Effects of scutellarin on liver function after brain ischemia/reperfusion in rats

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ABSTRACT

AIM: To investigate the effects of scutellarin (Scu) on liver function after brain ischemia/reperfusion in Wistar rats. **METHODS:** Rats were pretreated with Scu for 7 d and then subjected to a brain ischemia/reperfusion injury induced by a middle cerebral artery occlusion. The levels of nitric oxide (NO), xanthine oxidase (XOD), alanine transaminase (ALT), and aspartate aminotransferase (AST) in serum or liver tissues and the activities of antioxidant enzymes and cytochrome P450-dependent monooxygenases (CYPs) in liver tissues after brain ischemia/reperfusion were determined. **RESULTS:** In vehicle-treated rats, XOD, ALT, and AST activities ($P<0.01$) in serum and the MDA level ($P<0.05$) in liver tissues were all elevated but were significantly reduced ($P<0.05$) by Scu pretreatment. The NO levels in serum and liver tissues were decreased ($P<0.01$) dramatically in vehicle-treated rats and returned to the levels in the sham-operated animals when pretreated with Scu. SOD ($P<0.05$) and GSH-PX ($P<0.01$) activities in cytosol fraction were increased significantly by Scu pretreatment. Furthermore, a loss of CYP3A activity ($P<0.01$), but no changes of CYP1A1, CYP1A2, and CYP2E1 activities in liver were observed after brain ischemia/reperfusion in rats. Scu had no effect on them. **CONCLUSION:** These results demonstrated that pretreatment with Scu could attenuate hepatocellular damage elicited by brain ischemia/reperfusion in rats and this protection is in major part by its antioxidant activity.

INTRODUCTION

A devastating consequence of tissue reperfusion is the development of damage in organs uninvolved in the initial ischemic insult. Multiple organ dysfunction syndrome is a leading cause of death in critically ill patients[1] and is a documented consequence of gut[2-5], liver[6-8], burn injury[9] and skeletal muscle[10]. However,

there is no report on liver damage after brain ischemia/reperfusion (I/R). Most attention has been focused on the role of reactive oxygen species (ROS)[1], xanthine oxidase (XOD)[2,3,7,8] and nitric oxide (NO)[1,4,6] initiating the distant organ injury after ischemia/reperfusion (I/R). The activities of cytochrome P-450-dependent monooxygenases(CYPs) in rat brain and liver were also affected by the injury of I/R[11-13], especially CYP3A and CYP2E1 activities.

Scutellarin (Scu) with M_r 462.21 is one of the flavonoids isolated from the traditional Chinese medicine *Erigeron breviscapus*. *Erigeron breviscapus* has been used in clinic to treat cerebral vascular patients for many

years, but its therapeutic mechanism is not known very well[14]. It has been reported that Scu had neuroprotective effects on rat brain I/R[15].

This study was to investigate the effects of Scu on liver function after brain I/R in Wistar rats. For this purpose, the levels of NO, XOD, alanine transaminase (ALT), and aspartate aminotransferase (AST) in serum or liver tissues and the activities of antioxidant enzymes and CYPs in liver tissues after brain I/R were determined.

MATERIALS AND METHODS

Chemicals β-Nicotinamide adenine dinucleotide phosphate sodium salt (NADP+), glucose 6-phosphate (G6P), β-nicotinamide adenine dinucleotide phosphate (reduced form) tetrasodium salt (NADPH), glucose-6-phosphate dehydrogenase (G6PDH), dimethyl sulfoxide, methoxyresorufin, ethoxyresorufin, and resorufin were purchased from Sigma Chemical Co (St Louis MO, USA). All other chemicals and solvents were of highest purity analytical grade and obtained from Hubei Province Chemicals Co Ltd (Wuhan, China).

Scu (purity >96 %) was supplied by Yuxi Pharmaceutical Co Ltd (Kunming, China).

Biochemical assay kits NO, ALT, AST, XOD, MDA, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-PX) assay kits were all purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animal treatment and administration of Scu Male Wistar rats (Grade II, Certificate No 19-050) weighing 180-220 g were obtained from the Experimental Animal Center of Tongji Medical College, which were housed at a constant room temperature of 22 ºC under a 12 h light-dark cycle. The animals were allowed free access to food and drinking water. Animals were treated with vehicle (0.9 % NaCl) or Scu (50 mg/kg or 75 mg/kg) dissolved in 0.9 % NaCl by gavage for 7 d preceding ischemia or sham (sham-operated). Four treatment groups were studied: (a) sham-operated (sham control), (b) vehicle-treated I/R (I/R injury control, model group), (c) Scu (50 mg/kg)-treated I/R, (d) Scu (75 mg/kg)-treated I/R.

