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Effect of salvianic acid A on lipid peroxidation and membrane permeability in mitochondria

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Abstract

Salvia miltorrhiza Bunge is a traditional Chinese medicine and has long been used for treating liver and heart diseases in China. Salvianic acid A is the main active component of *Salvia miltorrhiza* Bunge. In the present study, the ability of salvianic acid A in scavenging free radicals, inhibiting lipid peroxidation and mitochondrial membrane permeability transition, as well as respiration and protein thiol oxidation in rat liver mitochondria, was evaluated. The results show that salvianic acid A scavenges superoxide anions in a dose-dependent manner (IC_{50} 52 μg/ml). Salvianic acid A could scavenge lipid free radicals and inhibit lipid peroxidation as effectively as Vitamin E. Salvianic acid A also inhibited the mitochondrial membrane permeability transition assessed as the extent of mitochondrial swelling. Salvianic acid A inhibited the oxidation of mitochondrial protein thiols involved in the mitochondrial membrane permeability transitions. We conclude that salvianic acid A is able to reduce lipid peroxidation in the mitochondrial membrane by scavenging free radicals, and inhibit mitochondrial membrane permeability transition by reducing protein thiol oxidation. These data indicated the pharmacological potential of salvianic acid A against pathological processes related to oxidative stress.

Keywords: Chinese traditional medicine; *Salvia miltorrhiza* Bunge; Salvianic acid A; Antioxidant activity

1. Introduction

Salvia miltorrhiza Bunge, a plant which belongs to the Labiatae family, has been widely used in Chinese traditional medicine for treating liver and heart disease, atherosclerosis and for protecting the heart against ischemia-reperfusion injury (Chen et al., 1979; Zhao et al., 1996). Previous studies had found that *Salvia miltorrhiza* showed a strong free radical scavenging activity (Zhao et al., 1996). According to several phytochemical reports, salvianic acid A is the main active component of *Salvia miltorrhiza* Bunge (Zhang et al., 1999). Its molecular structure is D(+) β-(3,4-dihydroxyphenyl) lactic acid (Fig. 1).

A variety of clinical pathological events have been found in recent years to be connected with oxygen free radical injury. Free radicals and other reactive oxygen species (ROS) are considered to be important causative factors in apoptosis, ischemia-reperfusion injury and inflammation. ROS mediated lipid peroxidation and DNA damage causing a variety of chronic health problems, such as cancer (Pard and Floyd, 1992), aging and atherosclerosis (Ames, 1983; Vaca et al., 1988). Plant and food derived antioxidants have been found to be beneficial in protecting against these diseases (Mukhtar and Ahmad, 1999), hence antioxidant therapy has become an attractive therapeutic strategy.

In the present study, we evaluated the ability of salvianic acid A to scavenge free radicals. The inhibitory effects of salvianic acid A on lipid peroxidation of mitochondrial membranes, mitochondrial membrane permeability transition, ability to inhibit protein thiol oxidation and effects on mitochondrial respiration as a parameter of mitochondrial function, were evaluated.

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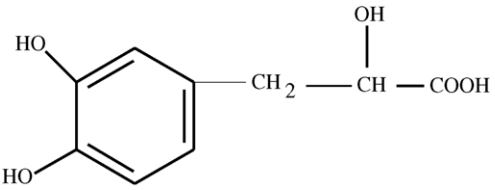

Fig. 1. Chemical structure of salvianic acid A.

2. Materials and methods

2.1. Preparation of salvianic acid A

Salvianic acid A, isolated from the root of *Salvia miltorrhiza* Bunge, was offered by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), (standard reagent). Salvianic acid A was dissolved in distilled water to reach appropriate concentrations, and stored at 4 °C.

