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Inhibitory mechanisms of YC-1 and PMC in the induction of iNOS expression by lipoteichoic acid in RAW 264.7 macrophages

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Abstract

In the present study, the signal pathways involved in NO formation and iNOS expression in RAW 264.7 macrophages stimulated by LTA were investigated. We also compared the relative inhibitory activities and mechanisms of PMC, a novel potent antioxidant of α-tocopherol derivatives, with those of YC-1, an sGC activator, on the induction of iNOS expression by LTA in cultured macrophages *in vitro* and LTA-induced hypotension *in vivo*. LTA induced concentration (0.1–50 μg/mL)- and time (4–24 hr)-dependent increases in nitrite (an indicator of NO biosynthesis) in macrophages. Both PMC (50 μM) and YC-1 (10 μM) inhibited NO production, iNOS protein, mRNA expression, and IκBα degradation upon stimulation by LTA (20 μg/mL) in macrophages. On the other hand, PMC (50 μM) almost completely suppressed JNK/SAPK activation, whereas YC-1 (10 μM) only partially inhibited its activation in LTA-stimulated macrophages. Moreover, PMC (10 mg/kg, i.v.) and YC-1 (5 mg/kg, i.v.) significantly inhibited the fall in MAP stimulated by LTA (10 mg/kg, i.v.) in rats. In conclusion, we demonstrate that YC-1 shows more-potent activity than PMC at abrogating the expression of iNOS in macrophages *in vitro* and reversing delayed hypotension in rats with endotoxic shock stimulated by LTA. The inhibitory mechanisms of PMC may be due to its antioxidative properties, with a resulting influence on JNK/SAPK and NF-κB activations. YC-1 may be mediated by increasing cyclic GMP, followed by, at least partly, inhibition of JNK/SAPK and NF-κB activations, thereby leading to inhibition of iNOS expression.

Keywords: YC-1; PMC; LTA; Cyclic GMP; iNOS; JNK/SAPK; NF-κB; Endotoxic shock

1. Introduction

In septic shock, a circulating bacterial endotoxin (Gram-negative, LPS) induces cardiovascular alterations including systemic arterial hypotension and a hyporesponsiveness to pressor agents [1]. The overproduction of NO generated by iNOS is a deleterious factor in inflammatory vasculopathies, and NO also contributes to vascular failure in endotoxic shock [1]. The contribution of NO to the pathophysiology of septic shock is highly heterogeneous, and inhibition of iNOS expression and/or activity still represents a rational therapeutic goal [2].

Traditionally recognized as a consequence of Gram-negative bacteremia, however, septic shock is also caused by Gram-positive organisms, fungi, and probably viruses and parasites. Although relatively rare in the 1970s, the incidence of Gram-positive septic shock has markedly increased over the past 15 years [1]. On the basis of this evidence, it seems reasonable to conclude that between one-third and one-half of all cases of sepsis are currently caused by Gram-positive organisms, and that the incidence of Gram-positive sepsis should continue to rise for at least the next few years [1].

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Abbreviations: BHA, butylated hydroxyanisole; LTA, lipoteichoic acid; LPS, lipopolysaccharide; IFN-γ, interferon-γ; IκBα, NF-κB inhibitory protein; iNOS, inducible nitric oxide synthase; ERK, extracellular signal-regulated kinase; JAK/STAT, janus kinases/signal transducers and activators of transcription; JNK/SAPK, c-Jun-N-terminal kinase/stress activated protein kinase; mAb, monoclonal antibody; MAP, mean arterial blood pressure; MAPK, mitogen-activated protein kinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NF-κB, nuclear factor-κB; ODQ, (1,2,4)oxadiazolo[4,3-*a*]quinozalin-1-one; PBS, phosphate-buffered saline; PKC, protein kinase C; PMC, 2,2,5,7,8,-pentamethyl-6-hydroxychromane; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; TNF-α, tumor necrosis factor-α; VSMC, vascular smooth muscle cell; YC-1, 1-benzyl-3-(5′-hydroxymethyl-2′-furyl) indazole.

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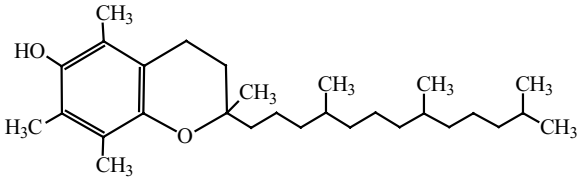
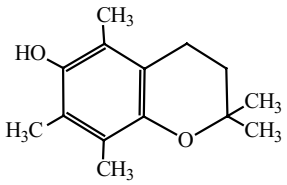
The formation of NO by iNOS is correlated with and primarily regulated at the level of iNOS mRNA in a variety of cell types [3]. The signal transduction cascades of expression of iNOS stimulated by LPS or cytokines such as TNF-α, interleukin-1, and IFN-γ are thought to be mediated through different signal pathways, including NF-κB, MAPK, and JAK/STAT [3]. iNOS mRNA is induced after encountering activated free or phosphorylated transcription factors (e.g. NF-κB, AP-1, and the ATF/cyclic AMP response element binding domain) in the nucleus where they activate transcription by binding to specific iNOS gene promoters [4,5]. Among these transcription factors, only NF-κB has been demonstrated to mediate the enhanced expression of the iNOS gene in macrophages stimulated by LPS [6]. On the other hand, LTA, a component associated with the membrane of Gram-positive bacteria [7], can induce the expression of iNOS in cultured VSMCs [8] or in macrophages [9] *in vitro*. LTA has therefore been implicated as the major component of the Gram-positive bacterial cell wall which by itself and/or via cytokines release is responsible for the induction of iNOS. However, the signal transduction events leading to the expression of iNOS by LTA are unclear and have only relatively rarely been compared with those of LPS.

