0 ± 94.3	2.6 ± 0.5
Scutellarin 50 mg kg^{-1}	13	410.7 ± 185.8*	224.4 ± 110.7**	
Scutellarin 100 mg kg^{-1}	10	402.2 ± 191.1*	229.9 ± 137.5**	1.6 ± 0.8*

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with Con A group

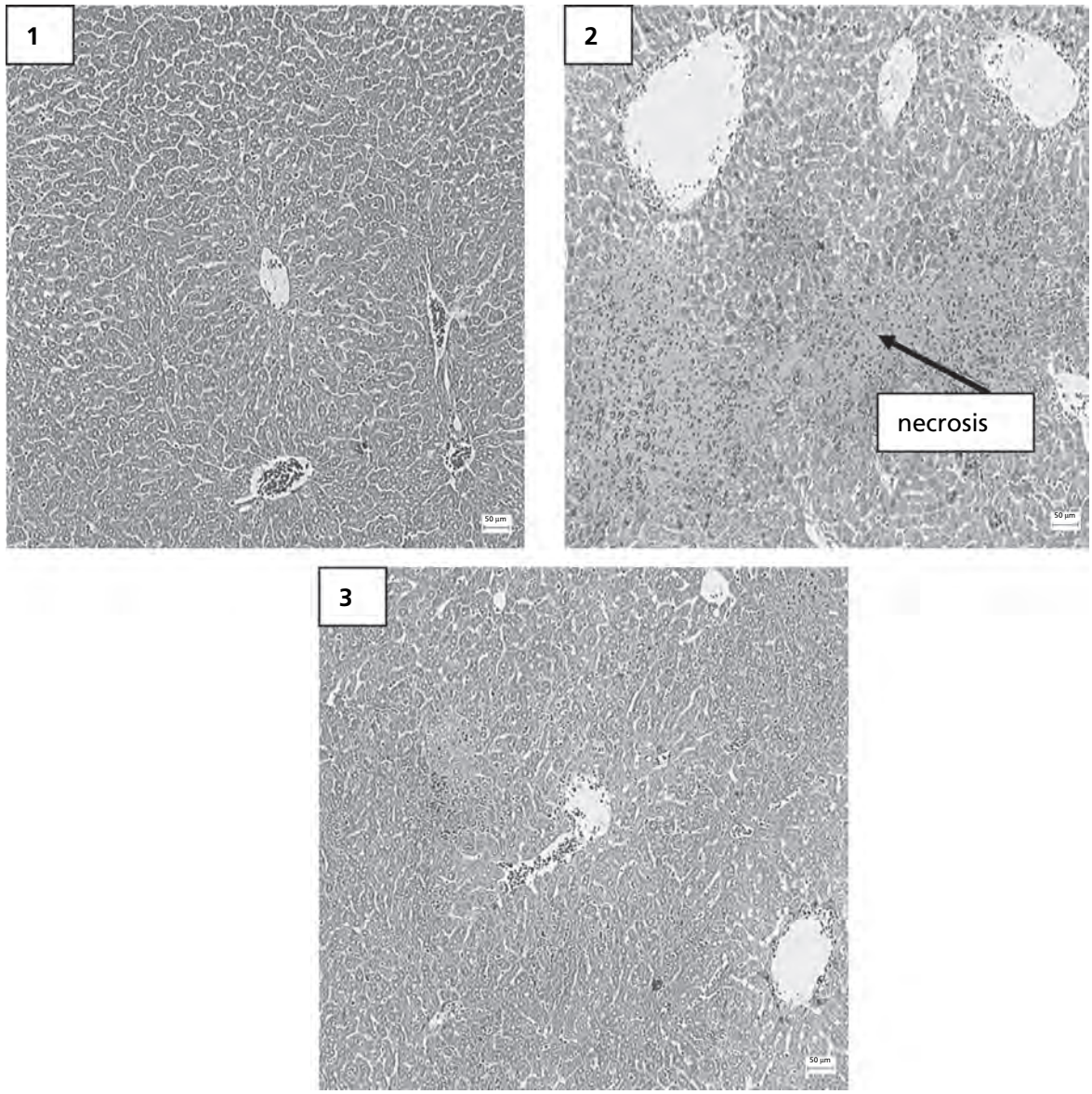

Figure 2 Effect of scutellarin on liver lesions induced by Con A in mice (H.E. staining, magnification 50 ∞). 1, normal mouse; 2, Con A-injected mouse; 3, scutellarin 100 mg kg^{-1} + Con A-treated mouse.

