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Protective effects of scutellarin on superoxide-induced oxidative stress in rat cortical synaptosomes[1]

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KEY WORDS scutellarin; synaptosomes; malondialdehyde; membrane fluidity; calcium; Na^+-K^+-exchanging ATPase

ABSTRACT

AIM: To evaluate the effects of scutellarin on superoxide-induced oxidative stress in rat cortical synaptosomes. **METHODS:** Oxidative damage model was established by incubation with xanthine (0.3 mmol/L) and xanthine oxidase (0.02U) at 37 ºC for 30 min. The extent of membrane oxidation was assessed by malondialdehyde (MDA). Membrane fluidity was measured by fluorescence anisotropy (polarization) of the lipophilic probe 1,6-diphenyl-1,3, 5-hexatriene (DPH). Intracellular Ca^{2+} concentration($[Ca^{2+}]_i$) was measured by fluorescent spectrophotometry. Fura 2-AM was used as an indicator for $[Ca^{2+}]_i$. Na^+/K^+-ATPase activity assay was based on the amount of inorganic phosphate (Pi) released during an enzymatic hydrolysis of ATP. **RESULTS:** Synaptosomes exposed to superoxide significantly elevated the levels of malondialdehyde (MDA) and $[Ca^{2+}]_i$ compared with those in normal group. These changes were accompanied by the decrease in membrane fluidity and Na^+/K^+-ATPase activity. Pretreatment with scutellarin (25-100 μmol/L) significantly ameliorated the oxidative damage of synaptosomes by reducing MDA levels and $[Ca^{2+}]_i$, up-regulating membrane fluidity and restoring Na^+/K^+-ATPase activity. **CONCLUSION:** Scutellarin exerts a potent protective effect against oxidative damage in synaptosomes induced by superoxide.

INTRODUCTION

Scutellarin (Scu) is an active extract of traditional Chinese medicine *Erigeron breviscapus* (Vant). In recent years, many studies have provided evidence for the neuroprotective effects of *Erigeron breviscapus*[1,2]. It has also been reported possessing anticoagulation effect[3]. Its preparations were used in clinic to treat cerebral insufficiency and peripheral circulatory problems. The results of our previous studies showed that Scu was an effective radical (including hydroxyl radicals, superoxide anion radicals, and hydrogen peroxide) scavenger *in vitro*[4]. Oxidative stress is believed to be one of the major causes of nerve cell death in many neurodegenerative diseases (eg, Alzheimer's disease, Parkinson's disease, and cerebral ischemia)[5]. Some natural flavonoids were shown a protective effect on stress-induced injury[6,7]. In order to investigate the relationship of neuroprotective effect of Scu with its antioxidant activity, we determined whether Scu could help protect the rat cortical synaptosomes from oxidative stress induced by superoxide. Oxidative stress was indicated by lipid peroxidation (thiobarbituric acid-re-

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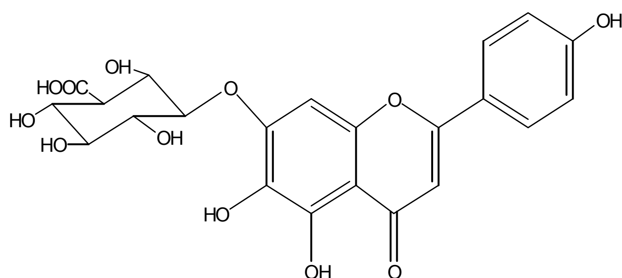

Scutellarin

active substances, TBARS), membrane fluidity, $[Ca^{2+}]_i$ and Na^+/K^+-ATPase activity.

MATERIALS AND METHODS

Chemicals Scu was provided by Yuxi Pharmaceutical Co Ltd (purity >96 %, HPLC). Fura-2 acetoxymethyl ester (Fura 2-AM), 1,6-diphenyl-1,3,5-hexatriene (DPH), HEPES, and ouabain, were purchased from Sigma (St Louis, MO, USA). All other reagents and solvents used in experiments were of analytical grade.

