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Salvianolate inhibits proliferation and endothelin release in cultured rat mesangial cells[1]

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KEY WORDS salvianolate; glomerular mesangium; cell division; endothelins; cytotoxicity

ABSTRACT

AIM: To study the effects of salvianolate, an aqueous extract of Radix Salviae Miltiorrhizae, on the proliferation and endothelin release of cultured rat mesangial cells. **METHODS**: The proliferation of mesangial cells was determined in terms of [^{3}H]thymidine uptake. The concentration of endothelin was measured by radioimmunoassay. The cytotoxicity of salvianolate was tested by tetrazolium (MTT) and lactic dehydrogenase (LDH) assay. **RESULTS**: Lipopolysaccharide (LPS) 10 mg/L increased the proliferation and endothelin release in cultured mesangial cells. When mesangial cells pretreated with 3, 10, and 30 mg/L of salvianolate were incubated for 4 h with LPS, salvianolate exhibited a concentration dependent inhibitory effect on proliferation and endothelin levels in the mesangial cells induced by LPS. Furthermore, the increased basal levels of mesangial cells proliferation and the endothelin release were also effectively inhibited by salvianolate 30 mg/L at 4, 8, and 12 h. Besides, no cytotoxicity of salvianolate was observed. **CONCLUSION**: These results indicate that salvianolate can inhibit mesangial cells proliferation, which may be related to the decrease of endothelin release.

INTRODUCTION

Radix Salviae Miltiorrhizae, a traditional Chinese medical herb known as "Danshen", has been widely used in clinics to improve blood circulation, relieve blood stasis and eliminate swelling, *etc*. In addition, it has been reported to have vasodilator, hypotensive, anticoagulant, and antibacterial activities and have a beneficial effect in rats with chronic renal failure[1,2]. Salvianolate is an aqueous extract, which is isolated from Radix Salviae Miltiorrhizae. It is demonstrated in a rat model of adenine-induced chronic renal failure (CRF) that salvianolate improved renal function by decreasing the serum levels of urea nitrogen and creatinine, and also inhibited the increase in the mean glomerular volume and the basement membrane thickness[3].

Mesangial cells (MC) are contractile cells that share features with smooth muscle cells and pericytes, which take part in the regulation of the glomerular hemodynamics, the processing of macromolecules and immunocomplexes. Several vasoactive substances, such as endothelin, platelet-activating factor (PAF), and cytokinins, may affect MC functions by stimulating cell proliferation, contraction or matrix production[4]. Mesangial cell proliferation has also been observed to contribute to the development of glomerular pathology[5].

Endothelin is a potent vasoconstrictor peptide of 21-amino-acid residues originally isolated and purified from the supernatant of cultured porcine aortic endothelial cells[6]. Recent studies have documented that besides aortic endothelium, glomerular and endothelial cells from renal vessels in culture also constitutively express preproendothelin gene and release the corresponding peptide in the cell supernatant[7]. In addition to its vasoactive properties, endothelin has a mitogenic effect on mesangial cells in culture[8]. By virtue of its vasoactive and mitogenic properties, endothelin may play a major role in the control of renal hemodynamics in normal and diseased conditions. Endothelin could be produced by phagocytic cells and B lymphocytes in response to lipopolysaccharide (LPS) and other bacterial products[9].

The purpose of this study was to investigate the effects of salvianolate on the proliferation and the endothelin release in cultured rat mesangial cells.

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MATERIALS AND METHODS

Reagents Salvianolate was provided by Department of Biotechnology of this institute. Lipopolysaccharide was obtained from Sigma Chemical Co (St Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, penicillin sodium and streptomycin were from Gibco (Grand Island, NY). Endothelin radioimmunoassay kit was purchased from Dongya Biotechnology Co (Beijing, China). LDH kit reagent was purchased from Jiangcheng Biotechnology Co (Nangjing, China). All other reagents were of high quality available.

Preparation and analysis of salvianolate Salvianolate was isolated and purified from an aqueous extract of Radix Salviae Miltiorrhizae produced in China, as described previously[10]. Through chromatography test, salvianolate contain 80 % magnesium lithospermate B and the chemical structure of this compound is shown in Fig 1.

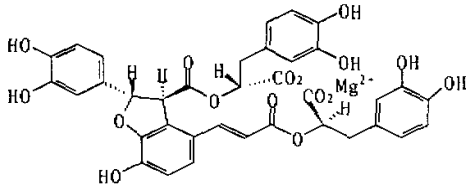

Fig 1. Structural formula of magnesium lithospermate.

