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Inhibition of Emodin on LPS-induced Nitric Oxide Generation by Suppressing PLC-γ Phosphorylation in Rat Peritoneal Macrophages

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Abstract: **Objective** To investigate the inhibitory mechanism of emodin on lipopolysaccharide (LPS)-induced nitric oxide (NO) generation in rat peritoneal macrophages. **Methods** NO production and iNOS expression were measured through nitrite assay and Western blotting assay, respectively. NF-κB activity and nuclei P65 expression were estimated by dual-luciferase and Western blotting assay, respectively. Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was detected using the ratiometric fluorescent calcium indicator dye, Fura-2, and a microspectrofluorometer. PLC-γ phosporylation was analyzed by Western blotting assay. **Results** First, emodin was found playing active roles in suppressing LPS-induced NF-κB activation in rat peritoneal macrophages. Second, emodin down-regulated transient $[Ca^{2+}]_i$ and could increase in NF-κB upstream signal. Finally, emodin suppressed phosphorylation of PLC-γ by LPS stimulation in the upstream of $[Ca^{2+}]_i$. **Conclusion** Suppression of PLC-γ phosphorylation is involved in emodin inhibiting NO generation by LPS stimulation in rat peritoneal macrophages.

Key words: emodin; lipopolysaccharide; nitric oxide; peritoneal macrophages; PLC-γ
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Introduction

Lipopolysaccharide (LPS), one of the membrane components of Gram-negative bacteria, can trigger varieties of response to severe infections. Macrophages respond to LPS early in the infection and thus play a pivotal role in host defense and generate cytokines. Excessive production of cytokines is harmful to the host and may cause serious results, such as multiple organ dysfunction syndromes (MODS) (Su, 2002). Besides cytokines, another important mediator of inflammatory response is nitric oxide (NO). NO is generated by nitric oxide synthetase (NOS) catalysed reactions. It is known that the expression of iNOS in macrophages increases rapidly following LPS and cytokines, such as IL-1 and TNF-α stimulation (Wei *et al*, 1995; MacMicking, Xie, and Nathan, 1997).

Emodin, a naturally occurring anthraquinone derivative extracted from Chinese medicine rhubarb, was mainly used as a purgative drug. However, it has long been known for its anti-inflammatory effects (Goel *et al*, 1991; Zhang *et al*, 2001) and was used in treating MODS patients. It is known that emodin could inhibit NO production and some other inflammatory mediators in LPS-stimulated macrophages (Mijatovic *et al*, 2004). However, the detailed mechanism has not been elucidated. A report revealed that emodin inhibited inflammation related gene expression, such as TNF-α and iNOS through inhibiting LPS activated NF-κB in RAW264.7 macrophages (Li *et al*, 2005). However, transcription data are not always consistent with translation data. It is also not known how emodin affects NF-κB activation in macrophages. Previously we proposed a signaling pathway for NF-κB activation in LPS-stimulated macrophages, which mainly included a PLC-γ > intracellular free Ca^{2+} ($[Ca^{2+}]_i$) > PKC- > NF-κB axis (Zhou, Yang, and Li, 2006). It is generally accepted and

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well known that the activated PLC catalyzes the hydrolysis of PtdIns (4,5) P2 (PIP2) into Ins (1,4,5) P3 (IP3) and DAG, and triggers the downstream signaling events, such as NF-κB activation. In this process, a transient $[Ca^{2+}]_i$ increase is often observed. The activation of PLC-γ is through the phosphorylation of its tyrosine residues. In the case of LPS stimulated macrophage, the activation of PLC-γ could be mediated by some upstream nonreceptor tyrosine kinase (Zhou, Yang, and, Li, 2006). The exact kinase is not known. In another experiment, we showed that emodin could inhibit $[Ca^{2+}]_i$ increase induced by LPS (Wang *et al*, 2002). Thus, it is possible that emodin inhibits LPS-induced NF-κB activation through its effects on PLC-γ. In this paper, our results support that suppression of PLC-γ phosphorylation is involved in inhibition of LPS-induced NO generation by emodin in rat peritoneal macrophages.