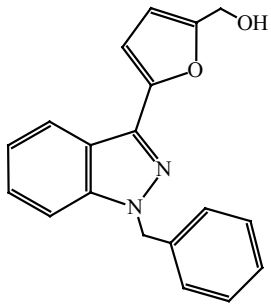
PMC is the most potent derivative of α-tocopherols in antioxidation and inhibits the activation of NF-κB [10–12] (Fig. 1). It is more hydrophilic than other α-tocopherol derivatives, and has potent free radical-scavenging activity [10]. On the other hand, the benzylindazole-derivative, YC-1, has been described as an NO-independent activator of sGC [13,14] (Fig. 1). In this study, we investigated the intracellular signal pathways in LTA-mediated iNOS expression in macrophages, and concurrently compared the relative inhibitory activities of PMC and YC-1 in this effect. We therefore utilized these findings to characterize the relationship between the inhibition of NO production *in vitro* and protective LTA-induced hypotension *in vivo* in both PMC and YC-1.



Alpha-tocopherol



PMC



YC-1

Fig. 1. Chemical structures of α-tocopherol, PMC, and YC-1.

2. Materials and methods

2.1. Materials

PMC was obtained from Wako Pure Chem. YC-1 was donated by Prof. C.M. Teng of the Pharmacological Institute, College of Medicine, National Taiwan University. Both drugs were dissolved in 0.2% DMSO. In each experiment, DMSO was employed at a constant final concentration (0.2%, v/v). LTA (from *Staphylococcus aureus* L-2515), MTT, HEPES, NP-40, SDS, phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol, cremophor EL, ODQ and leupeptin were purchased from Sigma. Murine anti-iNOS mAb was purchased from Transduction Lab. Anti-mouse IgG linked to horseradish peroxidase and the Western blotting detection system (ECL$^+$ plus) were purchased from Amersham.

2.2. Cell culture

RAW 264.7 cells, a murine macrophage cell line, were obtained from American Type Culture Collection (ATCC) and grown in DMEM supplemented with 10% fetal calf serum (FCS) and 100 units/mL penicillin G/100 mg/mL streptomycin in a humidified 37° incubator.

2.3. Determination of nitrite concentration

NOS activity was assessed by measuring the accumulation of nitrite in the supernatant of RAW 264.7 macrophages using the Griess reaction [15]. Briefly, RAW 264.7 cells (1×10^6 cells/mL; 500 μL per well) were dispensed onto 24-well plates until 80% confluency and then treated with various concentrations of LTA (0.1–50 μg/mL) for 24 hr or LTA (20 μg/mL) for the indicated times (4–24 hr). In some experiments, cells were pretreated with various concentrations of PMC (1–100 μM) or YC-1 (0.5–10 μM) for 30 min followed by the addition

of LTA (20 μg/mL) for stimulation for 24 hr. For determination of nitrite accumulation, supernatants were analyzed via a colorimetric reaction based on the Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 2.5% phosphoric acid). The optical absorbance at 550 nm was measured using a microplate reader (MRX microplate reader). Nitrite concentrations were calculated by regression with standard solutions of sodium nitrite prepared in the same culture medium.

2.4. Cell viability

RAW 264.7 cell viability after 24 hr of continuous exposure to LTA (50 μg/mL) was measured with a colorimetric assay based on the ability of mitochondria in viable cells to reduce the MTT as described previously [16]. The percentage of cell viability was calculated as the absorbance of treated cells/control cells × 100%.

2.5. Western blot analysis

For determination of the expression of iNOS, MAPKs, and NF-κB in RAW 264.7 cells, Western blot analyzes were performed as described previously [17]. Briefly, RAW 264.7 cells were cultured in 100-mm petri dishes. After reaching confluence, cells were pretreated with PMC (50 μM) or YC-1 (10 μM) for 30 min followed by the addition of LTA (20 μg/mL) for 24 hr or indicated times in a humidified incubator at 37°. After incubation, cells were washed with ice-cold PBS buffer (pH 7.3). Proteins were extracted with lysis buffer (10 mM Tris–HCl, 140 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 1 mM DTT, 2 mM PMSF, 1 μM aprotinin, and 1 μM leupeptin, pH 7.0) for 30 min. Additionally, phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate, and 10 mM sodium pyrophosphate) were added to the lysis buffer for phosphorylated MAPK analysis. Lysates were centrifuged, and the supernatant (50 μg protein) was subjected to SDS–PAGE, and electrophoretically transferred onto PVDF membranes (0.45 μm; Hybond-P; Amersham). After incubation in blocking buffer (50 mM Tris–HCl, 100 mM NaCl, 0.1% Tween 20, 5% dry skim milk, pH 7.5) overnight at 4° and being washed three times with PBS buffer, blots were treated with either an anti-iNOS mAb (1:2000; Transduction Lab.), anti-MAPK mAb (1:2000; Transduction Lab.), or a rabbit anti-human IκBα Ab (1:3000; Santa Cruz Biotech) in PBS buffer for 3 hr. They were subsequently washed three times with PBS buffer and incubated with peroxidase-conjugated goat anti-mouse or anti-rabbit Abs (Amersham) for 2 hr. Blots were then washed three times, and the band with peroxidase activity was detected using film exposure with enhanced chemiluminescence detection reagents (ECL^+ system; Amersham). Densitometric analysis of specific bands was performed with a Photo-Print Digital Imaging System (IP-008-SD) with analytic software (Bio-1Dlight, V 2000).

