Effects of chlorogenic acid, an active compound activating calcineurin, purified from *Flos Lonicerae* on macrophage[1]

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KEY WORDS calcineurin; macrophage; chlorogenic acid; *Flos Lonicerae*

ABSTRACT

AIM: To investigate the activation of chlorogenic acid (CHA) purified from *Flos Lonicerae* to calcineurin and its effects on macrophage functions *in vivo* and *in vitro*. **METHODS:** According to the screening results that *Flos Lonicerae* could activate calcineurin, the active component which could activate calcineurin was purified from *Flos Lonicerae* by column chromatography on silica gel and identified as CHA. The activation of CHA on calcineurin had been validated with both *p*-NPP and ^{32}P-labeled RII peptide as the substrates. The clearance of charcoal particles in normal mice and the cytotoxicity of U937 to MCF-7 were used together to determine the effects of CHA on macrophage functions. **RESULTS:** CHA could activate calcineurin, and the concentration of CHA on maximal activating calcineurin was 282.5 μmol/L. CHA administration (10 mg/kg, ig×7 d) significantly enhanced the macrophage functions in normal mice. CHA (70.6, 141.2, and 282.5 μmol/L) obviously increased the cytotoxicity of U937 to MCF-7. **CONCLUSION:** CHA could activate calcineurin and enhance the macrophage functions *in vivo* and *in vitro*, and its functions *in vivo* may be realized via the signal pathways of calcineurin.

INTRODUCTION

The phosphorylation-dephosphorylation of signal transduction protein plays a critical role in cell immune responses. Calcineurin, a Ca^{2+} and calmodulin (CaM)-dependent serine/threonine protein phosphatase[1,2], is an important mediator in signal transmission, connecting the Ca^{2+}-dependent signaling to a wide variety of cellular responses. The studies on biological roles of calcineurin have progressed to the important discovery that it is the common target of the immunosuppressant drugs cyclosporin A (CsA) and FK506[3]. They are pharmacological reagents that have been used to demonstrate calcineurin as a major player in Ca^{2+}-dependent eukaryotic signal transduction pathways. During the T cell activation, upon the binding of antigen to T cell receptor (TCR), intracellular Ca^{2+} is elevated through the actions of protein tyrosine kinases and phospholipase C. Calcineurin is activated by binding Ca^{2+}/CaM, and dephosphorylates the cytosolic forms of the NF-ATc transcription factors. The dephosphorylated NF-ATc transcription factors translocate to the nucleus, where they bind, either alone or in cooperation with AP-1 family members, to specific *cis* elements in the promoter/enhancer regions of cytokine genes, such as interleukin-2 (IL-2). This pathway makes an important contribution to the induction of IL-2 gene transcription, a hallmark of T cell activation[4]. CsA and FK506 inhibit the activity of calcineurin after forming complexes

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with cytoplasmic immunophilins, cyclophilins, and FKBP12, respectively[5-7]. These immunophilin-immunosuppressant complexes bind calcineurin and inhibit its functions by sterically hindering the access of substrates to the catalytic site. The inhibition of calcineurin is related to the adverse side effects of CsA and FK506[8,9].

Although calcineurin plays a critical role in cell immune responses, little is known about the functions of calcineurin in macrophages. Recently several studies have reported the important effects on macrophages by blocking calcineurin with CsA or FK506, such as macrophage proliferation and macrophage effector functions[10,11].

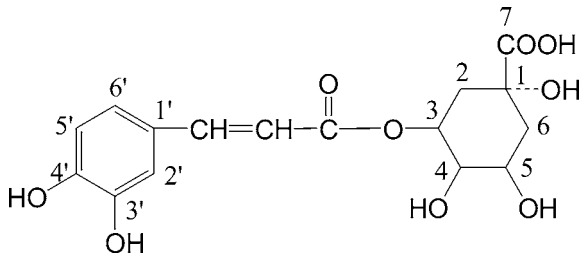
Except for some metal ions, such as Ca^{2+}, Mn^{2+} and Ni^{2+}[2], little attention has been paid to the activator of calcineurin. Based on the characteristics of calcineurin, an effective molecular screening model was established in our laboratory [12]. A lot of materials have been screened with this model, a few of Chinese traditional medicines, which could activate calcineurin and was related to immunity, have been obtained in our laboratory[13]. The active component which could activate calcineurin was purified from *Flos Lonicerae* (one of the activators we got) and identified as chlorogenic acid (CHA). Substrates *p*-NPP and ^{32}P-labelled RII peptide were used to validate the activation of CHA to calcineurin. Due to the connection between calcineurin and macrophages[10,11], the clearance of charcoal particles in normal mice and the cytotoxicity of U937 to MCF-7 were together used to determine the effects of CHA on the macrophage functions *in vivo* and *in vitro*.