Materials and methods

Reagents

Emodin (99% purity) was obtained from Tianjin Institute of Pharmaceutical Research (Tianjin, China). Dimethylsulfoxide (DMSO), LPS (*Escherichia coli* serotype 0127: B8 prepared by phenol extraction), Fura-2 acetoxymethyl ester (Fura-2/AM) and EGTA were from Sigma. RPMI1640 was from GIBCO. The rats were from Academy of Military Medical Sciences.

Isolation and culture of macrophages

Male Wistar rats (about 250 g), treated humanely in compliance with institutional guidelines, were killed. Hanks' balanced salt solution (HBSS) was injected into the abdomen of each rat. Macrophages in HBSS removed from the abdomen were centrifuged at 200 × *g* for 5 min. The cell pellet was collected, and the cells were cultured in RPMI 1640 and 10% fetal bovine serum for 6 h at 37 ℃ in 5% CO_2. Adherent cells were harvested, resuspended, and incubated for another 48 h before analysis. Nonspecific esterase staining showed that 95% of the adherent cells were macrophages.

Immunoblotting assay to detect PLC-γ phosporylation

Cells (10^7) were scraped from dishes into an cold lysis buffer [0.1 mol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P-40, 20 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L phenyl-methylsulphonyl fluoride (PMSF) and 1 μg/mL each of pepstatin and leupeptin] after being washed twice in cold PBS. Following centrifugation (10 000 × *g* for 10 min at 4 ℃), the soluble fraction containing PLC was collected. The proteins in the soluble fraction were separated by SDS-PAGE (7%) and transferred to polyvinylidene fluoride (PVDF) membranes. Immunoblotting was carried out by treating the membrane with 4.5 μg of antibody specific for activated phosphotyrosines of PLC-γ1 or PLC-γ2 (Cell Signaling Technology: ratio of antibody dilution, 1∶200) for 12 h. Biotinylated antibody specific for rabbit immunoglobulin (Amersham Biosciences) was employed as the secondary antibody. The chemi-luminescence of the streptavidin-horseradish peroxidase conjugates was detected by film.

Transient transfections and dual-luciferase assays for NF-κB activation

Transfection was done by using a standard procedure for calcium phosphate precipitation. Macrophages were transfected with 3 μg luciferase reporter plasmids that contained an NF-κB gene (pNF-κB-luc) and 3 μg of pRL-TK reporter plasmids (pRL-TK- Renilla). After 48 h, samples were harvested and prepared for luciferase assays according to the manufacturer's protocol (Promega Corp.). Firefly and Renilla luciferase activities were measured in each sample using the Dual Luciferase Assay kit (Promega Corp.) (Cao *et al*, 2002).

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was detected by using the ratiometric fluorescent calcium indicator dye Fura-2 and a micro-spectro fluorometer (Olympus Fluoview FV500 IX51). Cells were incubated in 3 μmol/L Fura-2/AM for 50 min at room temperature and washed twice with PBS. Changes in the fluorescence intensity of Fura-2 at excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm were monitored in individual peritoneal macrophages. $[Ca^{2+}]_i$ was calculated by using the following equation:

$$[Ca^{2+}]_i \approx k_d \times \frac{[Ca-Fura-2]}{Fura-2} \approx k_d \times \frac{F_{380}}{F_{360}}$$

k_d was the constant for Fura-2 chelating $[Ca^{2+}]_i$, and its value was about 135 nmol·L^{-1} when the temperature was 22 ℃. In the present experiment, F_{340}/F_{380} was directly related to $[Ca^{2+}]_i$

Western blotting assays for cytosolic fractions (IκB) in cytoplasm and nuclear fraction (P65) in nuclei

