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Effects of Salvianolic Acid A on Oxygen Radicals Released by Rat Neutrophils and on Neutrophil Function

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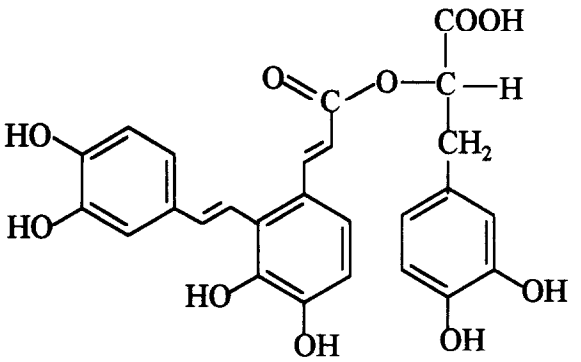
ABSTRACT. Salvianolic acid A (Sai A) has demonstrated potent antioxidant activity in previous studies. In the present study, the effects of Sai A on oxygen radicals released by rat neutrophils and on neutrophil function were investigated. Superoxide anion assayed by the nitroblue tetrazolium test and hydrogen peroxide detected with redox of scopoletin were scavenged concentration dependently by Sai A in *n*-formyl-methionyl-leucyl-phenylalanine (fMLP)- and phorbol myristate acetate (PMA)-stimulated rat neutrophils. Hydroxyl radicals generated in PMA-stimulated neutrophils, measured by HPLC, also were scavenged significantly by Sai A, whereas Sai A showed no significant effects on chemotaxis toward fMLP and phagocytosis of latex beads by rat neutrophils. In addition, the intracellular free calcium and cyclic nucleotide levels of neutrophils, when stimulated by fMLP, were not affected by Sai A. These results suggest that Sai A could significantly scavenge oxygen radicals released by activated neutrophils without affecting their functional ability. BIOCHEM PHARMACOL 51;9:1237–1241, 1996.

KEY WORDS. salvianolic acid A; neutrophils; free radicals; chemotaxis; phagocytosis; free calcium

Salvia miltiorrhiza Bge. has been used in traditional Chinese medicine for centuries. The water-soluble extract of *S. miltiorrhiza* has been used clinically in China to alleviate certain diseases such as angina pectoris, cerebral vascular diseases, and chronic hepatitis [1]. Among seven water-soluble compounds isolated from *S. miltiorrhiza* [2], Sai A† (see structure below) has shown the most potent action against peroxidative damage to biomembranes [3]. In addition, this compound was found to protect against oxygen radical-induced mitochondrial toxicity in rat heart and liver [4] and Adriamycin®-induced mitochondrial toxicity in rat heart [5].

A broad spectrum of neutrophil activities important for host defense includes chemotaxis, adhesiveness, phagocytosis, and killing of bacteria. Although the release of active oxygen species such as O_2^-, H_2O_2, and $OH^\cdot$ by activated neutrophils is associated with an antimicrobial mechanism, these active oxygen species may also become toxic to tissues such as heart, liver, lung, kidney, and gut [6–8]. There has been considerable interest in the role of oxygen-derived species as agents of tissue damage by activated neutrophils in a number of disorders, including reperfusion injury after ischemic cardiac damage, hepatic failure, chronic joint inflammation, adult respiratory distress syndrome, autoimmune disease, rheumatoid arthritis, and cancer [9]. In view of the fact that *S. miltiorrhiza* has been used to treat certain diseases related to injury of reactive oxygen species and to activation of neutrophils, the effects of Sai A on rat neutrophil chemotaxis, phagocytosis, and production of reactive oxygen species were investigated.