2.6. Isolation of total RNA and reverse-transcription polymerase chain reaction

Total RNA was isolated from RAW 264.7 cells by a commercially available kit according to the manufacturer's instructions (TRIzol, Gibco). For each RT-PCR reaction, 0.5 mg of the RNA sample and 0.2 μM of primers were reverse-transcribed and amplified in a 50-mL reaction mixture of commercially available reagents (Super Script On-Step RT-PCR system, Gibco) containing a 1× reaction mixture and 0.2 μM of an RT/Taq mixture in one cycle of 30 min at 50° for reverse transcription and one cycle at 94° for 2 min; followed by 35 cycles at 94, 60, and 72° for 15, 30 s, and 1 min, respectively; with a single extension step at 72° for 5 min followed by 4° for amplification in a thermal cycler (GeneAmp PCR system 2400, Perkin-Elmer). The primers used to target the iNOS mRNA were 5′-CTGGCAGCAGCGGCTCCATG-3′ (sense) at base positions 2987–3006 and 5′-GAAAAGACCGCACCGAAGAT-3′ (antisense) at base positions 3389–3409 of rat iNOS cDNA [18]. The GAPDH primers were 5′-GCCGCCTGGTCACCAGGGCTG-3′ (sense) and 5′-ATGGACTGTGGTCATGAGCCC-3′ (antisense). For visualization and quantification by densitometry of each RT-PCR reaction, a 10-μL aliquot was subjected to electrophoresis on a 1.0% agarose gel using a mini horizontal submarine unit (HE 33) containing 0.5 mg/mL ethidium bromide to allow UV-induced fluorescence (TCP-20.M, Vilber Lourmat). Densitometric analysis of the bands of the PCR products was performed as previously described. Preliminary experiments were performed to determine the range of amplification cycles and the beginning RNA substrate within the linear phase of the exponential increase of PCR products for each particular primer pair.

2.7. Endotoxic shock

Male Wistar rats (200–300 g) used in this study were obtained from the Department of Laboratory Animal Center of National Taiwan University. All animal experiments and care were performed according to the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, 1996). PMC (10 mg/kg) and YC-1 (5 mg/kg) were dissolved in cosolvent as cremophor EL/ethanol (1:1) before injection. Control rats were injected with an equivalent volume of the vehicle solution (cosolvent).

Rats were anesthetized by an intraperitoneal injection of a combination of urethane (0.5 g/kg) and chloral hydrate (0.4 g/kg). The trachea was cannulated to facilitate respiration, and the rectal temperature was maintained at 37° with a homeothermic blanket. A PE-50 tube was inserted into the right femoral artery for measuring hemodynamic parameters using a pressure transducer (P23ID). The left femoral vein was cannulated to administer the drug or vehicle solution. The hemodynamic parameters were displayed on a Grass polygraph recorder (model 7D; Grass

Instruments). Animals were allowed to stabilize for at least 30 min, during which time arterial pressure and heart rate were continuously monitored. To examine the effects of PMC and YC-1 on the hemodynamic parameters during Gram-positive-induced endotoxemia, PMC, YC-1, and the solvent control (cosolvent) were separately administered 30 min before LTA (10 mg/kg, i.v.) treatment, and animals were continuously monitored for 5 hr. In our preliminary test, we chose three doses (2, 5, and 10 mg/kg) of PMC and YC-1 to evaluate and compare the relative inhibitory activities of both drugs in LTA-induced septic shock *in vivo*. We found that PMC at 10 mg/kg and YC-1 at 5 mg/kg reach submaximal effects, respectively. Therefore, we fixed these doses of both drugs in the following experiments. At the end of the experiment, plasma was collected for the measurement of nitrite.

2.8. Statistical analysis

The experimental results are expressed as the means ± SEM and are accompanied by the number of observations. Data were assessed by Student's unpaired *t*-test. ANOVA followed by a multiple comparison test (Scheffe's test) was used to determine significant differences in the study of endotoxic shock. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of PMC and YC-1 on nitrite production and iNOS expression in LTA-stimulated RAW 264.7 cells

Within 24 hr, LTA (0.1–50 μg/mL) induced a concentration-dependent increase in nitrite production in the culture medium of RAW 264.7 macrophages of from 2.3 ± 0.1 μM (resting) to 6.8 ± 0.1 μM (20 μg/mL) (Fig. 2A). Furthermore, the increase in nitrite production was also time-dependent and reached a maximal level at 24 hr (Fig. 2B). There were no significant differences in cell viability in LTA-treated cells compared to control cells (normal saline treated) for 24 hr observed at the highest concentration of LTA in the MTT assay (50 μg/mL, >95% of control, N = 5). Therefore, cells were treated with LTA at 20 μg/mL for 24 hr in the following experiments.