2.2. Isolation of rat liver mitochondria

Male Sprague–Dawley rats (weighing approximately 300 g) were purchased from the Laboratory Animal Center (Chinese Academy of Sciences, Beijing, China). The animals were kept in the Animal Laboratory of the Institute of Biophysics (government license No. SYXK (Jing) 2003–0005) and maintained with free access to standard diet and tap water prior to experiments. The experiments were carried out according to the suggested international ethical guidelines for the care of laboratory animals. Animals were sacrificed by decapitation. The liver was immediately removed, sliced in isolation buffer containing 250 mM sucrose, 220 mM mannitol, 5 mM HEPES and 0.2% bovine serum albumin (BSA), pH 7.4, and homogenized. Mitochondria were isolated as described by Pedersen et al. (1978) with slight modifications. The homogenate was centrifuged at 1800 × *g* for 10 min, and the supernatant was centrifuged at 18,000 × *g* for 10 min. The resulting pellet was suspended in isolation buffer (without BSA), and centrifuged at 19,000 × *g* for 15 min. The final mitochondrial pellet was suspended in medium without BSA (pH 7.4). All procedures were performed at 4 °C. Mitochondrial protein was determined by the Bradford method (Bradford, 1976) using BSA as standard.

2.3. Measurement of mitochondrial superoxide anion generation

Mitochondria (1 mg/ml) were incubated for 1 h at room temperature with 0.1–200 μg/ml of salvianic acid A or 50 U/ml superoxide dismutase (SOD) in a solution containing 100 μM nitroblue tetrazolium (NBT), 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4) and 2 mM potassium phosphate. The reaction was initiated by the addition of 5 mM succinate in the assay cuvette, and the rate of NBT reduction was measured spectrophotometrically at 595 nm (Saturnino et al., 2003).

2.4. Analysis of lipid free radicals by electron spin resonance (ESR)

4-Pyrridyl-1-oxide-*N*-*t*-butylnitrone (4-POBN) was used to detect lipid free radicals produced from the linoleic acid system (Zhao et al., 1991). The analytical solution contained 3.6 mM linoleic acid, 0.04 mM 4-POBN, 0.1 mM DETAPAC (diethylene triaminepentaacetic acid), 0.5 mM $Fe(NH_4)_2SO_4{\cdot}6H_2O$ and 0.1–10 μg/ml salvianic acid A, SOD (200 U/ml) or Vitamin E (0.2 mM). The sample was mixed and transferred into a quartz capillary placed in the cavity of Varian E109C ESR spectrometer. The machine setting was: X band, microwave power 10 mW, scan width 200 G, modulation frequency 100 kHz, amplitude 2 G and time constant 0.125 s at room temperature.

2.5. Lipid peroxidation assay

The formation of malondialdehyde (MDA), due to the formation of thiobarbituric acid (TBA)-reactive compounds, was used to monitor lipid peroxidation (Sefantos et al., 1998). Rat liver mitochondria (1 mg/ml) were incubated in buffer containing 0.1 μM antimycin A, 50 mM NaH_2PO_4, 200 μM NADH and salvianic acid A (0.01–100 μg/ml), SOD (200 U/ml) or Vitamin E (0.4 mM) with shaking, for 30 min, at room temperature. The mitochondrial suspension mixed with 8% TBA, and then TBA-reactive compounds were incubated in boiling water for 1 h. After cooling with tap water, 1 ml methyl alcohol was added, and then MDA was measured spectrophotometrically at 532 nm as a measure of lipid peroxidation.

2.6. Mitochondrial swelling assay

Large-amplitude mitochondrial swelling attributable to the mitochondrial permeability transition was monitored by measuring the decrease in optical density at 540 nm (Cassarino et al., 1999). Rat liver mitochondria (1 mg protein) were pre-incubated with salvianic acid A in the presence of 70 mM sucrose, 220 mM mannitol, 5 mM succinate and 5 mM HEPES, pH 7.2, at 25 °C. The reaction was initiated by the addition of Ca^{2+} followed 2 min later by 0.5 mM potassium phosphate (Pi). Salvianic acid A and Ca^{2+} concentration were as indicated in the figure legends.

2.7. Protein thiol groups oxidation assay

After 30 min incubation under the swelling assay conditions (see above), the amount of free thiol groups in the membrane of mitochondria was assessed by exposure to DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] as described by Jocelyn (1987). Absorption was measured at 410 nm.