Salvianolic acid A (Sai A)

MATERIALS AND METHODS

Reagents

Sai A was isolated from the roots of *S. miltiorrhiza* and supplied by Professor N. L. Li of the Institute of Materia

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† *Abbreviations:* Sai A, salvianolic acid A; O_2^-, superoxide anion; $OH^\cdot$, hydroxyl radical(s); PMA, phorbol myristate acetate; fMLP, *n*-formyl-methionyl-leucyl-phenylalanine; NBT, nitroblue tetrazolium; FBS, fetal bovine serum; PBS(−), Dulbecco's phosphate-buffered solution free of calcium and magnesium; PBS(+), PBS containing 0.8 mmol/L Ca^{2+}, 0.5 mmol/L Mg^{2+}, and 5.5 mmol/L glucose; RIA, radioimmunoassay; cAMP, cyclic AMP; and cGMP, cyclic GMP.

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Medica, Chinese Academy of Medical Sciences. Its purity was over 98%, and it was water soluble. PMA, fMLP, fura-2/AM, glycogen, latex beads (0.8 μm), and scopoletin were purchased from the Sigma Chemical Co. (U.S.A.). NBT was purchased from the Fluka Co. (Switzerland). RPMI-1640 medium and FBS were obtained from the GIBCO Co. (U.S.A.). The cAMP and cGMP RIA kits were purchased from the Department of Isotopes, China Institute of Atomic Energy.

Preparation of Rat Neutrophils

Male Wistar rats, weighing 180–220 g, were used in the present study. Rat neutrophils were obtained from the peritoneal cavity after stimulation by glycogen. Briefly, each rat was injected intraperitoneally with 20 mL of a 0.2% solution of glycogen in sterile saline. After 18 hr, the peritoneal exudates containing neutrophils were collected by washing the peritoneal cavity of each rat with 30 mL of PBS(−). The cell suspension was centrifuged (400 *g*, 5 min) and the pellet was resuspended in PBS(−). The neutrophils were then adjusted to 1×10^7/mL in PBS(−) and preincubated with various concentrations of Sai A in a 37° water bath for 1 hr. The cells were then washed with PBS(−) once and suspended in PBS(+). Using the trypan blue dye staining assay, cell viability was determined to be greater than 95%.

O_2^- *Production*

O_2^- production was assayed by the formation of formazan in the NBT test [10]. A neutrophil suspension of 100 μL (2×10^7 cells/mL) and 100 μL NBT (0.2% w/v) were added to each well. The cells were mixed with fMLP (100 μmol/L) or PMA (100 ng/mL) and incubated at 37° for 1 hr. Then the supernatant was removed by centrifugation, and the sediment was resuspended in 200 μL DMSO. Absorbance at 570 nm was detected by a Bio-Rad MR700 Microplate Reader, with a reference wavelength at 450 nm.

$OH^\cdot$ *Production*

The formation of 2,3-dihydroxybenzoic acid (2,3-DHBA) from salicylic acid when attacked by $OH^\cdot$ was detected by HPLC [11]. The mobile phase was 80% 0.03 mol/L citric acid–0.03 mol/L acetic acid buffer, pH 3.6, and 20% methanol, at a flow rate of 1.0 mL/min. The detector was set at a wavelength of 315 nm. Neutrophils were incubated with 100 ng/mL PMA, 1 mmol/L salicylic acid and various concentrations of Sai A for 10 min in a volume of 1 mL. 2,3-DHBA, 40 μL in a concentration of 100 μmol/L, was used as an internal standard. After incubation, the sample was added with 50 μL of 1 N HCl and extracted with 10 mL of HPLC grade diethyl ether on a Vortex mixer for 90 sec. The diethyl ether layer was separated and evaporated completely in a water bath at 40°. The residue was dissolved in 50 μL of 1 N HCl and 32.5 μL of mobile phase. Twenty microliters of this solution was injected into the HPLC unit.

H_2O_2 *Production*

Neutrophils (5×10^6) in a volume of 1 mL were incubated with 20 μL of 1 mmol/L scopoletin and 10 μL of 1 μg/μL horseradish peroxidase for 1 hr at 37° in a water bath. The assay [12] was performed in a Hitachi model F-4010 fluorometer. PMA (100 ng/mL) and fMLP (100 nmol/L) were used to trigger H_2O_2 release.