After pretreatment of cells with various concentrations of PMC (1–100 μM), an α-tocopherol derivative, or YC-1 (0.5–10 μM), an sGC activator, for 30 min followed by the addition of LTA (20 μg/mL), we found that both PMC (20–100 μM) and YC-1 (0.5–10 μM) concentration-dependently respectively inhibited nitrite production stimulated by LTA (Fig. 3A and B). The IC_{50} values of PMC and YC-1 at inhibiting this reaction were about 47.8 ± 5.0 μM and 4.3 ± 1.3 μM, respectively. On a molar basis, YC-1 was about 11-fold more potent than PMC in this reaction. However, neither PMC (50 μM) or YC-1 (10 μM) nor

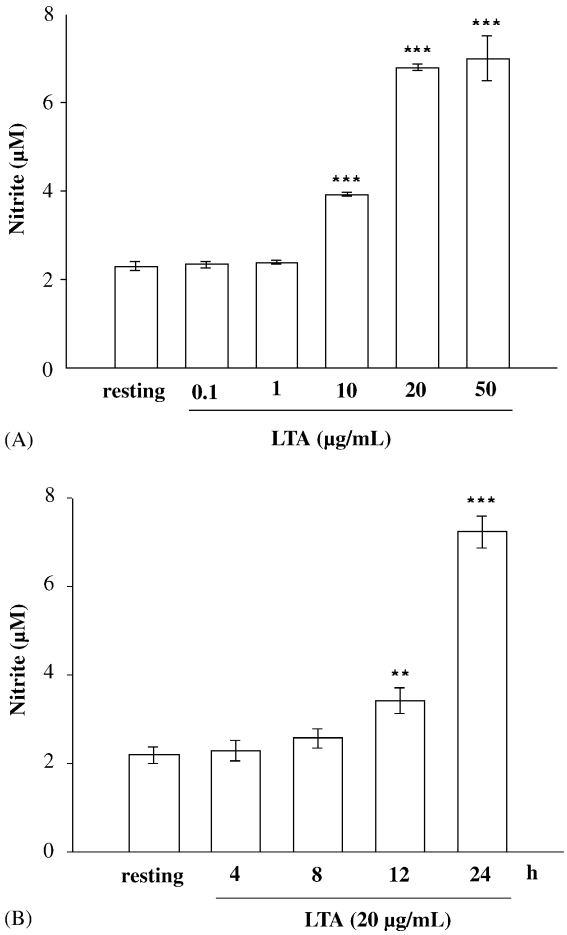


Fig. 2. Concentration- and time-dependent increases in nitrite formation stimulated by LTA in RAW 264.7 macrophages. Cells (1×10^6 cells/mL) were treated with (A) various concentrations of LTA (0.1–50 μg/mL) for 24 hr or (B) with LTA (20 μg/mL) for the indicated times (4–24 hr). Cell-free supernatants were assayed for nitrite production as described in Section 2. Data are presented as the means ± SEM (N = 5). $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared with the resting group.

the solvent control (0.2% DMSO) affected the cell viability of macrophages according to the MTT assays (data not shown). Furthermore, PMC and YC-1 neither interfered with the Griess reaction nor reacted with native NO. These results demonstrate that both PMC and YC-1 markedly suppress NO formation stimulated by LTA in RAW 264.7 cells.

3.2. Effects of PMC and YC-1 on the expression of iNOS protein and mRNA in LTA-stimulated RAW 264.7 cells

As shown in Fig. 4A, LTA (20 μg/mL)-induced expression of iNOS protein was markedly detectable as compared with that of the control group (resting) after a 24-hr treatment (Fig. 4A, lanes 1 and 2). Pretreatment with PMC or YC-1 for 30 min before LTA treatment revealed that both PMC (50 μM) and YC-1 (10 μM) markedly inhibited the expression of iNOS protein by about 75 and 92%, respectively (Fig. 4A, lanes 3 and 4). On the other hand, ODQ (6 μM), an inhibitor of sGC [19],