Preparation of mesangial cells and cell culture Rat mesangial cells were prepared by the method of Albrightson *et al*[11]. Glomeruli were isolated from Wistar rats (male, 150 – 200 g, SPF Grade, Certificate No 99 – 003, from the Department of Experimental Animals, Shanghai Institute of Materia Medica) by successive mechanical sieving (150 and 50 μm) treated with 300 kU/L collagenase. Cell colonies were subcultured in 60 mm culture dishes, and grown routinely in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10 % fetal calf serum (FCS), penicillin sodium (0.66 mg/L), streptomycin (60 mg/L), insulin (5 mg/L), transferrin (0.66 mg/L), and amphotericin B (2.5 mg/L) in 5 % CO_2 at 37 ℃. Mesangial cells surrounding the glomeruli were removed using cloning rings and were cultured under the above conditions. Cells between the 5th and 15th passage were used for the experiments.

Measurement of [^{3}H] thymidine incorporation in mesangial cells For studies of [^{3}H]thymidine incorporation, mesangial cells were grown in 24 well dishes (30 000 cells per well). When the cells were at or near confluence, fresh medium, conditioned, cultured medium without fetal calf serum was added. The dishes with various concentrations of salvianolate and LPS were incubated at 37 ℃ for 4, 8, and 12 h. As a positive control, a subset of cells were exposed to DMEM only. After incubation, 7.4×10^7 Bq/L of ^{3}H thymidine was added to each well for 6 h. Each well was then washed four times with ice-cold PBS and 1.0 mL of Hanks solution without calcium or magnesium. Trypsin/EGTA was then added to detach the cells. An aliquot was removed for cell counts before adding 1 mL of 20 % trichloroacetic acid (TCA). Following a 60-min incubation at 4 ℃, the TCA-insoluble fraction was collected on glass fiber filters using a cell fractionator and the radioactivity determined using liquid scintillation spectrometry.

Measurement of endothelins The primary culture medium was discarded and dishes were washed twice with PBS. Next, fresh, conditioned, cultured medium without fetal calf serum was added. The dishes with various concentrations of salvianolate and LPS were incubated at 37 ℃ for 4, 8, and 12 h. The addition of DMEM alone was treated as a control group. The medium was subsequently collected and the production of endothelins by cultured mesangial cells was measured by radioimmunoassay kit.

Evaluation of cell viability The cytotoxicity of savialonate was evaluated via both the reducing activity of MTT and the amount of lactic dehydrogenase (LDH) released. For the MTT test, mesangial cells grown in 96-well plates (1×10^4 cells per well) were incubated in the medium containing savialonate (3, 10, 30 mg/L) for 12 h. The medium was subsequently discarded and the cells were incubated for additional 24 h in 10 % FCS medium. This medium was then exchanged for 100 μL of a medium containing 0.5 g/L MTT and incubated for 4 h. DMSO 100 μL was subsequently added to each well to dissolve formazan crystals; the absorption at 550 nm was measured. For the LDH release test, mesangial cells grown in 96-well plates (3×10^4 cells per well) were incubated for 12 h in a medium containing salvianolate (3, 10, 30 mg/L). The medium was then collected and the cells were lysed with 1 % Triton X-100. LDH activity was measured with an LDH assay reagent. The percentage of LDH release was calculated from the ratio of LDH activity in the medium to the sum of the LDH

activity in the medium and in the cell lysate[12].

Statistical analysis Data were represented as $\bar{x} \pm s$. The results were analyzed using dunnett t-test to determine significance. Student's t-test was employed for analyzing the effect of salvianolate on the basal mesangial cell proliferation and endothelin release. A difference of $P < 0.05$ was considered to be significant.

RESULTS

Effects of salvianolate on cell viability The viability of cultured mesangial cells were evaluated by the reducing activity of MTT and by the amount of LDH release. After a 12-h of incubation, the number of formazan crystals reduced by mesangial cells and LDH released from the cells was not significantly affected by salvianolate or LPS (Tab 1).

Tab 1. Effect of salvianolate with or without LPS on cell viability. $n = 10$ in MTT test and $n = 8$ in LDH release test. $\bar{x} \pm s$.