Chemotaxis Assay

Chemotaxis was performed by the modified agarose plate method [13]. Briefly, agarose plates were prepared by mixing 10 mL of 2% agarose solution with 10 mL of 2× RPMI-1640 medium supplement with 20% heat-inactivated FBS. Three wells with a diameter of 3 mm were cut on a straight axis at 8-mm intervals. Ten microliters of neutrophil suspension with RPMI-1640 medium containing 10^8 cells/mL was added to the center well. To the outer well, 10 μL of 100 nmol/L fMLP was added as a chemoattractant. To the inner well, 10 μL of RPMI-1640 medium was added as a control. The plates were incubated at 37° in a humidified atmosphere containing 5% CO_2 in air for 4 hr. The distance traveled by neutrophils toward the outer well (d1, true chemotaxis) and inner well (d2, random migration) was measured with a microprojector. The chemotactic index (CI), which expresses the chemotaxis activity, was calculated as CI = d1/d2.

Phagocytosis Assay

One milliliter of latex particles was opsonized by incubating 0.2 mL of rat serum in 1 mL of Tris buffer (pH 8.5) at 37° for 1 hr. Neutrophils (1×10^7 cells) in 0.1 mL of PBS(+) were incubated with 10^8 particles at 37° for 5 min. The reaction was terminated by the addition of 0.4 mL of ice-cold PBS(−) containing 1 mmol/L EDTA. Cells were then diluted with saline and layered onto Ficoll-Paque (d = 1.077) and centrifuged at 400 *g* for 20 min. Cell pellets were collected, and the average number of latex beads internalized in 100 cells was determined. The number of cells containing at least one bead was counted by light microscopy.

Measurement of Cytosolic Free Calcium Concentration

The concentration of cytosolic free calcium in rat neutrophils stimulated by fMLP was measured by the fura-2 method [14]. Briefly, neutrophils suspended in PBS(−) containing 0.1% BSA and fMLP were incubated with 2 μmol/L fura-2/AM at 37° for 15 min and then at room temperature for another 20 min. After washing the loaded neutrophils three times with PBS(−), cell pellets were resuspended at 5×10^6 cells/mL with PBS(−) or PBS(+) containing 0.1% BSA. Fluorescence of fura-2-loaded neutrophils was mea-

sured at 37° using a Hitachi model F-4010 spectrofluorometer equipped with a magnetic stirrer. The ratio of emitted fluorescence at 510 nm over the 340 nm excitation wavelength was used to indicate change in cytosolic calcium. Calcium concentrations were calculated as described by Grynkiewicz *et al.* [15].

Measurement of Intracellular Cyclic Nucleotides

The concentration of intracellular cAMP and cGMP of rat neutrophils stimulated by fMLP was determined by using RIA. After incubation with 100 nmol/L of fMLP, the reaction of neutrophils was stopped by placing the mixture in an ice-cold water bath. After sonication, the contents of cAMP and cGMP in the supernatant of the samples were measured by use of RIA kits.

RESULTS

Effect of Sai A on O_2^- Production in Activated Rat Neutrophils

The scavenging effect of Sai A on O_2^- released by neutrophils is shown in Table 1. O_2^- production was increased when neutrophils were stimulated by fMLP, shown as the increase of the O.D. value at 570 nm. O_2^- production of the neutrophils stimulated by different concentrations of fMLP was inhibited by Sai A concentration dependently. PMA stimulated a greater production of O_2^-. Sai A at a high concentration (100 μmol/L) significantly inhibited O_2^- production, but Sai A at lower doses had no effect.

Effect of Sai A on OH· Production in Rat Neutrophils

OH· production of the neutrophils was increased upon stimulation by PMA. As shown in Table 2, OH· production by neutrophils stimulated by PMA was inhibited significantly by Sai A at 10–100 μmol/L (Table 2).

Effect of Sai A on H_2O_2 Production in Rat Neutrophils

As shown in Table 3, H_2O_2 release was triggered by PMA and fMLP. The release of H_2O_2 from the neutrophils under stimulation of PMA and fMLP was decreased concentration dependently by Sai A.