Brain ischemia/reperfusion (I/R) procedure The brain I/R injury was induced by a middle cerebral artery occlusion[16] under 10 % chloral hydrate anesthesia (350 mg/kg, ip). The basic surgical procedure consisted of blocking blood flow into the middle cerebral artery (MCA) with an intraluminal suture introduced

through the extracranial internal carotid artery (ICA). Additional exacranial vessels, including the left ICA, and right external carotid artery (ECA) were occluded to reduce collateral blood flow to the MCA territory. The body temperature was maintained at 37 ºC during the surgery with an infrared heat lamp and a heating pad. Sham-operated animals (sham), not exposed to ischemic insult, served as controls. At the end of 120 min of ischemia, withdrew the nylon suture and allowed the MCA ischemic territory to perfuse, after 180 min, the animals were sacrificed by cervical dislocation. The neurologic findings were scored on a five-point scale[17]: a score of 0 indicated no neurologic deficit, a scroe of 1 (failure to extend left forepaw fully) a mild focal neurologic deficit, a score of 2 (circling to the left) a moderate focal neurologic deficit, and a score of 3 (failing to the left) a severe focal deficit; rats with a score of 4 did not walk spontaneously and had a depressed level of consciousness.

Preparation of rat liver subcellular fractions The subcellular fractions of rat livers were separated by a standard differential centrifugation procedure[12,18]. Blood samples were obtained from rat carotid artery for the determination of NO, ALT, AST, XOD. Then rats were killed by cervical dislocation, the entire livers were perfused immediately with chilled 0.9 % NaCl solution and removed, blotted, weighed, minced and homogenized in ice-cold 0.05 mol/L Tris buffer (pH 7.4), containing 0.15 mol/L KCl to yield a 10 % (w/v) homogenate, which was then centrifuged at 4 ºC, $1000 \times g$ for 15 min, a sample of this supernatant was used for MDA and XOD, while the remainder continued to be centrifuged at 4 ºC, $12\,500 \times g$ (Beckman, AvantiTmJ-25 centrifuge, USA) for 15 min. The resultant supernatant was centrifuged at 4 ºC, $105\,000 \times g$ (Beckman, OptimaTmXL-100K Ultracentrifuge, USA) for 60 min. The supernatant (cytosol fraction), after discarding any floating lipid layer, was used for NO, XOD, and cytosolic antioxidant enzymes: SOD, CAT and GSH-PX. The pellet representing microsome was used for assaying CYPs activities. These liver subcellular fractions were all suspended in 0.05 mol/L Tris-0.15 mol/L KCl buffer (pH 7.4) and immediately frozen in liquid nitrogen, stored at -80 ºC until required.

Assays of aminopyrine *N*-demethylase (APND) and aniline hydroxylase (ANHD) activities Aminopyrine *N*-demethylase activity was determined by estimation of formaldehyde production by use of Nash reagent[19]. Aniline hydroxylase activity was measured fol-

lowing the formation of the *p*-amiophenol from aniline, according to the method of Lee[12].

Measurements of methoxyresorufin *O*-demethylase (MROD) and ethoxyresorufin *O*-deethylase (EROD) activities Reaction was carried out in fluorimeter cuvettes at 25 ºC using a spectro-fluorophotometer (Shimadzu, Japan). The reaction mixture, containing microsomal protein (between 0.2 mg and 1.0 mg), 5 μmol/L substrate (10 μL of a 1 mmol/L solution in dimethyl sulfoxide) and 0.05 mol/L Tris-HCl buffer (pH 7.4), was equilibrated for 1 minute at 25 ºC. The reaction was then started by the addition of 250 μmol/L NADPH (20 μL of a 25 mmol/L solution). The final reaction volume was 2 mL. The fluorimeter settings were: excitation and emission slits, 5 nm; excitation and emission wavelengths, 530 and 585 nm, respectively. After a suitable period of linear reaction, the extent of fluorescence increase was calibrated by comparing the rate of increase in relative fluorescence to the fluorescence of known amounts of resorufin[20].