Table 2 Effects of scutellarin on serum TNF-α and NO_2^-/NO_3^- levels in Con A-injected mice

Group	No. mice	TNF-α (pg mL^{-1}) (2 h after injection of Con A)	NO_2^-/NO_3^- (μmol L^{-1}) (8 h after injection of Con A)
Control	6	23.5 ± 17.1***	19.3 ± 7.1
Con A	8	1067.9 ± 477.8	24.8 ± 10.4
Scutellarin 50 mg kg^{-1}	7	1082.6 ± 535.1	21.1 ± 8.8
Scutellarin 100 mg kg^{-1}	8	477.1 ± 157.8*	13.5 ± 8.1*

*$P < 0.05$, ***$P < 0.001$ as compared with Con A group.

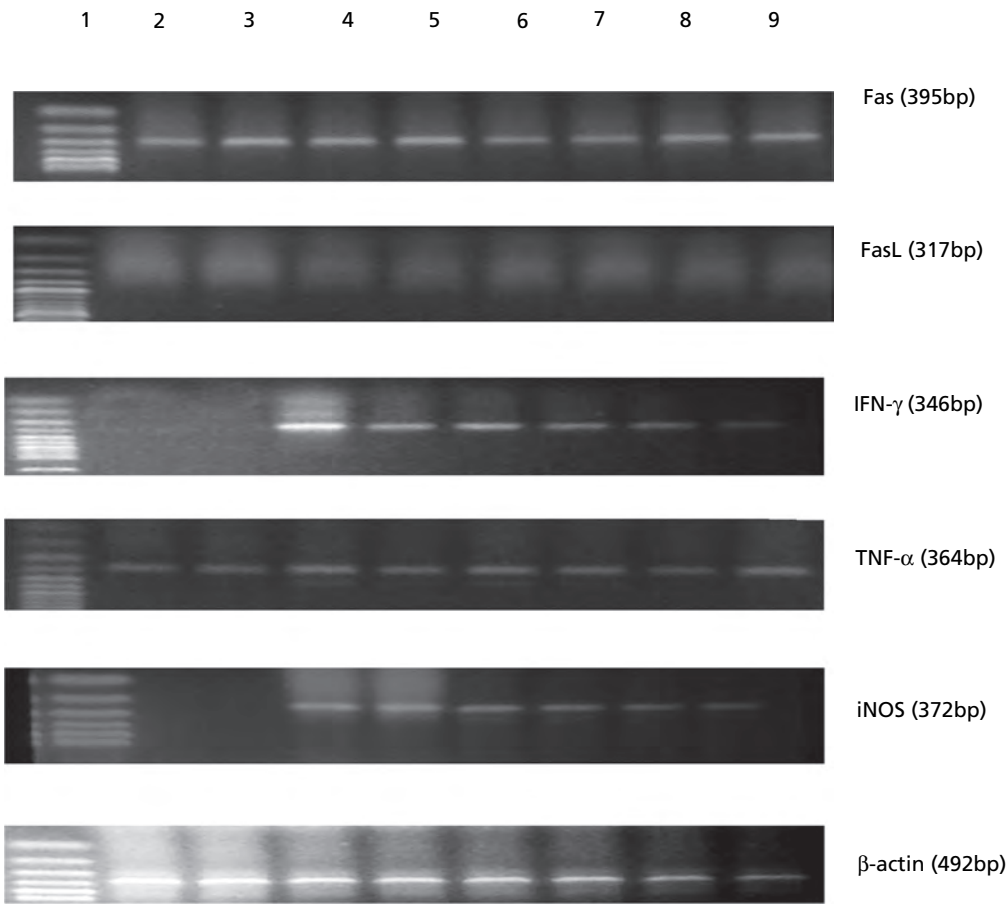

Figure 3 Effect of scutellarin on the expression of TNF-α, INF-γ, iNOS and Fas mRNA in livers of Con A-treated mice detected by RT-PCR. 1, marker; 2, 3 normal; 4, 5, Con A; 6, 7, Con A + scutellarin 50 mg kg^{-1}; 8, 9, Con A + scutellarin 100 mg kg^{-1}.

Table 3 Effects of scutellarin on the expression of TNF-α, INF-γ, iNOS, Fas and FasL mRNA in the liver of Con A-treated mice determined by RT-PCR (at 2 h after Con A injection)

Group	iNOS/β-actin	IFN-γ/β-actin	Fas/β-actin	TNF-α/β-actin
Control	0.02 ± 0.01**	0.34 ± 0.09**	3.60 ± 1.13	2.25 ± 0.79
Con A	2.29 ± 0.18	2.50 ± 0.90	2.82 ± 0.44	3.00 ± 0.79
Scutellarin 50 mg kg^{-1}	1.48 ± 0.42**	1.94 ± 0.83	2.05 ± 0.75	2.03 ± 0.70
Scutellarin 100 mg kg^{-1}	0.95 ± 0.05**	1.85 ± 0.58	1.81 ± 0.53	1.13 ± 0.19**

The data are means of 4 liver determinations. *$P < 0.05$, **$P < 0.01$ as compared with Con A.