TABLE 1. Effect of Sai A on O_2^- production in rat neutrophils stimulated by fMLP and PMA

Sai A (μmol/L)	O_2^- (ΔO.D.$_{570\ nm}$)		
	None	fMLP	PMA
0	0.054 ± 0.002	0.093 ± 0.007	0.157 ± 0.010
1	0.056 ± 0.002	0.084 ± 0.005*	0.142 ± 0.021
10	0.047 ± 0.002*	0.063 ± 0.004†	0.144 ± 0.013
100	0.020 ± 0.002†	0.021 ± 0.002†	0.078 ± 0.010†

Values are means ± SD, N = 6.
*† Significantly different from the respective control (Sai A at 0): *$P < 0.05$, and †$P < 0.01$.

TABLE 2. Effect of Sai A on (OH·) production in rat neutrophils stimulated by PMA

Sai A (μmol/L)	OH· (nmol/10^7 cells)	
	None	PMA
0	23.37 ± 9.47	47.94 ± 9.72
1	ND*	31.57 ± 3.60
10	ND	23.75 ± 7.14†
100	ND	25.14 ± 7.90†

Values are means ± SD, N = 6.
* ND = not detected.
† $P < 0.05$ vs control group (Sai A 0 with PMA).

Effect of Sai A on Rat Neutrophil Chemotaxis and Phagocytosis

The maximum effect of fMLP on neutrophil chemotaxis was observed at a concentration of 100 nmol/L (data not shown). The results in Table 4 demonstrate that neutrophil chemotaxis toward the chemotactic peptide fMLP was not affected by Sai A. In addition, neither the average number of latex beads internalized in 100 cells nor the number of cells containing at least one bead was affected by Sai A. These data indicated that neither of the neutrophil activities of chemotaxis and phagocytosis is influenced by Sai A.

Effect of Sai A on Cytosolic Free Calcium and Intracellular cyclic Nucleotides of Rat Neutrophils

The data in Fig. 1 indicate that the intracellular calcium mobilization of rat neutrophils was not influenced by Sai A. In Fig. 2, the decrease of the cAMP level when stimulated by 100 nmol/L fMLP also was not affected by Sai A.

DISCUSSION

There is considerable evidence that neutrophils are implicated in a number of disorders as a result of the release of active oxygen species [6–8]. A series of neutrophil activities including chemotaxis, orientation, adhesiveness, phagocytosis, and metabolic changes are important in host defense and are also involved in tissue injuries. When exposed to chemoattractant agents, neutrophils respond rapidly with a

TABLE 3. Effect of Sai A on H_2O_2 production in rat neutrophils stimulated by fMLP and PMA

Sai A (μmol/L)	H_2O_2 (nmol/10^7 cells)		
	None	fMLP	PMA
0	20.67 ± 0.36	40.42 ± 0.42	46.16 ± 0.22
1	ND*	36.36 ± 2.26	45.34 ± 0.40
10	ND	23.20 ± 2.70†	36.46 ± 1.74†
100	ND	19.60 ± 0.84‡	22.64 ± 1.32‡

Values are means ± SD, N = 6.
* ND = not detected.
†‡ Significantly different from the respective control group (Sai A at 0): †$P < 0.05$, and ‡$P < 0.01$.

TABLE 4. Effect of Sai A on rat neutrophil chemotaxis toward fMLP and on phagocytosis of latex beads

Sai A (μmol/L)	Chemotaxis (index)	Phagocytosis	
		%	Index
0	2.14 ± 0.22	61.0 ± 2.5	4.09 ± 0.10
1	1.84 ± 0.34	64.0 ± 2.8	3.89 ± 0.36
10	1.81 ± 0.35	59.3 ± 2.1	3.91 ± 0.37
100	1.80 ± 0.38	58.7 ± 0.5	3.34 ± 0.71

Values are means ± SD, N = 6. There were no significant differences between the Sai A-treated groups and the control groups (Sai A 0).