Synaptosome preparation Synaptosomes were prepared as previously described by Campbell[8], with minor modifications. Adult male and female Sprague-Dawley rats (150-200 g, Grade II, Certificate No 190-084) were killed by cervical dislocation and their brains were rapidly removed. The cortex was dissected immediately on ice from cerebellum, cord matter and other structures. Each cerebral cortical tissue was homogenized in 10 vol of cold sucrose 0.32 mol/L containing HEPES 10 mmol/L (pH 7.4), and subsequently centrifuged at 3000×*g*, 4 ºC for 10 min. The resulting supernatant was centrifuged at 15 000×*g*, 4 ºC for 20 min after which the supernatant was discarded and the final pellet was resuspended in an ice-cold physiological Ca^{2+}-free salt solution (PSS, NaCl 133 mmol/L, KCl 4.8 mmol/L , HEPES 10 mmol/L, Na_2HPO_4 1.2 mmol/L, $MgSO_4$ 1.2 mmol/L, glucose 10 mmol/L; pH=7.4).

Determination of membrane fluidity The quantitative measurement of membrane fluidity employed the fluorescence polarization technique[9] with minor modifications. DPH was used as a fluorescence probe. Membrane preparations (50 μg protein) were suspended in Tris-HCl buffer 50 mmol/L (pH 7.4), mixed with DPH prepared from a stock solution of DPH 5 mmol/L solubilized in tetrahydrofurans, and incubated at 37 ºC for 30 min. Fluorescence polarization was determined using a Shimadzu-RF 540 fluorescence spectrophotometer equipped with rotating polarizing filters (Shimadzu Co, Kyoto, Japan). The excitation and emission wavelengths set at 362 nm and 430 nm, respectively. The temperature of the sample was maintained at 25 ºC. Anisotropy (*r*) was calculated using the following equation[10]:

$$r=(I_{VV} - GI_{VH})/(I_{VV}+2GI_{VH})$$

Where, I_{VV} and I_{VH} represent the intensity of vertically and horizontally polarized fluorescence light when excitation light is vertically polarized. The correction factor $G=I_{HV}/I_{HH}$. I_{HV} and I_{HH} represent the intensity of vertically and horizontally polarized fluorescence light when excitation light is horizontally polarized.

Measurement of $[Ca^{2+}]_i$ Synaptosomes were pretreated with Scu for 30 min. After various treatments, synaptosomes were loaded with 5 mmol/L (final concentrations) Fura 2-AM, 0.1 % dimethyl sulfoxide, and 1 % BSA at 37 ºC for 30 min. The resultant was washed two times with Krebs-Ringer buffer and centrifuged at 15 000×*g* for 5 min. The final pellet was resuspended in 3 mL ice-cold Krebs-Ringer buffer, pH 7.4. Fluorescence measurements were carried out with a Shimadzu-RF 540 spectrofluorophotometer. Fura 2-AM loaded synaptosomes were exposed sequentially to an excitation wavelength of 340 and 380 nm (bandwidth 10 nm) and the emission signal was monitored at a wavelength of 500 nm (bandwidth 10 nm). The intracellular free calcium concentration $[Ca^{2+}]_i$ was calculated according to Gryenkiewicz[11]. Fluorescence ratios were converted into calcium concentrations by using the equation: $[Ca^{2+}]_i=K_d\ (R-R_{min})/(R_{max}-R)\ B$.

Na^+/K^+-ATPase activity assay Na^+/K^+-ATPase activity of the synaptosomes was determined by spectrophotometric measurement of inorganic phosphate liberated as described previously[12]. The reaction was carried out at 37 ºC for 10 min in 200 μL of medium containing NaCl 100 mmol/L, KCl 20 mmol/L, $MgCl_2$ 5 mmol/L, ATP 3 mmol/L and Tris buffer 50 mmol/L and synaptosomes protein 20 μg , pH 7.4 in the presence and absence of ouabain 1 mmol/L . The liberated inorganic phosphate was estimated spectrophotometrically, enzyme activity was determined from the inorganic phosphate content by the method[13].

Measurement of lipid peroxidation The concentration of free MDA was assessed using a lipid peroxidation assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to manufacturer's instructions. This assay is based on the reaction of MDA with thiobarbituric acid (TBA), forming a stable

thiobarbituric acid-reactive substances (TBARS), which absorbs at 532 nm. Lipid peroxidation activity was expressed as nmol of MDA per mg protein.