The assays were performed as described previously (Zhou, Yang, and Li, 2006). Briefly, cells were washed in cold PBS and proteins were harvested in cold lysis buffer containing protease inhibitors and stored at −70 ℃. IκB and P65 were separated by SDS–PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). After non-specific binding sites were blocked by incubation overnight at 4 ℃ with 1% bovine serum albumin, the membranes were then incubated with polyclonal antibodies specific for IκB or P65 (Santa Cruz Biochemicals). Biotinylated antibody to rabbit immunoglobulin (Amersham Life Science) was employed as the secondary antibody. The chemiluminescence of the streptavidin-HRP conjugates was detected by film (Haddad *et al*, 2001).

Immunoblotting assay to detect iNOS expression

After treatment, macrophages (10^6) were lysed in SDS sample buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-ME, and 0.02% bromophenol blue] and boiled for 5 min at 100 ℃. Aliquots (20 μg/lane) underwent electrophoresis in a 10% polyacrylamide gel, and the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). Membranes were treated with 10% nonfat milk for 1 h to block nonspecific binding sites, rinsed, and incubated with rabbit polyclonal antibody to iNOS (Santa Cruz Biotechnology) overnight at 4 ℃. The membranes were then treated with HRP-conjugated antirabbit IgG (dilution ratio, 1∶2000) for 1 h. Immune complexes were detected as described (Ajizian, English, and Meals, 1999).

Measurement of NO by nitrite assay

NO production was determined by measuring the stable nitrite product NO_2^- using a colormetric assay. Briefly, 100 μL supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl) ethylenediamine/2HCl in 2.5% H_3PO_4) at room temperature for 10 min. Samples were analyzed at 540 nm on a microplate spectrophotometer. Nitrite concentration was calculated from a $NaNO_2$ standard curve prepared with sodium nitrite 0–50 μmol/L) (Ajizian, English, and Meals, 1999).

Statistical analysis

All assays were repeated a minimum of three times, and transient $[Ca^{2+}]_i$ changes' representative results from a single measurement were shown. Some results were expressed as mean ± SD. We used *t*-test as a measure of statistical significance. Significance was determined based on $P < 0.05$.

Results

Emodin reduced LPS induced NF-κB activation, iNOS expression, and NO production

To confirm emodin's inhibition on LPS-stimulated NF-κB activation in non-cell line macrophage and in translation level, emodin at different concentrations was co-administered with 10 μg/mL LPS to the cells. Fig. 1 shows that LPS-induced NO generation was greatly decreased by addition of emodin, especially during 8 to 14 h. Emodin 10 μmol/L had greater inhibitory effect than emodin 1μmol/L. The inhibitory effect is time-dependent. However, there was a basic level of NO in the cells treated with LPS, which could not be overcome by emodin in either concentration. Nevertheless, these results indicated that NOS activity was inhibited.

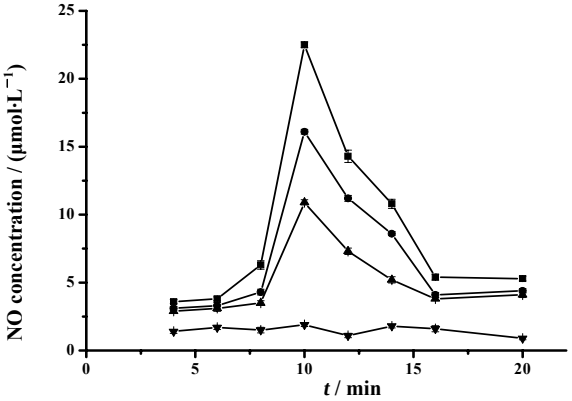

Fig. 1 Reduction of emodin on production of NO in rat peritoneal macrophages

■ Cells were treated with 10 μg·mL^{-1} LPS only

● 10 μg·mL^{-1} LPS + 1 μmol·L^{-1} emodin

▲ 10 μg·mL^{-1} LPS + 10 μmol·L^{-1} emodin

▼ control, no LPS or emodin. NO concentrations were measured at 4, 6, 8, 10, 12, 14, 16, and 20 h with the method described in material and methods. At time points 8–16 h, the difference between the samples with different emodin concentrations is significant ($P < 0.05$)