TNF-α and NO_2^-/NO_3^- levels and the liver lesions were also ameliorated. The ALT exists in cytosol and AST in mitochondria of hepatocytes. Scutellarin reduced both serum ALT and AST levels in Con A hepatitis mice, indicating that scutellarin protected the mitochondria and plasma membrane of hepatocytes from Con A-induced damage.

To study the mechanism of the protective action of scutellarin against liver injury, besides serum ALT and AST, the pro-inflammatory cytokine TNF-α and NO_2^-/NO_3^- levels in serum, and related iNOS-, TNF-α, IFN-γ and Fas mRNA and their proteins in liver, were also measured. As indicated in this paper, the injection of Con A significantly up-regulated the expression of iNOS mRNA and its protein in the liver, although the serum NO_2^-/NO_3^- level only slightly increased and was not significant as compared with the normal group of mice. The question arose as to why the NO_2^-/NO_3^- level in serum did not parallel the increase of iNOS mRNA expression in the liver. The possible answer might be the time difference of determination of serum NO_2^-/NO_3^- level and liver iNOS mRNA expression; the serum NO_2^-/NO_3^- level was determined 8 h following Con A injection, while iNOS mRNA expression was detected 2 h after Con A injection. Eight hours after Con A injection might be not the best

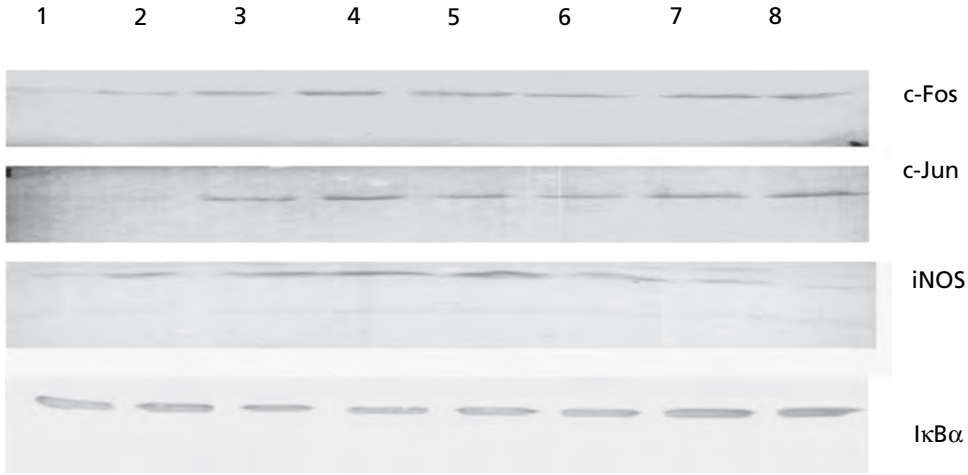

Figure 4 Effect of scutellarin on the expression of c-Jun, c-Fos, iNOS and IκBα protein in livers of Con A-treated mice detected by Western blotting. 1, 2, normal; 3, 4, Con A; 5, 6, Con A + scutellarin 50 mg kg^{-1}; 7, 8, Con A + scutellarin 100 mg kg^{-1}.

time for determination of serum NO_2^-/NO_3^- level. Nevertheless, pretreatment with 100 mg kg^{-1} scutellarin not only markedly decreased the levels of NO_2^-/NO_3^- in serum (and even reduced them to lower than those of normal group mice), but also significantly down-regulated the expression of iNOS mRNA and protein in the liver. So, we speculated that NO plays a major role in the protection against Con A hepatitis by scutellarin. This speculation is also based on evidence from many other papers. Various cell types, including macrophages and Kupffer cells, are able to generate NO from L-arginine catalysed by nitric oxide synthase (NOS). NO interacts with superoxide to produce peroxynitrite ($ONOO^-$) in-vivo. The peroxynitrite reacts with free or protein-bound tyrosine residues to form nitrated tyrosine, which correlates with cell necrosis. At least three different isoforms of NOS are known. Only inducible nitric oxide synthase (iNOS) can be induced by cytokines (e.g. TNF-α and IFN-γ) (Droge 2002). It was reported that iNOS expression has hepatotoxic effects in haemorrhagic shock, ischaemia/reperfusion injury, endotoxaemia and D-galactosamine plus LPS poisoning (Thiemermann et al 1995; Isobe et al 1999; Menezes et al 1999). Pretreatment of wild-type mice with a potent and specific inhibitor of iNOS significantly reduced transaminase levels after Con A injection, while Con A could not induce liver damage in iNOS (–/–) mice (Sass et al 2001).