Statistical analysis Data were expressed as mean±SD. Statistical analysis of each set of data was carried out using Student's unpaired *t*-test, $P<0.05$ was considered statistically significant.

RESULTS

Effects of Scu on the contents of MDA After incubation with xathine (0.3 mmol/L) and xanthine oxidase (0.02 U) at 37 ºC for 30 min, MDA immediately increased to significance (>5-fold). The addition of Scu at the concentration of 25-100 µmol/L significantly attenuated the rise of MDA (Tab 1).

Tab 1. Effect of scutellarin (Scu) on the elevation of MDA and the decrease of membrane fluidity induced by superoxide in rat cortical synaptosomes. Normal: without treatment with superoxide and Scu. Control: without treatment with Scu. *n*=5. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control.

Group	Dose/µmol·L^{-1}	MDA/ µmol·g^{-1} (protein)	Fluorescent anisotropy
Normal	-	4.69±0.46	0.164±0008
Control	-	27.49±2.58	0.190±0.006
Scu	5	24.72±1.23	0.189±0.004
	25	17.09±2.34^c	0.180±0.006^b
	50	14.41±0.81^c	0.174±0.004^c
	100	6.93±0.73^c	0.173±0.006^c

Effects of Scu on the membrane fluidity The xanthine/xanthine oxidase system (0.3 mmol/L/0.02 U, 37 ºC, 30 min) resulted in a decrease in fluidity, because the fluorescence anisotropy significantly increased. When Scu was added to the system, the anisotropy values decreased with increasing concentration of Scu (25-100 µmol/L) (Tab 1).

Effects of Scu on the $[Ca^{2+}]_i$ The basal levels of synaptosomes $[Ca^{2+}]_i$ in rat cortex was (155±26) µmol·L^{-1}·g^{-1} (protein). In response to superoxide, there was a significant increase (211 %) in the basal $[Ca^{2+}]_i$ concentration (326±35) µmol·L^{-1}·g^{-1} (protein). At low concentration (0.05 mmol/L), Scu was less potent in antagonizing the effect of superoxide anion-induced the increase of $[Ca^{2+}]_i$ (309±26) µmol·L^{-1}·g^{-1} (protein). However, this increase was significantly inhibited by Scu 25, 50 and 100 µmol/L [(251±16), (212±15) and (187±21) µmol·L^{-1}·g^{-1} (protein)] (Fig 1).

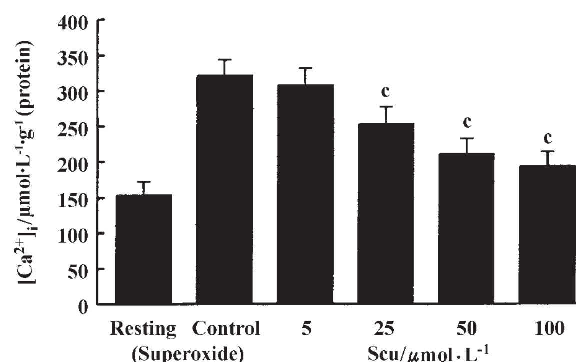

Fig 1. Inhibitory effects of scutellarin (Scu) on superoxide induced $[Ca^{2+}]_i$ increase in rat cortical synaptosomes. Resting: without treatment with superoxide and Scu. Control: without treatment with Scu. *n*=4. Mean±SD. $^cP<0.01$ *vs* control.

Effects of Scu on the activity of Na^+/K^+-ATPase Exposure to superoxide resulted in an decrease in Na^+/K^+-ATPase activity in rat cortex synaptosome from (6.48±0.49) to (2.42±0.33) mmol·g^{-1} (protein)·h^{-1}. There was no significant elevation in Na^+/K^+-ATPase activity following treatment with Scu 5 µmol/L [(2.9±0.4) mmol·g^{-1} (protein)·h^{-1}]. But at the dose of 25, 50 and 100 µmol/L , the activies of Na^+/K^+-ATPase increased significantly [(3.85±0.46), (4.21±0.45) and (4.56±0.48) mmol·g^{-1} (protein)·h^{-1} respectively] (Fig 2).