To see if emodin's inhibitory effect on NO production was a result of iNOS inhibition, we examined the expression of iNOS in the time range of 4–18 h. In Fig. 2, the Western blotting results revealed that LPS-induced iNOS expression was decreased by addition of 1 or 10 μmol/L emodin. The inhibitory effect was also time-dependent, and was most significant at 10 h, when NO production reached a peak. Similar to the case of NO production, there was a basic

iNOS expression induced by LPS, which could not be abrogated by emodin. The correlativity between the inhibitory effects on iNOS expression and NO production was strong.

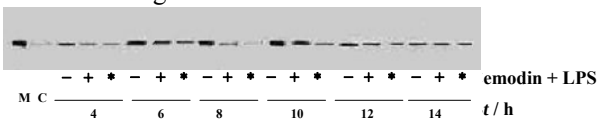

Fig. 2 Effect of emodin on the expression of iNOS induced by LPS in rat peritoneal macrophages

Cells were collected after treated with 10 μg·mL^{-1} LPS or 10 μg·mL^{-1} LPS + emodin (–: 0 μmol·L^{-1}, +: 1 μmol·L^{-1} or ∗: 10 μmol·L^{-1}) for 4, 6, 8, 10, and 14 h. iNOS was measured by Western blotting assay described in material and methods. The expression of iNOS was induced by LPS and emodin depressed it in a dose- and time-dependent manner. The column diagram displayed the density of the Western boltting lane (the density was described as the folds of the control lane where cells were treated with medium only)

M: Marker lane; C: control lane where cells treated with only medium

To confirm that the inhibitory effect on iNOS expression was due to inhibition of NF-κB activation, dual luciferase assays of NF-κB activation at different time points were performed. Fig. 3A shows that emodin (10 μmol/L) caused a decrease of luciferase activity compared with that in cells under LPS stimulation only. The changes of luciferase activity were indications of the activation of NF-κB. It's most prominent at 6 and 4 h prior to the iNOS expression and NO generation reached their climax. Similar results were observed in Fig. 3B using the level of P65 in nucleoli as a designator. Not surprisingly, the addition of emodin also reduced the accumulation of IκB in cytoplasm (data not shown). Interestingly, the LPS-induced activation of NF-κB could not be totally eliminated by emodin either.

Taken together, the above results indicated that the inhibitory effect of emodin on NO production in LPS-stimulated macrophages (non-cell line) was a result of the inhibition of iNOS and NF-κB activation. But there still exist NF-κB activation pathways which can not be suppressed by emodin.

Emodin inhibited LPS-stimulated transient increase in $[Ca^{2+}]_i$

The activation of NF-κB involved phosphorylation of IKK and its upstream kinases by PKCs, which can be activated in Ca^{2+} dependent or independent manner. To test if the upstream signal of $[Ca^{2+}]_i$ is involved in emodin's inhibition to LPS-stimulated macrophages, $[Ca^{2+}]_i$ was detected by using fluorescent indicator dye Fura-2. Emodin at different concentrations (10 or 1 μmol/L) was co-administered with 10 μg/mL LPS to the cells with or without extracellular calcium. Fig. 4 shows some representative traces of the transient response of $[Ca^{2+}]_i$. After a latent time of about 110 s, LPS-induced $[Ca^{2+}]_i$ increased with a time course of about 300 s. After that, the $[Ca^{2+}]_i$ recovered to normal