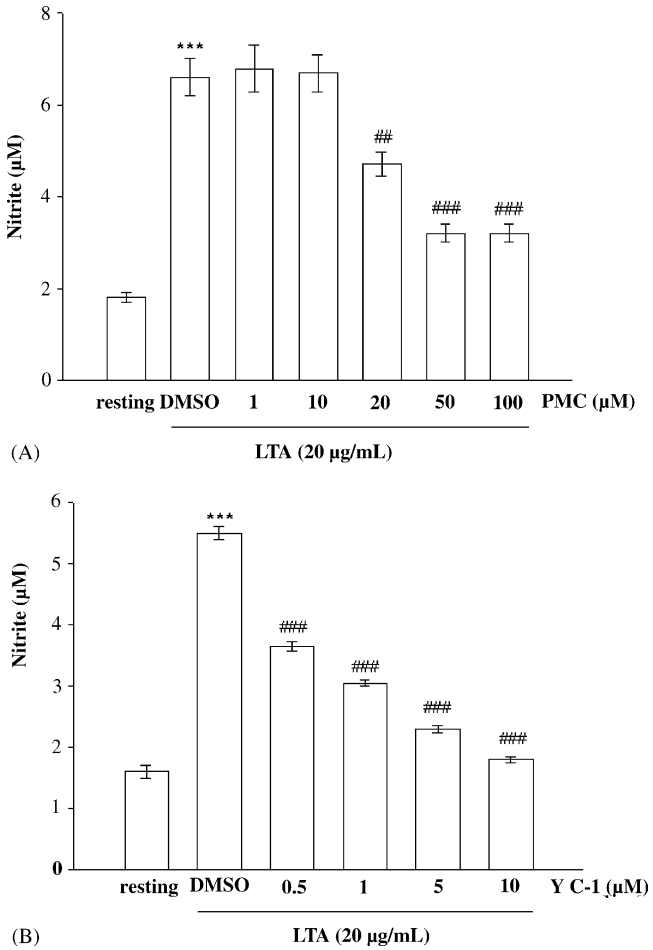


Fig. 3. Effects of PMC and YC-1 on LTA-induced nitrite formation in RAW 264.7 macrophages. Cells (1×10^6 cells/mL) were treated with solvent control (0.2% DMSO) or various concentrations of (A) PMC (1–100 μM) or (B) YC-1 (1–10 μM) for 30 min followed by the addition of LTA (20 μg/mL) for 24 hr. Cell-free supernatants were assayed for nitrite production as described in Section 2. Data are presented as the means ± SEM (N = 5). $^{***}P < 0.001$ as compared with the resting group; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ as compared with the LTA group.

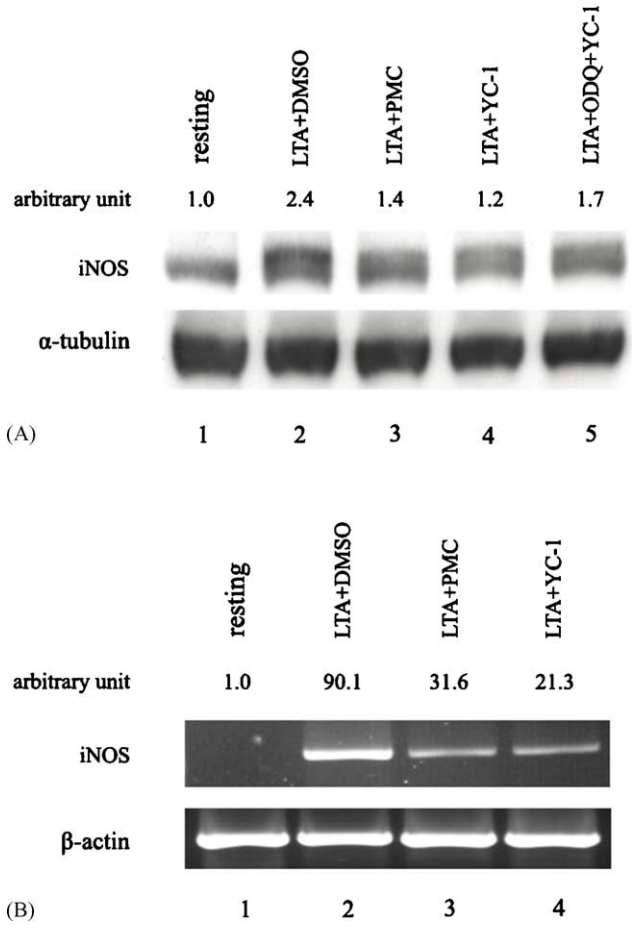


Fig. 4. Effects of PMC and YC-1 on LTA-induced expression of iNOS protein and mRNA in RAW 264.7 macrophages. Cells (1×10^6 cells/mL) were treated with solvent control (0.2% DMSO), PMC (50 μM), or YC-1 (10 μM), or ODQ (6 μM) plus YC-1 (10 μM) for 30 min followed by the addition of LTA (20 μg/mL) for 24 hr. Cells were collected and subcellular extracts were analyzed for the expression of (A) iNOS protein and (B) mRNA. Lane 1, cells treated with normal saline only (resting group); cells pretreated with solvent control (lane 2, 0.2% DMSO), PMC (lane 3, 50 μM), YC-1 (lane 4, 10 μM) or ODQ (6 μM) plus YC-1 (10 μM) (lane 5) followed by the addition of LTA (20 μg/mL). The densitometric data was expressed by the relative arbitrary unit on the top of bands. The results are representative examples of four similar experiments. (A) Equal loading in each lane is demonstrated by similar intensities of α-tubulin. (B) The β-actin levels were used to normalize the amount of the cDNA template used in each PCR reaction.

significantly reversed the inhibitory effect of YC-1 in this effect (Fig. 4A, lane 5). Furthermore, 8-bromo-cyclic GMP (80 μM) also markedly inhibited the expression of iNOS protein induced by LTA (data not shown). These results indicate that YC-1 stimulates, at least partly, the cyclic GMP-dependent mechanism, leading to inhibition of iNOS expression.

Furthermore, LTA (20 μg/mL) markedly stimulated an increase in iNOS mRNA in RAW 264.7 cells as compared with the resting control (Fig. 4B, lanes 1 and 2). Pretreatment with PMC (50 μM) or YC-1 (10 μM) for 30 min markedly reduced the expression of iNOS mRNA stimulated by LTA, respectively (Fig. 4B).

3.3. Effects of PMC and YC-1 on NF-κB and MAPK activations

To further investigate the inhibitory mechanisms of PMC and YC-1 on the reduction of iNOS expression in LTA-stimulated RAW 264.7 cells, we detected several signaling molecules including IκBα and MAPKs, which we refer to as p38 MAPK, p42/44 MAPK (ERK 1/2), and p46/54 MAPK (JNK/SAPK). The immunoblotting analysis revealed that treatment with LTA (20 μg/mL) caused a rapid and time-dependent disappearance in the immunoreactive bands of IκBα (Fig. 5A). IκBα protein was markedly degraded within 30 min after LTA stimulation, and returned to basal levels after 40 min. Both PMC (50 μM) and YC-1 (10 μM) significantly attenuated the degradation of the immunoreactive bands of IκBα with 30 min of LTA stimulation (Fig. 5B).