Assay of nitrite and nitrate The amount of NO in serum and liver tissues was determined by using an NO assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. Briefly, the method involved measuring the amount of NO metabolites (nitrite and nitrate), which were more stable than NO. Nitrate in the tissues and serum was reduced first to nitrite by the action of nitrate reductase. Then the reaction was initiated by the addition of Griess reagent, and absorbance of the mixture at 550 nm was determined[21].

Other biochemical index assay The MDA levels were evaluated in order to estimate the extent of lipid peroxidation in the liver tissue after brain I/R. MDA, ALT, AST levels in serum, XOD activities in serum and liver tissues, SOD, CAT, and GSH-PX activities in liver

cytosol fraction were assayed by using commercially available kits according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The protein content was estimated by the dye binding assay of Bradford[22], with Bovine serum albumin used as a standard.

Statistics Data were expressed as mean±SD and analyzed with Microsoft Excel 2002. Statistical analyses were performed by Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Alterations of the liver/body ratio and neurologic score after Scu treatment in rats Compared with sham-operated animals, the liver weight and liver/body weight ratio were increased significantly in the animals with Scu treatment (Tab 1). The rats in vehicle-treated and Scu (50 mg/kg) groups showed a significant increase of neurologic score as compared with the rats in sham-operated group, but no change in Scu (75 mg/kg) group; treatment with Scu decreased neurologic score significantly as compared with vehicle-treated control (Tab 1).

Changes of XOD, NO, ALT, and AST levels in serum after Scu treatment in rats An elelation of ALT and AST activities in serum was shown in the animals treated with vehicle (Tab 2). When treated with Scu, ALT, and AST activities were decreased significantly, returning to the levels in sham-operated animals (Tab 2). Compared with sham-operated animals, XOD activities in serum in vehicle-treated rats were increased significantly, however, the total nitrite and nitrate levels (NO_2^-+NO_3^- levels) were decreased significantly (Tab 2). Compared with vehicle-treated animals, the total nitrite and nitrate levels in serum were increased significantly

Tab 1. Effects of scutellarin (Scu) on rat body weight, liver weight, and neurologic score after brain I/R. *n*=6. Mean±SD. Sham: sham-operated (not I/R control); Model: vehicle-treated (I/R control). [b]*P*<0.05, [c]*P*<0.01 *vs* Sham. [f]*P*<0.01 *vs* Model.

Groups	Body weight/g	Liver weight/g	Liver/body ratio (wt/wt) %	Neurologic score Total	Average
Sham	195±5	5.9±0.4	3.02±0.25	1 (0,0,1,0,0,0)	0.2±0.4
Model	194±8	6.4±0.6	3.27±0.28	18 (4,3,2,2,4,3)	3.0±0.9[c]
Scu (50 mg/kg)	194±7	6.7±0.3[b]	3.44±0.24[b]	8 (1,1,1,2,2,1)	1.3±0.5[cf]
Scu (75 mg/kg)	190±7	6.5±0.4[b]	3.41±0.15[b]	5 (1,1,0,0,1,2)	0.8±0.8[f]

Tab 2. Effects of scutellarin (Scu) on XOD, NO, ALT, and AST levels in rat serum after brain I/R. *n*=5-6. Mean±SD. Sham: sham-operated (not I/R control); Model: vehicle-treated (I/R control). $^c P<0.01$ *vs* Sham. $^e P<0.05$, $^f P<0.01$ *vs* Model. $^h P<0.05$ *vs* Scu (50 mg/kg).

Groups	XOD (U/L)	NO (μmol/L)	ALT (U/L)	AST (U/L)
Sham	43.7±2.3	240±85	25±2	158±18
Model	54±5^c	90±21^c	46±7^c	319±61^c
Scu (50 mg/kg)	49.4±1.5^c	147±51^e	31±7^f	197±57^f
Scu (75 mg/kg)	41±8^{fh}	171±28^f	30±6^f	169±53^f

Tab 3. Effects of scutellarin (Scu) on MDA (nmol·mg^{-1} protein), NO (nmol·mg^{-1} protein), and XOD (U·g^{-1} protein) levels in rat liver tissues after brain I/R. n=5-6. Mean±SD. Sham: sham-operated (not I/R control); Model: vehicle-treated (I/R control). $^b P<0.05$, $^c P<0.01$ *vs* Sham. $^e P<0.05$ *vs* Model.