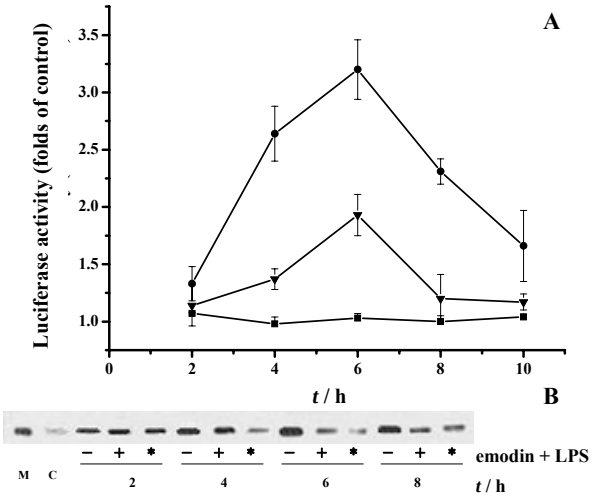

Fig. 3 Reduction of emodin on NF-κB activation induced by LPS in rat peritoneal macrophages

A: Dual luciferase assay of NF-κB activation at different time

● Reporter plasmids were transiently transfected into rat peritoneal macrophages for 48 h and further treated with 10 μg·mL^{-1} LPS

▼ 10 μg·mL^{-1} LPS + 10 μmol·L^{-1} emodin for 2, 4, 6, 8, and 10 h. The luciferase activity at 0 h in the control group was defined as 1.

■ control

B: Immunoblot assay of P65 translocation into nucleoli at different time. Cells were treated with 10 μg·mL^{-1} LPS only, or 10 μg·mL^{-1} LPS + emodin (–: 0 μmol·L^{-1}, +: 1 μmol·L^{-1} or ∗: 10 μmol·L^{-1}) and sampled at 2, 4, 6, and 8 h. M: marker lane; C: control lane where cells were treated with only medium. The difference of luciferase activity by LPS with different emodin concentration after 2 h were significant ($P < 0.05$)

level. For both situations (with or without extracellular calcium), emodin decreased the transient increase in $[Ca^{2+}]_i$ stimulated by LPS dose-dependently. In Fig. 4, traces II and V show the intracellular calcium released from calcium store could be influenced by LPS and emodin. Thus, the mechanism of emodin inhibited LPS induced macrophage activation might involve an inhibition of calcium release from calcium store. This inhibition might be a result of inhibition of PLC-γ so that IP_3 generation is decreased.

Emodin inhibited LPS induced phosphorylation of PLC-γ

To investigate whether PLC-γ was influenced by

emodin, we examined emodin's effect on LPS-induced tyrosine phosphorylation of PLC-γ2 (Fig. 5). The rat peritoneal macrophages were treated with 10 μg/mL LPS or 10 μg/mL LPS + 1 or 10 μmol/L emodin for 5, 10, 20, and 30 min. LPS-induced tyrosine phosphorylation of PLC-γ2 for about 20 min. The phosphorylation reached a maximum at 10 min. Emodin suppressed LPS-induced phosphorylation of PLC-γ2 in a dose-dependent manner. These results indicated that emodin inhibited LPS-induced phosphorylation of PLC-γ2, although the exact kinase responsible for this phosphorylation is not known.

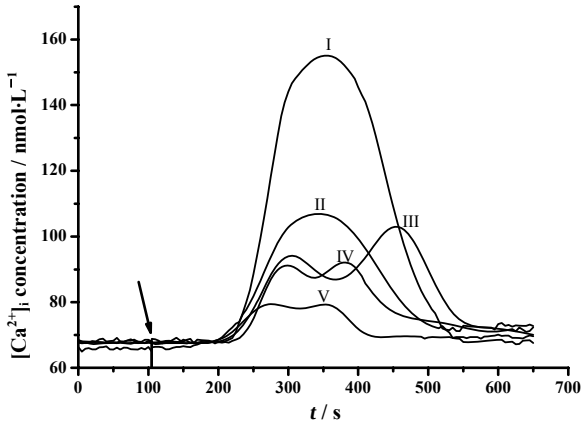

Fig. 4 Emodin inhibited the transient increase in $[Ca^{2+}]_i$ evoked by LPS in rat peritoneal macrophages