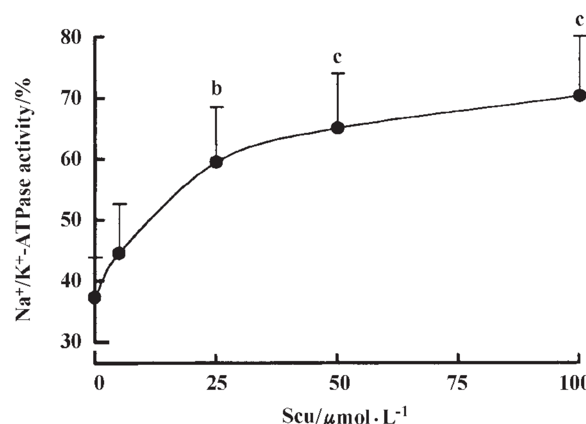

Fig 2. Effect of scutellarin (Scu) on superoxide-induced decrease of Na^+/K^+-ATPase activity in rat cortical synaptosomes. *n*=4. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control group (without treatment with Scu).

DISCUSSION

Scu is a polyphenolic flavonoid compound. Flavonoids exist naturally and show a high free radical

scavenging activity[14]. These activities were dependent on their chemical structures. Our previous studies demonstrated that Scu was a good ROS scavenger *in vitro*[4].

An important target of free radicals was the polyunsaturated fatty acids of lipid bilayer membrane. Their peroxidation may impair membrane functions, increase permeability, reduce the membrane fluidity, inhibit the signal transduction over the membrane and inactivate membrane-bound enzymes and receptors[15]. The present results have clearly demonstrated that the exposure of synaptosomes to superoxide at 37 ºC for 30 min increased lipid peroxidation as measured MDA. These changes induced by superoxide were significantly ameliorated by Scu. The mechanism of this effect may be by directly scavenging radicals, chelating the transition of metal ions such as Fe and Cu (prevent the formation of OH· from H_2O_2 via the Fenton reaction) and inhibiting the lipooxygenase enzyme[16] .

The main determinant of membrane fluidity is the dynamic properties of the lipid bilayer[17]. Peroxidation of these constituent lipids by free radicals can alter membrane fluidity[18,19]. In our study there was a marked decrease in synaptosomes membrane fluidity in superoxide-treated group. Membrane fluidity of the superoxide+Scu (25-100 μmol/L) group increased significantly when compared to the control group and the superoxide group. The reason of Scu increasing membrane fluidity is possibly due to the ROS scavenging effect[20].

Na^+/K^+-ATPase is a membrane integral enzyme responsible for neuronal homeostasis through the membrane. This enzyme activity has been used as a potential indicator for membrane structure and function[21]. Its inhibition will result in a decline of Na^+ and K^+ electrochemical gradients and ultimately lead to cell death as a result of osmotic damge as well as the accumulation of intracellular Ca^{2+} secondary to a decrease in active Ca^{2+} influx by the Na,Ca-exchange system[22]. The synaptosome Na^+/K^+-ATPase was quite sensitive to the superoxide, showing significantly loss of activity when exposed to superoxide for 30 min (Fig 2). In contrast, a significant recover in the activity was observed in the presence of Scu. This protective effect of Scu might also be due to the ability to scavenge superoxide which prevent the oxidative damage of synaptosomes.

An increase in $[Ca^{2+}]_i$ has been commonly associated with oxidative injury of neuronal cell[23]. Oxidative injury may activate phospholipase C, leading to diacylglycerol generation, as well as to IP3 production and subsequent calcium release[24]. Radical also results in membrane lipid peroxidation which alters the permeability of membrane to ions and activities of membrane-bound enzymes[25,26]. These changes markedly increased $[Ca^{2+}]_i$. Scu inhibited the synaptosomes lipid peroxidation by scavenging superoxide which prevented the elevation of $[Ca^{2+}]_i$ induced by oxidative damage.

In conclusion, these findings suggest that Scu possess a significant antioxidation activity against superoxide-induced oxidative stress in rat cortical synaptosomes. Furthermore, the results are in good agreement with each other. These data demonstrate that the neuroprotective effects of Scu are probably due to its ability to scavenge free radicals. Our results may partly explain and support the use of *Erigeron breviscapus* as a neuroprotector in traditional medicine.

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