The central role of TNF-α in the Con A hepatitis model was confirmed in various studies using either TNF-α or TNF-α receptor knockout mice or soluble TNF-α receptor fusion proteins (Küsters et al 1997; Ksontini et al 1998). The results of this paper indicated that the injection of Con A induced a 50-fold elevation of serum TNF-α level as compared with the normal group of mice, although there was no significant up-regulation of TNF-α mRNA in the liver. Scutellarin significantly lowered the elevated serum TNF-α level and liver TNF-α mRNA in Con A hepatitis mice. Combining the results of iNOS mRNA and TNF-α mRNA expressions in the liver, it appears that both iNOS mRNA and TNF-α mRNA are the main sites of anti-liver injury action of scutellarin in Con A-induced hepatitis in mice.

The inflammatory response of cells induced by TNF-α is mediated, in part, though the regulation of gene expression by the apoprotein (AP-1) and NF-κB groups of transcription factors (Li et al 2002). AP-1 is a complex composed of proteins of the Fos (c-Fos, FosB, Fra-1 and Fra-2) and Jun (c-Jun, JunB and JunD) proto-oncogene families. Fos and Jun family proteins function as dimeric transcription factors that bind to AP-1 regulatory elements in the promoter and enhancer regions of genes (Chinenov & Kerppola 2001). NF-κB plays a pivotal role in communicating the signals from cytokines, toxins and various stressors to regulation of gene expression. NF-κB is a family of dimeric transcription factors involved in immune and inflammatory responses and cell apoptosis. In unstimulated cells, the IκB proteins localize in NF-κB dimers in the cytoplasm by masking the nuclear localization sequence of NF-κB. Various stimulations, including cytokine stimulation and oncogenic signals, can activate NF-κB. Once IκB is degraded, the nuclear localization sequence of NF-κB is unmasked, allowing nuclear accumulation, DNA binding and transcriptional activation of target genes (Ghosh & Karin 2002). TNF-α-mediated induction of JNK involves activation of AP-1. JNK phosphorylates the activation domains of the transcription factors c-Jun, ATF-2 and Elk-1. c-Jun and ATF-2 heterodimers induce transcription of the c-jun gene, whereas Elk-1 transactivates c-fos (Ventura et al 2003). Stimulation of iNOS expression by TNF-α involves activation and nuclear translocation of NF-κB, where it interacts with κB sequences in the promoter of the iNOS gene (Xie & Nathan 1994). The iNOS gene is predominantly regulated at the level of transcription via several putative NF-κB response elements. However, this gene also contains the transcription factor binding sites for the AP-1 (Taylor et al 1998b; Spitsin et al 1997). Apparently, NF-κB, iNOS and TNF-α genes are a network, and they interact. As shown in Table 3 and Figure 4 of this paper, the injection of Con A enhanced the expression of c-Jun and iNOS protein, and decreased the IκBα protein in the

mouse liver. Prior administration of scutellarin reduced the expression of c-Jun protein, and the degradation of IκBα from NF-κB, expressed as the increase of IκBα protein in the livers of Con A-injected mice. There is a possibility that scutellarin down-regulates the expression of c-Jun and enhances the dissociation of IκBα by reduction of AP-1 expression and NF-κB activation, although the AP-1 expression in liver was not directly determined in this study.

Finally, it should be pointed out that the TNF-α and Fas mRNA expressions in the liver appeared to be not significantly up-regulated following Con A injection. However, we still examined the effects of scutellarin pretreatment on those parameters. In the original design of this study, theoretically, the TNF-α and Fas mRNA expressions should be enhanced after Con A injection in mice. As a result, Con A injection did not up-regulate either biomarker's expression, and scutellarin showed no effect on the up-regulated IFN-γ expression. A possible explanation for this is that the sensitivity of IFN-γ and Fas mRNA to the action of Con A and scutellarin is not the same, like iNOS and TNF-α. In past years, we have performed many studies on the effect of natural and synthetic compounds on liver injury induced by various hepatotoxins, and we always met the same problem, as with scutellarin in this study: the responses of various biomarkers to a drug were not the same (Li & Liu 2004). The unparalleled responses of different biomarkers to a drug might be dependent on the specific target of the drug action.

Conclusions

Scutellarin is a natural compound from a Chinese herb, and has been used as a drug in the treatment of cardio-cerebral diseases for more than twenty years. This study first demonstrated that scutellarin has a protective action against Con A-induced immunological liver injury in mice. The mechanism of its action may be related to the inhibition of the NF-κB–TNF-α–iNOS transduction pathway.

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