	MTT / A_{550}	LDH release / %
Control	0.52 ± 0.04	35 ± 10
Salvianolate (30 mg/L)	0.54 ± 0.07	34 ± 3
Salvianolate (3 mg/L) + LPS (10 mg/L)	0.51 ± 0.04	33 ± 6
Salvianolate (10 mg/L) + LPS (10 mg/L)	0.52 ± 0.06	31 ± 11
Salvianolate (30 mg/L) + LPS (10 mg/L)	0.53 ± 0.08	35 ± 4

Effects of salvianolate on mesangial cell proliferation induced by LPS When mesangial cells were incubated with or without the presence of LPS 10 mg/L for 4 h, the effect of salvianolate on mesangial cell proliferation was examined in terms of [^{3}H] thymidine incorporation. In Fig 2A, compared to normal control, LPS significantly increased the mesangial cell proliferation ($P < 0.01$) and salvianolate dose-dependently suppressed LPS-induced mesangial cell proliferation ($P < 0.01$ in 30 mg/L). The IC_{50} (the concentration that produces half of the maximal inhibitory effect) was 26.1 mg/L and 95 % confidence limit was 19.4 – 43.1 mg/L.

Effects of salvianolate on endothelin release from mesangial cells induced by LPS When mesangial cells were incubated in the presence of LPS

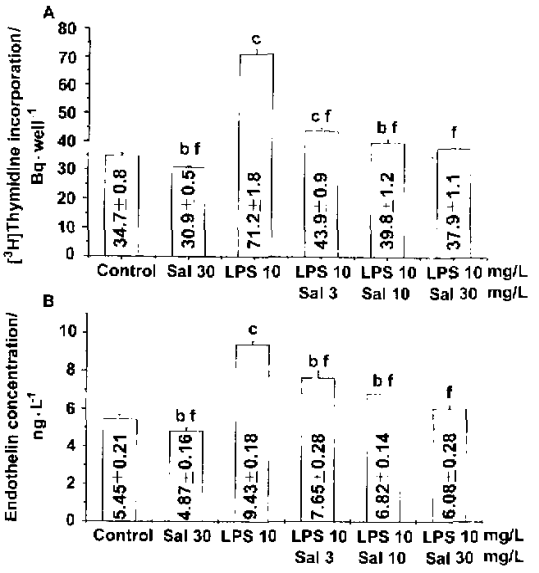

Fig 2. Effects of salvialonate (Sal) on rat mesangial cells induced by LPS. A) Effects of salvialonate on LPS-induced proliferation in rat mesangial cells. B) Effects of salvialonate on LPS-induced endothelin release in rat mesangial cells. Rat mesangial cells were treated with or without LPS 10 mg/L in the medium containing various concentrations of salvialonate for 4 h. $n = 3$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* normal control. $^fP < 0.01$ *vs* LPS 10 mg/L.

10 mg/L for 4 h, the concentration (ng/L) of endothelin in the conditioned medium increased from 5.18 ± 0.28 to 9.43 ± 0.18 ($n = 10$, $P < 0.01$). After mesangial cells were pretreated with 3, 10, and 30 mg/L salvianolate, the concentration of endothelin in the conditioned medium was significantly decreased ($P < 0.01$ in salvianolate 30 mg/L groups). The decrease in endothelin levels was in a concentration dependent manner (As shown in Fig 2B). Our results indicate that salvianolate inhibited LPS-induced endothelin release from rat mesangial cells.

Effects of salvianolate on basal proliferation of mesangial cells Salvianolate 30 mg/L effectively inhibited the proliferation of cultured rat mesangial cells after the 4-h incubation without LPS (Fig 2A). When mesangial cells were incubated with salvianolate 30 mg/L for 8 h and 12 h, salvianolate also effectively suppressed the proliferation with time (As shown in Fig 3A). After 4-, 8-, and 12-h incubation with salvianolate, the mesangial cell proliferation was inhibited by 10.7 %, 22.7 %, and 27.4 %, respectively.

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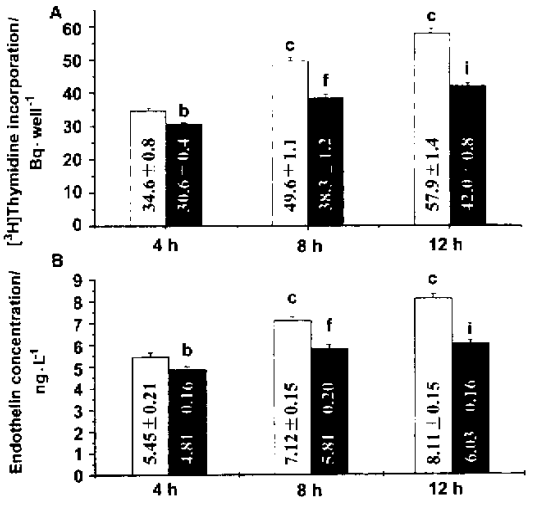

Fig 3. Effects of salvialonate on rat mesangial cells without LPS. A) Effects of salvialonate on basal mesangial cells proliferation. B) effects of salvialonate on basal endothelin release in mesangial cells. Mesangial cells were treated with salvialonate 30 mg/L for 4, 8, and 12 h. □ control; ■ salvianolate 30 mg/L. $n=3$. $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs* control at 4 h. $^fP<0.01$ *vs* control at 8 h. $^iP<0.01$ *vs* control at 12 h.