Groups	MDA$^{1)}$	NO$^{2)}$	XOD$^{1)}$	XOD$^{2)}$
Sham	1.98±0.09	2.1±0.3	25.0±1.6	20.1±2.5
Model	2.27±0.17^b	1.43±0.17^c	25.6±0.6	19.5±1.2
Scu (50 mg/kg)	1.99±0.05^e	1.56±0.06^c	24.3±1.4	19.9±1.3
Scu (75 mg/kg)	1.96±0.25^e	1.70±0.20^e	24.8±1.2	19.8±2.5

$^{1)}$ post-1000×*g* supernatant. $^{2)}$ cytosol fraction.

in the animals pretreated with Scu (Tab 2). A significant reduction of the XOD activities in serum was observed when pretreated with Scu (75 mg/kg) in rats (Tab 2).

Effects of Scu on MDA and NO levels, XOD activities in rat liver tissues An increase of MDA levels in liver tissues was shown in animals treated with vehicle as compared with sham-operated animals, however, the total nitrite and nitrate levels were decreased significantly (Tab 3). Compared with vehicle-treated animals, the MDA levels in liver tissues were decreased significantly and the total nitrite and nitrate levels were increased significantly in the animals pretreated with Scu (Tab 3). But in liver tissues, compared with sham-operated animals, the XOD activities had no changes in vehicle-treated and Scu-treated animals (Tab 3).

Effects of EB on CAT, SOD, and GSH-PX activities in rat liver cytosol fraction Compared with vehicle-treated animals, GSH-PX activities were increased significantly in Scu-treated animals and a significant increase of the SOD activity was also observed when pretreated with Scu (75 mg/kg) in rats (Tab 4). No change of the CAT activity was observed in Scu-treated groups as compared with sham-operated and

vehicle-treated groups.

Effects of Scu on various CYP-dependent mono-oxygenases in rat liver microsomes Compared with sham-operated animals, the MROD (CYP1A2), EROD (CYP1A1) and ANHD (CYP2E1) activities did not show any change in vehicle-treated and Scu-treated animals, but the APND (CYP3A) activities were decreased significantly. Compared with vehicle-treated animals, the MROD, EROD, APND, and ANHD activities did not show any change in Scu-treated animals (Tab 5).

DISCUSSION

It has been reported that Scu had neuroprotective effects on rat brain I/R by elevating the activities of SOD, GSH-PX and CAT[15] in rat brain. In the present work we studied the effects of Scu on liver function after brain I/R in rats.

Membrane lipid peroxidation is induced by oxidative stress (such as I/R injury) and will cause changes in structure of the membrane, which usually result in a more rigid membrane that will cause changes in the

Tab 4. Effects of scutellarin (Scu) on CAT, SOD, and GSH-PX activities in rat liver cytosol fraction after brain I/R. *n*=5-6. Mean±SD. Sham: sham-operated (not I/R control); Model: vehicle-treated (I/R control). $^e P<0.05$, $^f P<0.01$ *vs* Model.

Group/enzymes	Sham	Model	Scu 50 mg·kg^{-1}	Scu 75 mg·kg^{-1}
CAT (U·g^{-1} protein)	72±11	70±12	72±10	71±14
SOD (NU·mg^{-1} protein)	1187±56	1164±88	1206±21	1285±44^e
GSH-PX (nmol·mg^{-1} protein·min^{-1})	35±4	30±5	38.3±2.9^f	37.9±2.2^f

Tab 5. Effects of scutellarin (Scu) on various CYP-dependent monooxygenases in rat liver microsomes after brain I/R. *n*=5-6. Mean±SD. ^cP<0.01 *vs* sham.

Group/enzymes	Sham	Model	Scu 50 mg·kg⁻¹	Scu 75 mg·kg⁻¹
Methoxyresorufin O-demethylase[1)	31±14	31±4	33±5	28±7
Ethoxyresorufin O-demethylase[1)	21±9	17±3	17.4±1.7	15±6
Aminopyrine N-demethylase[2)	24±3	11±5[c]	13.8±1.1[c]	12±4[c]
Aniline hydroxylase[2)	11±5	9±5	14.4±0.8	14±4

Sham: sham-operated (not I/R control); Model: vehicle-treated (I/R control). [1)]pmol·mg⁻¹ protein·min⁻¹; [2)]nmol·mg⁻¹ protein 30 min⁻¹.

activity of essential membrane proteins such as antioxidant enzymes and CYPs[11-13,23,24]. Malondialdehyde (MDA), a product of lipid peroxidation, has been shown to react with critical biomolecules such as nucleic acids, thus damaging the cells[23,24]. Our results showed that ALT and AST activities in serum and MDA levels in liver tissue were elevated significantly when treated with vehicle after brain I/R in rats, which indicated that the liver function was damaged. When rats were treated with EB, the elevated serum ALT and AST activities and MDA levels in liver tissues were decreased significantly, returning to the sham-operated animals levels (activities). The activities of GSH-PX and SOD in cytosol fraction were increased significantly in Scu-treated animals. These findings suggested pre-treatment with Scu could attenuate the brain I/R-induced liver damage in rats by increasing the antioxidant enzymes activities. Similar results were found in other I/R models[5,9].