MATERIALS AND METHODS

Enzyme and reagent RII peptide was purchased from Biomol Lab. *p*-Nitrophenyl-phosphatase (*p*-NPP) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma. [γ-^{32}P]-ATP was purchased from Beijing Furi Biology Engineering Corp. cAMP-dependent protein kinase and catalytic subunits were purchased from Promega. U937 (Human monocytic cells) and MCF-7 were provided by Research Institute of Cellular Biology of Beijing Normal University. RPMI-1640 and DMEM were purchased from Hyclone Corp. FCS was purchased from Yuanheng Shengma Biotechnology Research Center of Beijing. All other reagents used were of analytical grade.

Material *Flos Lonicerae* was purchased from Beijing Tongrentang Pharmaceutical Group and identified as *Lonicera japonica* Thunb. The voucher sample was deposited in the department of biochemistry and molecular biology of Beijing Normal University.

Extraction and isolation *Flos Lonicerae* (3.0 kg) was extracted with EtOH:H_2O (75:25, v:v) and concentrated in vacuum. The concentrated extract was dissolved in water and adjusted to pH 9-10 with $NH_3 \cdot H_2O$, then partitioned with chloroform ($CHCl_3$) for 4 times. After that, the pH of the water layer was adjusted to 3-4 with HCl, and then partitioned with ethyl acetate (EtOAc) for 4 times. The activation assay showed that the EtOAc layer was the major activation partition; and then it was evaporated to dry in vacuum and carefully subjected to silica gel column chromatography and fractionated with an EtOAc-MeOH solvent system. EtOAc:MeOH (100:5) fractions, showing stronger activation on calcineurin, were further purified by re-crystallizing with $CHCl_3$ and MeOH, respectively. A white crystal was obtained and its structure was identified as CHA by means of ^{1}HNMR/^{13}CNMR, MS, and IR.



Chemical structure of CHA

Assay of CHA to calcineurin activation Calcineurin and calmodulin were isolated from fresh bovine brain as previously described[14]. The activation assay of CHA to calcineurin was assayed by using *p*-NPP as substrate[15]. Ten μL enzyme solution and 10 μL CHA solution or 10 μL water (as lack-CHA control) were mixed and pre-incubated in ice bath for 10 min, then added 180 μL assaying buffer (Tris-HCl 50 mmol/L pH 7.4, DTT 0.5 mmol/L, $CaCl_2$ 0.2 mmol/L, $MnCl_2$ 0.5 mmol/L, BSA 0.2 g/L, calmodulin 0.3 μmol/L, and *p*-NPP 20 mmol/L) and incubated in water-bath at 30 ºC for 20 min in a final volume of 200 μL; the reaction was terminated by adding 1.8 mL solution (Na_2CO_3 0.5 mol/L, edetic acid 20 mmol/L), and then the absorbance at 410 nm was measured by using a lack-CHA/enzyme control; and measured values were corrected by a lack-enzyme control. The units (U) of calcineurin activity are defined as nanomoles of *p*-NPP hydrolyzed per mil-

ligram enzyme per minute at 30 ºC.