highly coordinated series of events. In response to fMLP, for example, activation occurs as a consequence of the stimulus binding to the receptors on the cell membrane. Secondary changes in neutrophil metabolism occur immediately; these include activation of phospholipase C, increase in free cytoplasmic Ca^{2+}, activation of protein kinase, and changes of cAMP and cGMP levels. The data in the present study indicate that the above neutrophil activities when stimulated by fMLP or PMA were not affected by Sai A. In our previous studies, Sai A significantly inhibited oxygen radical-induced lipid peroxidation and directly scavenged O_2^- and OH· generated in the xanthine-xanthine oxidase system and adriamycin reacting with H_2O_2 in isolated rat heart mitochondria *in vitro* [4, 5]. In accord with our previous results, O_2^-, OH· and H_2O_2 released from the activated rat neutrophils were reduced concentration dependently by Sai A. The migration and phagocytosis of neutrophils may be related to changes on their surface, especially the appearance of pseudopods [16]. The lack of effect of Sai A on cytosolic free calcium, intracellular cyclic nucleotide, and the surface of the neutrophils may explain the inability of Sai A to inhibit chemotaxis and phagocytosis of neutrophils. In this aspect, Sai A is quite different from schisanhenol, which not only shows a free radical scavenging effect like Sai A, but also affects the functional activities of rat neutrophils [17].

It has been reported that the tablets and injections made

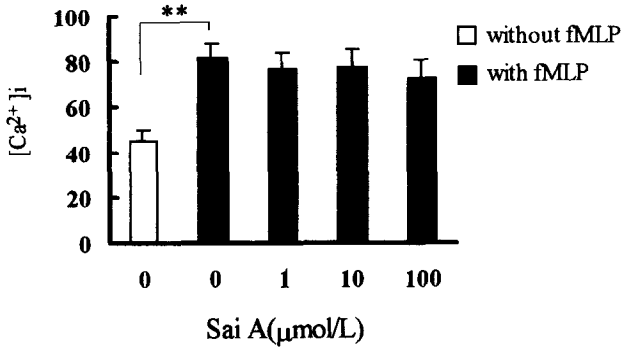

FIG. 1. Effect of Sai A on cytosolic free calcium in rat neutrophils stimulated by fMLP (100 nmol/L). Values, expressed in nmol/L, are means ± SD, N = 6. See Materials and Methods for the experimental conditions. Key: (**) $P < 0.01$.

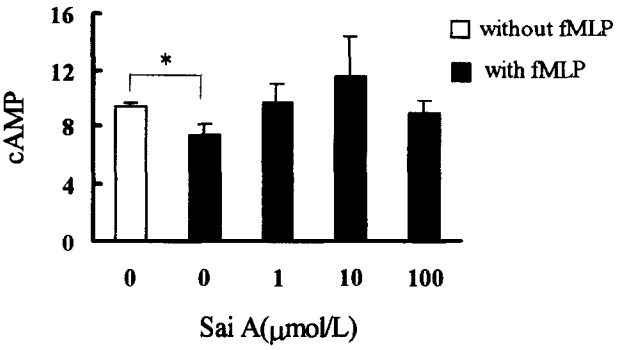

FIG. 2. Effect of Sai A on the level of cytosolic cAMP in rat neutrophils stimulated by fMLP (100 nmol/L). Values, expressed in pmol/10^7 cells, are means ± SD, N = 6. See Materials and Methods for the experimental conditions. Key: (*) $P < 0.05$.

from *S. miltiorrhiza* exhibit some therapeutic effects on cardiac and cerebral ischemia as well as on liver diseases [9]. It is well known that neutrophils, as a result of the release of active oxygen radicals, are involved in the damage of reperfusion after organ ischemia [1, 6, 7] and liver injury [8]. The proposed role of the neutrophil in myocardial reperfusion injury is strengthened by the knowledge that free radical scavengers, antioxidant enzymes, and neutrophil depletion are associated with a significant reduction in the extent of irreversible myocardial injury [6]. The data in the present investigation may be helpful in understanding the clinical use of *S. miltiorrhiza* in the treatment of heart and liver diseases.

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