Effects of salvianolate on basal endothelin release from mesangial cells When mesangial cells were incubated without the presence of LPS for 4, 8, and 12 h, the endothelin concentration (ng/L) also increased significantly, compared to the 4-h incubation, from 5.45 ± 0.21 to 7.12 ± 0.15 and 8.11 ± 0.21. As shown in Fig 2B, salvianolate 30 mg/L inhibited the endothelin release from mesangial cells after a 4-h incubation ($P < 0.05$). And after 8- and 12-h incubation, the increased basal endothelin release was effectively decreased by salvianolate 30 mg/L respectively to (5.81 ± 0.20) ng/L and (6.03 ± 0.16) ng/L (Fig 3B).

DISCUSSION

Our results of this study showed for the first time, salvianolate inhibited the LPS-induced mesangial cells proliferation. Moreover, the basal increased proliferation of the mesangial cells was also effectively suppressed by salvianolate, which was consistent with the reported study for magnesium lithospermate B[13]. It was reported that the proliferation of mesangial cells play an important role in the regulation of renal function. Several hormones, growth factors, and inflammatory cytokines have been shown to modulate mesangial cell turnover in culture, indicating an important implication in nephropathies associated with mesangial cells proliferation[14,15]. Salvianolate can suppress the proliferation of the mesangial cells in the LPS-induced renal pathophysiology, which may be the molecular mechanism that salvianolate improve the renal function in chronic renal failure rats.

It has been reported that the aqueous extract of Radix Salviae Miltiorrhizae can improve renal failure in rats[1] and it modulates the vasoconstrictor effect. The results of this experiment showed a marked inhibitory effect of salvianolate on the release of endothelin from rat mesangial cells. Endothelin is known to possess a proliferative effect and as a potent vasoconstrictor[16]. Though the mechanism of the inhibition of endothelin released by salvianolate is not clear now, the current results suggest that salvianolate can strongly inhibit the endothlin release in cultured mesangial cells, which agree well with our previous work in renal failure rats[3].

Several researchers have reported that endothelins, especially endothelin-1, stimulates DNA synthesis, cell division, and hypertrophy in rat mesangial cells[17]. The mechanism by which ET-1 produces this potent proliferation synergism is yet to be established and it may involve both the opening of cell membrane voltage-gated Ca^{2+} channels and activation of phospholipase C[18]. Our previous work showed that salvianolate reduced plasma endothelin and calcium content of renal cortex in adenine-induced renal failure rats. We further found salvianolate inhibited the free cytosolic calcium concentration in LPS-induced mesangial cells (data not shown).

These results taken together suggested that salvianolate suppressed the mesangial cells proliferation and inhibited the endothelin release.

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丹参多酚酸盐抑制培养大鼠系膜细胞增殖及内皮素释放[1]

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关键词 丹参多酚酸盐；肾小球系膜；细胞分裂；内皮素类；细胞毒性

目的：研究丹参多酚酸盐对体外培养的大鼠系膜细胞增殖及内皮素释放的影响。**方法**：细胞增殖的测定用[^{3}H]-TdR 掺入法；用放射免疫法测定细胞上清液中内皮素浓度的变化；细胞毒性的测定用 MTT 及 LDH 法。**结果**：LPS 10 mg/L 能诱导系膜细胞增殖及内皮素释放的增多。丹参多酚酸盐 3、10 及 30 mg/L 与系膜细胞孵育 4 h，丹参多酚酸盐能浓度依赖性抑制由 LPS 诱导引起的系膜细胞增殖及内皮素释放的增多。丹参多酚酸盐 30 mg/L 也能显著抑制系膜细胞 4、8 和 12 h 时的增殖及内皮素的释放。丹参多酚酸盐对系膜细胞没有细胞毒作用。**结论**：丹参多酚酸盐能抑制大鼠系膜细胞的增殖，其机制可能与抑制内皮素的释放有关。

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