When assaying phosphatase activity with substrate ^{32}P-labeled RII peptide[16], the experimental protocol was, according to the previous description[17], modified as below: calcineurin (1 μL) and CHA solution (1 μL) were mixed and pre-incubated for 10 min in ice bath, then added 18 μL assaying buffer with 20 μmol ^{32}P-labelled RII peptide and incubated in water bath at 30 ºC for 20 min. Finally, 180 μL 83.3 mmol H_3PO_4 was added to terminate the reactions. Total 175 μL reaction mixture was subjected to a cation-exchanged column for separating ^{32}P-released from RII peptide and quantitated by liquid scintillation spectrometry. The units (U) of protein phosphatase activity are defined as ^{32}P nanomoles of RII peptide released per milligram enzyme per minute at 30 ºC.

Clearance of charcoal particles Male Kunming mice (18-22g, Grade II, Certificate No SCXK11-02-0004. Experimental Animals Center of Peking University, Beijing, China) were housed under controlled conditions (temperature: 20±1 ºC and lighting: 08:00 AM-20:00 PM) with food and water *ad libitum,* except during experiments. Animals were treated in accordance with current laws and the NIH Guide for Care and Use of Laboratory Animals. CHA was dissolved in saline prior to administration at doses of 5, 10, and 20 mg/kg and orally administered every day at the same time for 7 d. The oral volume was kept constant at 20 mL/kg irrespective of dose. From d 8 after the first administration, mice were injected iv with 1:10 diluted Yidege ink 10 mL/kg. At 1 min (T_1) and 12 min (T_2), blood 20 μL was taken from retro-ocular venous plexus and resolved in 2 mL 0.1% Na_2CO_3 solution. The absorbance (A) was measured at 675 nm with 720 spectrophotometer[24]. The clearance rate K and clearance index α were calculated:

$$K=(\lg C_1-\lg C_2)/(T_1-T_2)$$

$$\alpha=(W/WLS)\cdot K^{1/3}$$

(W: weight of rat; WLS: liver weight+spleen weight; C_1, C_2: the concentration of charcoal particles in blood at T_1 and T_2, respectively)

Cell Culture and experimental treatment Human monocytic cells (U937) were used as effector cells (E), while human breast cancer cells (MCF-7) were used as target cells (T). U937 cells and MCF-7 cells were incubated in 96-well plate-bottom at a ratio of (20:1) with mixed culture medium of 90 % RPMI-1640 and 10 % FCS. CHA was dissolved in this mixed culture medium and added into the well-cultured U937 and MCF-7. The final concentrations of CHA were 70.6, 141.2, and 282.5 μmol/L, respectively. Controls were established in every group. The cells were incubated for 24 h at 37 ºC, 5 % CO_2 and 90 % relative humidity. Then the U937 cell with the medium was aspirated from the wells carefully, and the 10 μL *MTT* (5 mg/mL) and 100 μL of fresh medium were added into those wells. Cultured at the previous conditions for 4 h, the medium was aspirated and 100 μL ethanol was added into every well. After 20 min, the absorbance was determined with an ELISA reader at a measured wavelength of 570 nm and a referenced wavelength of 630 nm[18].

Statistical analysis The data were expressed as mean±SD. Statistical analysis of the data for multiple comparisons was performed by one-way ANOVA followed by the Dunnett's test.

RESULTS

Activation of CHA to calcineurin The activation of CHA to calcineurin was investigated by using *p*-NPP as the substrate. CHA significantly activated calcineurin at concentration ranges of 2.25-1412.4 μmol/L. The maximal activation concentration of CHA to calcineurin was 282.5 μmol/L (Tab 1).

Tab 1. Effects of CHA on calcineurin with *p*-NPP as substrate. Three separated experiments were accomplished for each concentration and triplicate in every assay. *n*=3. Mean±SD. $^cP<0.01$ *vs* calcineurin control.