Stimulation of cells with LTA (20 μg/mL) at various time points (5–40 min) resulted in marked time-dependent

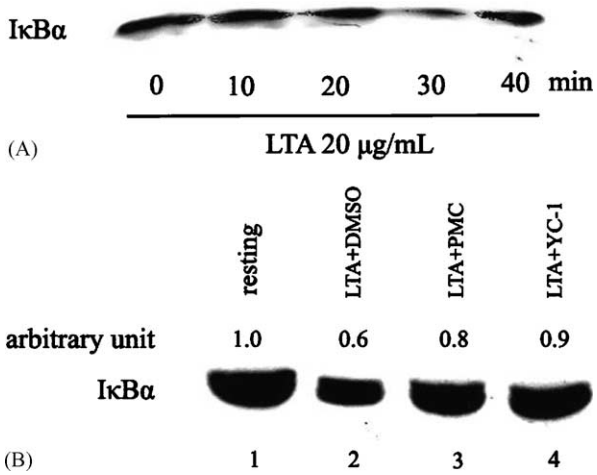


Fig. 5. Effects of PMC and YC-1 on the degradation of immunoreactive IκBα stimulated by LTA in RAW 264.7 macrophages. (A) Cells (1×10^6 cells/mL) were treated with LTA (20 μg/mL) for the indicated times (10–40 min) or (B) cells were pretreated with normal saline only (lane 1, resting group), solvent control (lane 2, 0.2% DMSO), PMC (lane 3, 50 μM), or YC-1 (lane 4, 10 μM) for 30 min followed by the addition of LTA (20 μg/mL) for 30 min. Cells were then collected, and subcellular extracts were analyzed for the degradation of immunoreactive IκBα as described in Section 2. The densitometric data was expressed by the relative arbitrary unit on the top of bands. The results are representative examples of four similar experiments.

phosphorylation of p46/54 JNK/SAPK (Fig. 6A), while having no effect on the activation of p38 MAPK or p42/44 MAPK (ERK 1/2), even with stimulation using a higher concentration of LTA (40 μg/mL) or a prolonged stimulation time (2 hr) (data not shown). The peak activation of p46/54 JNK/SAPK by LTA occurred after 30 min of stimulation, and returned to basal levels after 40 min. After pretreatment with PMC (50 μM) or YC-1 (10 μM) followed by stimulation with LTA (20 μg/mL) for 30 min, we found that PMC (50 μM) completely inhibited the activation of p46/54 JNK/SAPK about 92%, whereas YC-1 (10 μM) only partially inhibited its activation by about 19% (Fig. 6B, lanes 3 and 4).

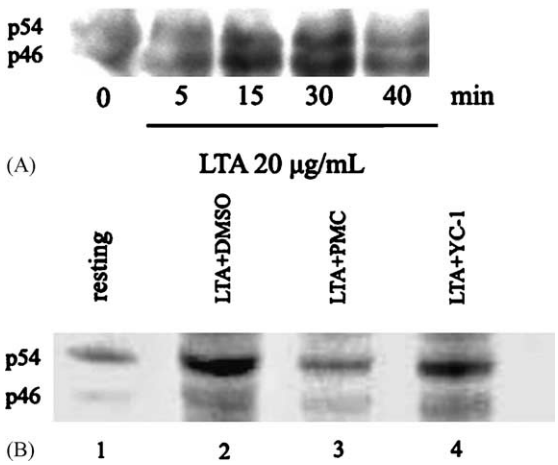


Fig. 6. Effects of PMC and YC-1 on the LTA-induced activation of JNK/SAPK in RAW 264.7 macrophages. (A) Cells (1×10^6 cells/mL) were treated with LTA (20 μg/mL) for the indicated times (10–40 min) or (B) cells were pretreated with normal saline only (lane 1, resting group), solvent control (lane 2, 0.2% DMSO), PMC (lane 3, 50 μM), or YC-1 (lane 4, 10 μM) for 30 min followed by the addition of LTA (20 μg/mL) for 30 min. Cells were then collected, and subcellular extracts were analyzed for the phosphorylation of JNK/SAPK. JNK/SAPK activation was determined by Western blotting with an mAb which recognizes only phosphorylated JNK/SAPK (p46/54). The results are representative examples of four similar experiments.

3.4. Effects of PMC and YC-1 on LTA-induced septic shock in vivo

As shown in Fig. 7, there was a rapid fall of about 28% in MAP within 60 min after administration of LTA (10 mg/kg, i.v.); it then increased, but thereafter continuously decreased until 5 hr. At 5 hr after LTA injection, MAP was markedly decreased from 110 ± 6 mmHg to 58 ± 4 mmHg. However, in the control group (cosolvent), there was no significant change in MAP during the experimental period. The effect of PMC (10 mg/kg, i.v.) attained significance at 300 min as the effect of YC-1 (5 mg/kg, i.v.) attained significance at 240–300 min (Fig. 7). The MAP of PMC- and YC-1-treated groups were significantly elevated by about 21% (from 58 ± 4 mmHg to 70 ± 3 mmHg) and 29% (from 58 ± 4 mmHg to 75 ± 3 mmHg), respectively, as compared with the LTA-treated group at 300 min after LTA administration. In addition, when PMC (10 mg/kg, i.v.) was combined with YC-1 (5 mg/kg, i.v.), the MAP was greater than that produced by the individual drug alone at 300 min after LTA administration (84 ± 7 mmHg, $N = 3$, data not shown). Furthermore, pretreatment of L-NAME