$[Ca^{2+}]_i$ changes were monitored fluorometrically with the fluorescence indicator Fura-2 as described in Materials and Methods. The conditions were: 10 μg/mL LPS only (I), + 1 μmol·L^{-1} Emodin (III) or 10 μmol·L^{-1} emodin (IV) in normal medium; 10 μg·mL^{-1} LPS only (II), or + 10 μmol·L^{-1} emodin (V) in calcium-free medium '↓' designated the time point when reagents were administrated

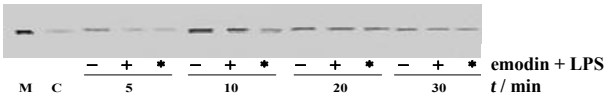

Fig. 5 Emodin decreased LPS-induced phosphorylation of PLC-γ2 in rat peritoneal macrophages

Cells were collected after treated with 10 μg·mL^{-1} LPS or 10 μg·mL^{-1} LPS + emodin (–: 0 μmol·L^{-1}, +: 1 μmol·L^{-1} or ∗: 10 μmol·L^{-1}) for 5, 10, 20, and 30 min. Phosphatized PLC-γ2 was measured by immunoblotting assay described in material and methods. Phosphorylated PLC-γ2 was up-regulated by LPS and emodin suppressed the effect in a dose- and time- dependent manner

M: marker lane; C: control lane where cells were treated with medium only

Discussion

The PLC-γ2 isoform had previously been associated with LPS-induced signalling pathway (Aki *et al*, 2008). Our previous work showed that activation of PLC-γ2 might be modulated by LPS triggered PTK activation (Zhou, Yang, and Li, 2006). Emodin was an effective component of rhubarb, which was clinically used mainly for its' purgative effect. It was also used as anticancer agent because it can inhibit some protein kinases' activity, such as p65lck, CKII (Jayasuriya *et al*, 1992; Yim *et al*, 1999). Structure analysis shows that emodin can bind competitively to the ATP binding site in catalyzing domain of CKII and inhibit or decrease CKII's activity (Battistutta *et al*, 2000). Moreover, emodin was used as anti-inflammatory agent because it reduced the production of some cytokines such as IL-2, TNF-α in cells (Huang *et al*, 1992; Kuo *et al*, 2001). As indicated in the introduction section, this is concerned with suppression of NF-κB activation.

In this paper, we proved that emodin could inhibit LPS-induced phosphorylation of PLC-γ2 in rat peritoneal macrophages. The suppression of PLC-γ2 weakened the activation of its downstream, such as the transient increase in $[Ca^{2+}]_i$ and PKC activation. There are evidences that PKC may participate in phosphorylation of serine in MEKK1 in LPS-stimulated rat peritoneal macrophages and thus affect the activation of NF-κB (Zhou, Yang, and Li, 2006). Sequentially, NF-κB activation was reduced when PKC activity was down-regulated. Then, the expression of iNOS and thus the production of NO were also suppressed. Since there is no report that emodin can act on PLC-γ2 directly, one possible target of emodin might be a PLC-γ upstream PTK, although the exact kinase is not known. This is inferred from its' anticancer effect. Our results also show that LPS has a basic elevation of NO, iNOS, and NF-κB, which could not be totally blocked by emodin. This means there are some targets of LPS or their downstream that could not be affected by emodin. But at least, in the regulatory net, suppression of PLC-γ2 phosphorylation is surely an important event, as shown by this work.

In general, this work showed that emodin could inhibit NF-κB activation in LPS-stimulated peritoneal macrophages of rats by suppressing the phosphorylation of PLC-γ2. Through PLC-γ2-$[Ca^{2+}]_i$-NF-κB pathway, LPS-reduced elevation of NO production could be decreased by emodin, which may partly account for its anti-inflammatory effect.

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