CHA concentration/ μmol·L^{-1}	Calcineurin activity/ nmol·min^{-1}·mg^{-1}	Comparative activation of CHA to calcineurin/%
0 (calcineurin control)	544±23	100.0
2.25	572±10	105.2
11.3	593±11^c	109.0
56.5	654±11^c	120.2
282.5	719±6^c	132.2
1412.4	675±5^c	124.1

It was reported that the inhibition of immunosuppressant drugs to calcineurin *in vitro* only occurred when a physiological substrate, such as phosphocasein or phospho-RII peptide, was used to assay the calcineurin activity, but this inhibition would be presented to activation when using *p*-NPP as substrate[19,20]. To validate the activation of CHA to calcineurin, the activation of

CHA to calcineurin with the substrate ^{32}P-labelled RII peptide was designed and assayed. The pre-assayed results with substrate ^{32}P-labelled RII peptide showed that the maximal activation concentration of CHA to calcineurin was 282 μmol/L (data not shown). At this CHA concentration, three independent experiments had been conducted to investigate the activation of CHA on calcineurin. The results showed that CHA could significantly up-regulate the activity of calcineurin from 388.8 U to 485.4 U (P<0.01). The comparative activation of CHA to calcineurin was 24.9 %.

Effects of CHA on clearance of charcoal particles in normal mice Effects of CHA on clearance of charcoal particles in normal mice were shown in Tab 2. CHA produced a significant increase on K and α at 10 mg/kg.

Tab 2. Effects of CHA on clearance of charcoal particles in normal mice. n=10. Mean±SD. ^{b}P<0.05, ^{e}P<0.01 *vs* saline.

Treatment/mg·kg^{-1}	10^3×Clearance rate (K)	Clearance index (α)
Saline	29.4±8.8	3.61±0.32
CHA 5	24.3±3.8	3.59±0.28
CHA 10	37.2±9.8^{b}	4.47±0.19^{e}
CHA 20	26.6±3.8	3.75±0.19

Effects of CHA on cytotoxicity of U937 The effects of CHA on cytotoxicity of macrophage were assayed by using U937 as effector cell and MCF-7 as target cell. CHA significantly enhanced the cytotoxicity of U937 to MCF-7 at 70.6, 141.2, and 282.5 μmol/L (Tab 3).

Tab 3. Effects of CHA on cytotoxicity of U937 to MCF-7. n=4. Mean±SD. ^{b}P<0.05, ^{c}P<0.01 *vs* CHA control.

CHA/μmol·L^{-1}	OD_{570nm}	
	CHA alone	CHA+U937
0	0.77±0.04	0.54±0.10
70.6	0.75±0.06	0.36±0.07^{c}
141.2	0.72±0.05^{b}	0.24±0.04^{c}
282.5	0.71±0.05^{c}	0.24±0.10^{c}

DISCUSSION

Calcineurin plays an important role on regulation of immunity through activating T cells. Those functions of calcineurin on immunity regulation have been proved through inhibiting the activity of calcineurin with immunosuppressant, such as CsA and FK506. But the classical immunosuppressants have a practical defect because of their high toxicity. In our laboratory, it had been found that immunosuppressive components from traditional Chinese medicines, ZIP1, could directly inhibit calcineurin without drug binding protein[13] and provide a new clue for the further development of immunosuppressants with lower toxicity. Then, the modulators screened from traditional Chinese medicines may bring extensive attention on immunity.

Macrophages function critically in the immune system. They behave as regulators of homeostasis and as effector cells in infection, wound healing and tumor growth[21]. Clearance of charcoal particles is a classical experiment for testing the functions of macrophage on foreign substance *in vivo*. And the macrophage cytotoxicity of U937 to MCF-7 could explain the cytotoxic functions of macrophage on tumor cells. In the present study, it has been found that during the traditional Chinese medicines screening, the phosphatase activity using *p*-NPP as a substrate was coincident with the result using ^{32}P-labelled RII peptide as a substrate. Our results clearly demonstrate that CHA activates calcineurin and increases the ability of macrophage functions. It may be absorbed by macrophage and activate its calcineurin. The activated calcineurin plays subtle roles in immune systems. The calcineurin-mediated effects of CHA on macrophage are just one possible explanation for the observed results[22,23].

In a word, CHA could activate calcineurin, and the administration of CHA significantly improves the functions of macrophage in normal mice. The reason remains somewhat speculative, it is thus imperative to investigate the changes of activity and content of calcineurin. Our present study provides evidence for activators screening from Chinese herbal medicine. More important is that calcineurin is an efficient enzyme for screening[24]. The natural products obtained from our screening offer the clues for further development of leading compounds.

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