2.8. Oxygen consumption assay

Oxygen consumption of mitochondria was measured polarographically using an oxygraph equipped with a Clark-type oxygen electrode (Gilson Medical Electronics, Middleton, WI, USA). The respiratory parameters were determined as described by Simon et al. (1997). Malate (10 mM) plus glutamate (10 mM) and succinate (10 mM) were used as substrate for complexes I and II, respectively.

2.9. Statistical analysis

Data are expressed as mean ± S.E. Significance testing was performed by means of Student's *t*-test. Differences between groups were considered significant at a value of $P < 0.05$.

3. Results

3.1. Effect of salvianic acid A on superoxide anions generated from liver mitochondria

Mitochondria are the major place for the production of intracellular ROS. As shown in Fig. 2, SOD (50 U/ml) scavenged 84% of superoxide anions production in isolated liver mitochondria measured by the reduction of NBT. Salvianic acid A (0.1–200 μg/ml) scavenged superoxide anions in a dose-dependent manner with an IC_{50} of 52 μg/ml.

3.2. Scavenging of lipid free radicals produced by the linoleic acid system

The ESR spectrum of lipid free radicals produced by the linoleic acid system and trapped with 4-POBN is shown in Fig. 3. Addition of salvianic acid A (0.1–10 μg/ml) scavenged lipid free radicals in a dose-dependent manner. Vitamin E (0.2 mM) scavenged 78% of lipid free radicals, but the addition of SOD (200 U/ml) didn't show significant effect.

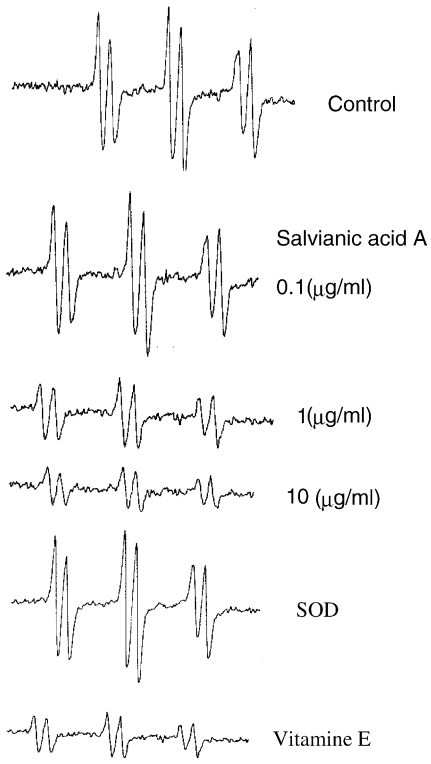

Fig. 3. Effect of salvianic acid A on lipid free radicals. The ESR spectrums show lipid free radicals produced by the linoleic acid system and trapped with 4-POBN.

3.3. Effects of salvianic acid A on lipid peroxidation

As shown in Fig. 4, mitochondrial lipid peroxidation was inhibited by the addition of salvianic acid A (0.1–100 μg/ml) in a dose-dependent manner. Since 100 μg/ml salvianic acid

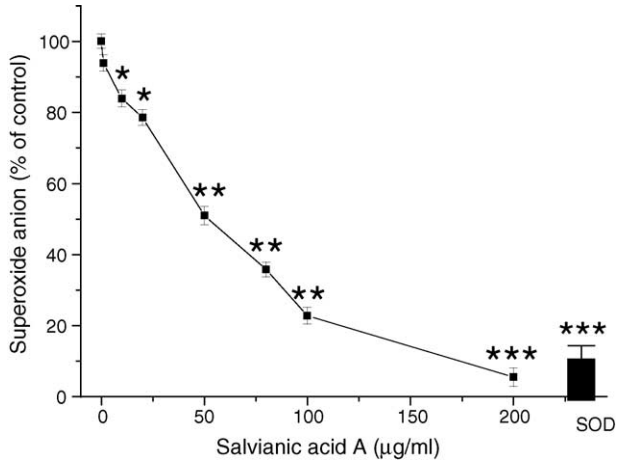

Fig. 2. Scavenging effect of salvianic acid A on superoxide anion. Superoxide anion generated in rat liver mitochondria was measured as the reduction of NBT detected spectrophotometrically at 595 nm. Values are mean ± S.E. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ vs. control.