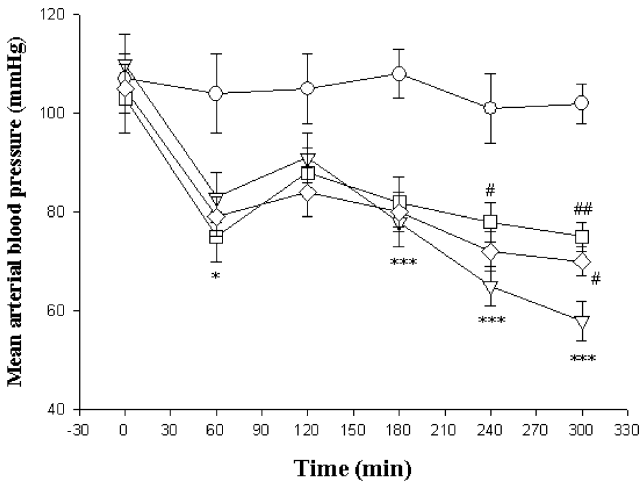


Fig. 7. Effects of PMC and YC-1 on MAP in rats treated with LTA. Depicted are the changes in MAP during the experimental period in different groups of rats that received injections of cosolvent (cremophor EL/ethanol) only as the control group (○), or cosolvent plus LTA (10 mg/kg, i.v.) (▽), YC-1 (5 mg/kg, at 30 min prior to LTA treatment) plus LTA (□), or PMC (10 mg/kg, at 30 min prior to LTA treatment) plus LTA (◇). Data are presented as the means ± SEM from eight separate animals. $^{*}P < 0.05$ and $^{***}P < 0.001$ as compared with the control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ as compared with the cosolvent plus LTA group.

(5 mg/kg, i.v.), an inhibitor of iNOS [20], and 8-bromo-cyclic GMP (6 mg/kg, i.v.) for 30 min followed by the administration of LTA (10 mg/kg, i.v.), the MAP of L-NAME- and 8-bromo-cyclic GMP-treated groups were also significantly elevated by about 22% (from 58 ± 4 mmHg to 70 ± 7 mmHg, $N = 3$) and 24% (from 58 ± 4 mmHg to 72 ± 6 mmHg, $N = 3$) as compared with the LTA-treated group at 300 min after LTA administration (data not shown). Additionally, plasma nitrite concentrations markedly increased from 2.5 ± 0.6 μM in control rats to 14.7 ± 1.8 μM in LTA-treated rats ($N = 5$, $P < 0.001$, data not shown). The increase was significantly inhibited by pretreatment of animals with PMC (10 mg/kg) (5.2 ± 0.9 μM, $N = 5$; $P < 0.05$) and YC-1 (5 mg/kg) (6.3 ± 0.7 μM, $N = 5$; $P < 0.01$) at 300 min after LTA administration (data not shown). This result indicates that YC-1 is more potent than PMC at attenuating the delayed phase of hypotension in rats with endotoxemia stimulated by LTA.

4. Discussion

The present study demonstrates that the increase in NO formation stimulated by LTA in RAW 264.7 macrophages is a consequence of the induction of iNOS and indicates that JNK/SAPK and NF-κB are involved in the signal transductions leading to the expression of iNOS mRNA and protein stimulated by LTA. The major findings of the study are to show that PMC, a novel potent antioxidant of an α-tocopherol derivative, and YC-1, an sGC activator, both markedly inhibited LTA-stimulated NO formation in macrophages and significantly attenuated the delayed phase of hypotension stimulated by LTA.

iNOS is expressed in macrophages in response to stimulation by bacterial LPS, LTA, and/or certain inflammatory cytokines [6,8,21]. Once expressed, iNOS is maximally activated and remains for several hours, generating high levels of NO. Overproduction of NO has been implicated in the pathogenesis of several important disease states, most notably septic shock [2]. It is well known that LPS causes a receptor protein (CD 14) to initiate the induction of iNOS. It is not fully clear whether LTA uses a similar CD 14-like protein for the induction of iNOS. Nevertheless, the induction of NO synthesis and iNOS gene expression stimulated by LTA was significantly inhibited by an anti-CD 14 mAb [22]. Therefore, it is possible that both LTA and LPS bind to the same receptor, CD 14.

The iNOS gene is precisely regulated at the level of transcription in mammalian cells [4]. However, the effects of the upstream signaling pathways utilized by stimulation of LTA on the induction of iNOS are not fully understood. Recently, it was reported that LTA may activate phosphatidylcholine-phospholipase C (PC-PLC) and phosphatidylinositol-phospholipase C (PI-PLC) to induce PKC activation, which is followed by initiation of NF-κB activation, and finally induces iNOS expression and NO formation [21]. NF-κB is constitutively present in cells as a heterodimer, consisting of a p50 DNA-binding subunit and a p65 transactivating subunit. NF-κB is normally held in the cytoplasma in an inactivated form by the inhibitor protein IκBα [23]. LPS or cytokines activate cell receptors resulting in dissociation of IκBα from the NF-κB complex, and then proteolytic degradation of IκBα by IκBα protease, followed by translocation of the activated NF-κB into the nucleus and attachment to relevant DNA binding sites on the promoter region of genes, ultimately leading to initiation of iNOS transcription. Our work has also proven that NF-κB is involved in transcriptional regulation of LTA-induced iNOS expression in marcophages.