Although a variety of mechanisms have been proposed to explain remote organ injury induced by a local organ I/R[1], a xanthine oxidase-mediated injury has received the most attention[1-3,7,8]. Xanthine oxidase is capable of reducing molecular oxygen to both superoxide and hydrogen peroxide, it has been invoked to be the major source of reactive oxygen metabolites generated during I/R[2,3,7,8,23,24]. Plasma xanthine oxidase activity was increased dramatically after liver or gut I/R[2,3,7,8]. This increase was associated with remote pulmonary, hepatic and myocardial injury[2,3,7,8], when treated with XOD inhibitors or antisera to XOD, the remote organ damage was attenuated significantly[2,3,7,8], which suggested that XOD or its products might contribute to I/R-induced distant organ injury[2,3,7,8]. But no change of XOD activity was observed in the tissues[2,3,7,8]. Our experiments in vehicle-treated animals demonstrated that XOD activity in serum was increased significantly but

there was no changes in liver tissues. When pre-treated with Scu, the elevated XOD activity was reduced and returned to the levels in the sham-operated animals. These findings indicated that the protective effects of Scu on liver injury elicited by brain I/R might be due to reduction of the elevated circulating XOD activity.

Depletion and/or inactivation of nitric oxide have also been implicated as a contributing factor in the pathogenesis of I/R-induced remote organ injury[1,4,6]. Liu *et al* reported[6] when the NO level was decreased by administering NO inhibitor to rats subjected to hepatic I/R, the liver injury and the lung injury induced by hepatic I/R were exacerbated. Horie Y *et al* also showed[4] a similar result. Our data showed that the NO levels in serum and liver tissues were reduced dramatically in vehicle-treated animals, but were increased and returned to the sham-operated animals levels by pre-treatment with Scu. This may be another mechanism that Scu protects liver from injury evoked by brain I/R.

Several models of injury demonstrated tissue-specific alterations in cytochrome P450 activity. A loss of liver CYP3A activity and an increase of CYP2E1 activity were observed in hepatic I/R of rats[12]. Brain CYP2E1 activity in rats was induced by a brain ischemia injury[13]. Furthermore, rat liver CYP2E1 activity was reduced significantly by a traumatic brain injury[25] and liver CYP1A1 and CYP2E1 activities were all down-regulated during lipopolysaccharide-evoked localized inflammation in rat brain[26]. Liver EROD (CYP1A1), MROD (CYP1A2), and ANHD (CYP2E1) activities did not show any change, only APND (CYP3A) was decreased dramatically after brain I/R in rats in our experiment. No significant alterations of these enzymes were observed following brain I/R with Scu pre-treatment animals. Hepatocytes are involved in the metabolism and activation of drugs, toxins, or endogenous compounds by the cytochrome P450 superfamily[27,28]. The

cytochrome P450 3A sub-family (CYP3A) is the major one expressing in the liver and is predominantly implicated in the metabolism of a vast variety of drugs[27,28]. This might help us to choose suitable doses of drugs metabolized by CYP3A to the patients with a brain I/R-induced disease and avoiding drug-drug interactions or drug side effects.

In summary, our data confirmed that the liver function was damaged after brain I/R in rats. Moreover, our finding also demonstrated that Scu, one of the flavonoids isolated from Herba erigeron, could attenuate hepatocellular damage induced by brain I/R in rats and that this protection was, in major part, caused by elevating the activities of antioxidant enzymes, decreasing XOD activity in serum while increasing NO levels in serum and liver tissues, and thus decreasing lipid peroxidation. Furthermore, the modulation of CYP3A activity in liver by brain I/R may result in altered metabolism of coadministered drugs with important implications for their disposition. However, this is only the first time to study the remote organ injury elicited by brain I/R. Further study needs to be carried out in this field.

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