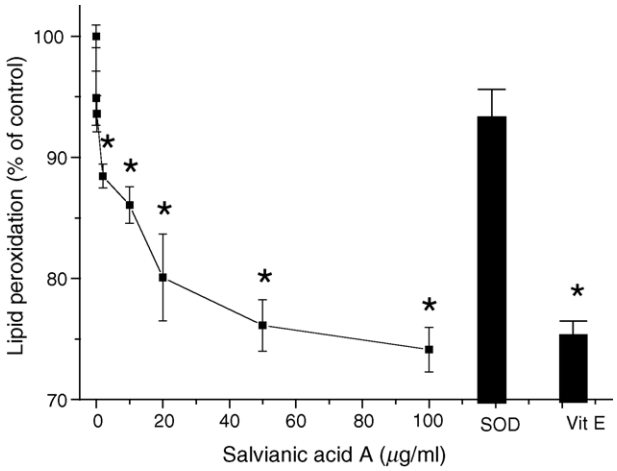

Fig. 4. Concentration–response curves for the inhibitory effects of salvianic acid A on lipid peroxidation in isolated rat liver mitochondria. Values are mean ± S.E. $^{*}P < 0.05$ vs. control.

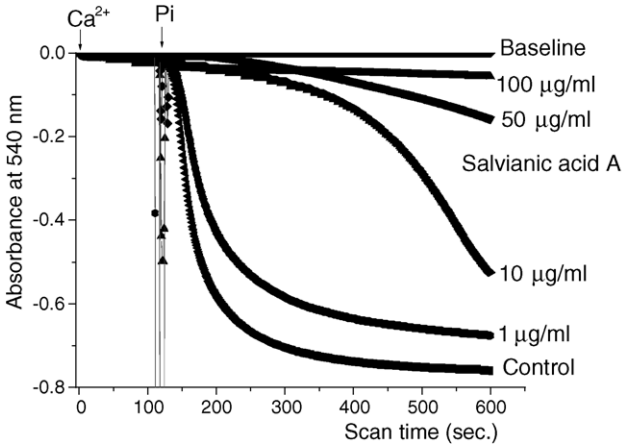

Fig. 5. Effect of salvianic acid A on mitochondrial swell. Representative recordings show the effect of salvianic acid A (1–100 μg) on the mitochondrial swelling induced by 100 μM Ca^{2+}/500 μM Pi and measured spectrophotometrically as a decrease in absorption at 540 nm.

A showed an effect similar to that of Vitamin E (0.4 mM) in inhibiting lipid peroxidation ($P < 0.05$), but the addition of SOD (200 U/ml) showed only a minor inhibitory effect.

3.4. Effects of salvianic acid A on mitochondrial membrane permeability transition

Fig. 5 shows the effects of salvianic acid A on mitochondrial swelling in isolated rat liver mitochondria, as an evaluation of membrane permeability transition. Salvianic acid A (1–100 μg/ml) inhibited the 100 μM Ca^{2+}/500 μM Pi-induced swelling in a dose-dependent manner. The swelling was inhibited by approximately 40–50% in the presence of 10 μg/ml salvianic acid A.

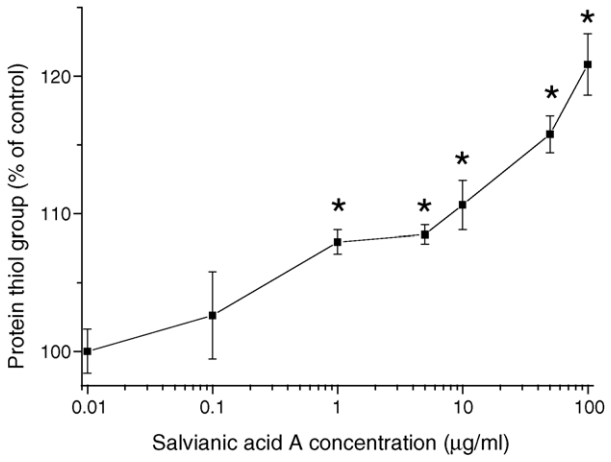

Fig. 6. Effects of salvianic acid A on mitochondrial protein thiol group oxidation. The content of free thiol groups was determined with DTNB and absorption was measured at 410 nm. Values are mean ± S.E. *$P < 0.05$ vs. control.