One of the major intracellular signal transduction pathways stimulated by inflammatory cytokines such as TNF-α and IFN-γ are the MAPKs. The MAPKs are comprised of three principal family membranes, namely ERK 1/2, JNK/SAPK, and p38 MAPK with different isoforms existing within each member. In this study, JNK/SAPK but not ERK 1/2 or p38 MAPK was activated by LTA in RAW 264.7 macrophages. This result is consistent with prior work by Chan and Riches [24] who suggest that only JNK/SAPK but neither ERK 1/2 nor p38 MAPK was involved in signal transduction stimulated by cytokines (TNF-α or IFN-γ) in the induction of iNOS in macrophages. There is increasing evidence that JNK/SAPK may be associated with the c-Rel subunit of NF-κB and may directly enhance the activation of NF-κB [25]. The mechanism through which JNK/SAPK is involved in the activation of NF-κB is not known, although it has been hypothesized to behave as a tethering protein that brings necessary components together or perhaps to play a more direct role by phosphorylating c-Rel-associated proteins [25]. Therefore, there are convincing data to suggest that JNK/SAPK, at least partly, is involved in the activation of NF-κB, a potential transcriptional element in iNOS induction by LTA in macrophages.

One of the determinants of NF-κB activation is the redox status of cells [26]. This status is determined by the concentration of ROS such as superoxide, hydrogen peroxide, and hydroxy radicals. There is ample evidence to suggest that cytokines increase the formation of cellular ROS by causing alterations in electron flows in mitochondria [26]. The antioxidants rotenone and BHA prevented iNOS induction by LTA, suggesting that ROS are generated by activation of macrophages by LTA [26,27]. Thus, ROS, presumably by their ability to activate NF-κB, play an important role in the series of events leading to the expression of iNOS induced by LTA. In this study, LTA-mediated NO formation and iNOS expression were concentration-dependently inhibited by PMC (IC_{50}, 47.8 ± 5.0) and YC-1 (IC_{50}, 4.3 ± 1.3). Furthermore, both PMC and YC-1 significantly reversed the LTA-mediated degradation of IκBα, indicating that PMC and YC-1 possibly inhibit iNOS gene expression through stabilizing

the association between IκBα and NF-κB. α-Tocopherol is known to effectively inhibit cytokine-induced NF-κB activation [12]. Therefore, PMC inhibition of LTA-mediated iNOS expression, JNK/SAPK phosphorylation, and IκBα degradation may occur through its antioxidation and/or inhibition of NF-κB. On the other hand, YC-1 not only decreased iNOS and NO formation, but also partially inhibited JNK/SAPK activation in macrophages stimulated by LTA. However, the discordance between the marked inhibition of iNOS mRNA levels and the incomplete inhibition of JNK/SAPK by YC-1 suggests that the inhibitory effect of YC-1 on iNOS transcription is only partially dependent on its ability to inhibit JNK/SAPK. In addition, the attenuation of iNOS mRNA by YC-1 was paralleled by a reversal in IκBα degradation. This implies that activation of IκBα protease is more important than phosphorylation of JNK/SAPK in the induction of iNOS caused by LTA.

A number of events precede the activation of NF-κB after stimulation of cells by LPS or cytokines. For example, PC-PLC controls the activation of NF-κB in response to LTA [24]. Kuo *et al*. [21] reported that LTA activates PC-PLC to induce PKC activation, and finally induces iNOS expression in marcophages. Increased intracellular cyclic GMP negatively inhibits agonist-induced PKC activation and iNOS expression in murine macrophage [28–30]. Therefore, the inhibitory mechanisms of YC-1 may be mediated by increasing cyclic GMP formation, resulting in the inhibition of PKC activation, followed by inhibition of NF-κB activation.

In anesthetized rats, injection of LTA results in the elevation of plasma NO and induction of iNOS activity associated with delayed circulatory failure [31]. iNOS-generated NO is an important causal factor in septic shock syndrome, characterized by peripheral vasodilation and profound hypotension. Septic shock results in poor tissue perfusion, which leads to multiple organ failure and ultimately death [32]. In the present study, both PMC and YC-1 significantly attenuated the delayed hypotension in rats with septic shock. YC-1 (5 mg/kg) exhibiting more-potent activity than PMC (10 mg/kg) *in vivo* is consistent with the results of LTA-mediated iNOS expression *in vitro*. Therefore, we propose that the improvements in septic hypotension by PMC and YC-1 were due to their inhibition of iNOS-dependent NO generation.

In conclusion, YC-1 shows more-potent activity than PMC at abrogating the expression of iNOS in macrophages *in vitro* and reducing the delayed hypotension in rats with endotoxic shock stimulated by LTA. The inhibitory mechanism of PMC may be due to its antioxidative properties, with a resulting influence on JNK/SAPK followed by inhibition of NF-κB activation and subsequent inhibition of iNOS expression. On the other hand, YC-1 may mediate the increasing cyclic GMP formation, resulting in the inhibition of PKC activation, followed by, at least partly, inhibition of JNK/SAPK and NF-κB activations, thereby leading to inhibition of iNOS expression.

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