3.5. Effects of salvianic acid A on protein thiol group oxidation

To investigate the role of thiol group modification in mitochondrial swelling, we measured the abundance of free thiol groups in isolated liver mitochondria. Salvianic acid A (0.01–100 μg/ml) significantly increased the amount of protein thiol groups in a dose-dependent manner (Fig. 6), suggesting that salvianic acid A could inhibit protein thiol oxidation under mitochondrial swelling assay conditions.

3.6. Effects of salvianic acid A on oxygen consumption

Concentrations between 1 and 100 μg/ml of salvianic acid A had no significant effect on respiration in isolated rat liver mitochondria when malate/glutamate and succinate were used as substrates, respectively.

4. Discussion

Mitochondria are the most important intracellular source of ROS. ROS are implicated in many diseases, including atherosclerosis, liver injury, aging, inflammation, neurodegenerative disease and cancer. Scavengers of active oxygen radicals might be beneficial for the prevention or cure of such diseases. A number of Chinese herbs have been found to be very good scavengers of oxygen free radicals. Recent studies have shown that salvianic acid A has multiple biological activities including potent anti-inflammatory, anti-atherosclerosis and anti-hepatic fibrosis (Zhou et al., 2003), so we try to study the mechanism of salvianic acid A in the light of this theory.

In the present study, we found that salvianic acid A could effectively scavenge superoxide anions in a dose-dependent mode with an IC_{50} of 52 μg/ml. Salvianic acid A scavenged lipid free radicals and protected liver mitochondrial membranes from lipid peroxidation as effectively as Vitamin E. Free radicals can initiate lipid peroxidation that may cause tissue damage. The protective effect of salvianic acid A on mitochondrial membranes may work as a preventive antioxidant by scavenging superoxide anions, or it may work as a chain-breaking antioxidant by scavenging lipid free radicals.

The mitochondrial permeability transition pores are multiprotein complexes that have been shown to possess redox-sensitive sites of critical vicinal thiols. The oxidation of protein thiols caused opening of the permeability transition pore, a thiol-dependent channel and facilitated the membrane permeability transition (Reed, 1994). Salvianic acid A (1–100 μg/ml) has been shown to inhibit the mitochondrial swell and the oxidation of thiol groups under the swelling assay conditions, which suggested that salvianic acid A might inhibit membrane permeability transition via reducing oxidation of protein thiols of the permeability transition pore complex.

In addition to being the main intracellular source of ROS, the mitochondrial membrane is particularly susceptible to the action of oxygen radicals, which may impair mitochondrial function by lipid peroxidation and/or mitochondrial membrane permeability transition (Gunter and Pfeiffer, 1990). It is increasingly apparent that mitochondrial membrane permeability transition is a key event in the course of a variety of toxic, hypoxic and oxidative forms of cell injury, as well as apoptosis (Bernardi, 1996; Petit et al., 1996). Therefore, agents that inhibit the mitochondrial membrane permeability transition, as well as lipid peroxidation on the mitochondrial membrane, may be of high pharmacological potential.

The structure of salvianic acid A (Fig. 1) includes two hydroxyl groups in the benzene ring, which may explain its ability to scavenge free radicals. The two hydroxyl groups in salvianic acid A make it hydrophilic and allowing easier access to the area where the lipid free radicals are generated, and facilitating reaction with lipid free radicals. Concentrations of salvianic acid A that affected lipid peroxidation and mitochondrial membrane permeability transition, showed no significant effect on mitochondrial respiration. Concentrations between 0.01 and 200 μg/ml of salvianic acid A had no toxic effect on cell growth as observed by the MTT test (data not shown).

In conclusion, our results show that salvianic acid A has a high anti-lipoperoxidant activity and inhibits mitochondrial permeability transition without significant toxic effects, suggesting the pharmacological potential of salvianic acid A against pathological processes related to oxidative stress.

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