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# A novel antioxidant, octyl caffeate, suppression of LPS/IFN-γ-induced inducible nitric oxide synthase gene expression in rat aortic smooth muscle cells

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**Abstract**

In the present study, we investigated the effects and mechanisms of a novel potent antioxidant, octyl caffeate, on the induction of iNOS expression by lipopolysaccharide (LPS) and interferon-γ (IFN-γ) in cultured primary rat aortic smooth muscle cells (RASMCs) *in vitro* and LPS-induced hypotension *in vivo*. Octyl caffeate (0.1–1.0 μM) exerted a concentration-dependent inhibition of iron-catalyzed lipid peroxidation in rat brain homogenates. Furthermore, octyl caffeate (20, 50, and 100 μM) concentration-dependently diminished the initial rate of superoxide-induced NBT reduction and the enzymatic activity of xanthine oxidase. It also concentration-dependently (1–50 μM) inhibited the NO production, iNOS protein and messenger RNA expressions upon stimulation by LPS (100 μg/mL)/IFN-γ (100 U/mL) in RASMCs. In addition, we found that octyl caffeate did not significantly affect IκBα degradation stimulated by LPS/IFN-γ in RASMCs. On the other hand, octyl caffeate (10 and 50 μM) significantly suppressed activation of c-Jun-N-terminal kinase and extracellular signal-regulated kinase. Moreover, octyl caffeate (10 mg/kg, i.v.) significantly inhibited the fall in mean arterial pressure stimulated by LPS (7.5 mg/kg) in rats. In conclusion, we demonstrate that a novel potent antioxidant, octyl caffeate, significantly ameliorates circulatory failure of endotoxemia *in vivo* by a mechanism involving suppression of iNOS expression through inactivation of mitogen-activated protein kinases in RASMCs.


*Keywords:* Octyl caffeate; Antioxidant; iNOS; LPS/IFN-γ; MAPK; Endotoxic shock

## 1. Introduction

There is increasing evidence that overproduction of NO generated by the iNOS is a deleterious factor in inflammatory vasculopathies, and NO also contributes to vascular failure in endotoxic shock [1]. The systemic inflammatory condition induced by LPS or cytokines is associated with the generation of peroxynitrite, a potent and vasotoxic oxidant formed from the reaction of NO and superoxide. The contribution of NO to the pathophysiology of septic shock is highly heterogeneous, inhibition of iNOS expression and/or activity still represents a rational therapeutic goal [2].

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]. Vascular smooth muscle cells have been shown to express iNOS when stimulated with bacterial endotoxin (LPS) or cytokines such as tumor necrosis factor-α (TNF-α), interleukine-1, or IFN-γ. Furthermore, the complete induction of NO production requires a

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*Abbreviations:* LPS, lipopolysaccharide; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; RASMCs, rat aortic smooth muscle cells; JNK, c-Jun-N-terminal kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; TBARS, thiobarbituric acid-reactive substances; ERK, extracellular signal-regulated kinase; JAK/STAT, janus kinases/signal transducers and activators of transcription; NF-κB, nuclear factor κB; MAPK, mitogen-activated protein kinases; DMSO, dimethyl sulfoxide; NBT, nitroblue tetrazolium; TLR-4, toll-like receptor-4.

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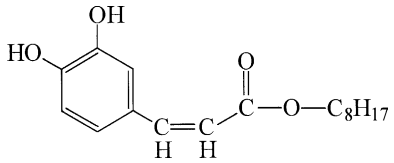


Fig. 1. Chemical structure of octyl caffeate.

combination of one or more cytokines plus LPS [4]. The signal transduction cascades of such co-stimulation are thought to be mediated through different signal pathways, including NF-κB, MAPK, and JAK/STAT [5]. iNOS mRNA is induced after encountering activated free or phosphorylated transcription factors in the nucleus where they activate transcription by binding to specific *cis*-response elements in iNOS gene promoters [6].

Octyl caffeate is a semi-derived compound from caffeic acid (Fig. 1). Caffeic acid and some of its derivatives show antioxidant and anti-inflammatory activities [7–9]. In addition, caffeic acid was revealed to inhibit LPS/IFN-γ-induced NO production in C6 astrocyte cells [10]. In this study, we investigated the antioxidative activity of octyl caffeate as revealed by its protective effect against lipid peroxidation, and by inhibitory mechanisms on the signal pathway of iNOS gene expression in cultured RASMCs. We therefore utilized these findings to characterize the relationship between the inhibition of NO production *in vitro* and protective LPS-induced hypotension of octyl caffeate *in vivo*.

## 2. Materials and methods

### 2.1. Materials

Octyl caffeate was prepared from caffeic acid by exhaustive octanol-esterization, after which it was dissolved in DMSO. In each experiment, DMSO was employed at a constant final concentration (0.1%, v/v). 2-Thiobarbituric acid, tetramethoxypropane, α-tocopherol, bovine serum albumin (BSA), xanthine oxidase (Grade IV, from buttermilk), 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron), superoxide dismutase (SOD, type I, from bovine liver), lucigenin, LPS (*Escherichia coli*, serotype 0127:B8), sodium nitrite, sulphaniamide, MTT, β-mercaptoethanol, leupeptin, and diethylpyrocarbonate were purchased from Sigma Chemical Co. Recombinant rat IFN-γ was purchased from Pepro Technol. Murine anti-iNOS monoclonal antibody was purchased from Transduction Lab. Anti-mouse IgG antibody linked to horseradish peroxidase and the Western blotting detection system (ECL$^{+}$plus) were purchased from Amersham.

### 2.2. Antioxidant activity in rat brain homogenate

Rat brain homogenates was prepared from the brains of freshly killed Wistar rats, and the peroxidation induced by ferrous methods was measured for TBARS, as described by Hsiao *et al.* [11]. Tetramethoxypropane was used as a standard, and the results are expressed as nanomoles of malondialdehyde equivalents per milligram of protein of both preparations. The protein contents of the brain homogenates and other cellular preparations were determined using the Bio-Rad method [12], with bovine serum albumin as a standard.

### 2.3. Quenching of superoxide anion

The superoxide scavenging activity of the test compounds was determined by spectrophotometric monitoring of their competition with NBT for superoxide anions generated by the xanthine/xanthine oxidase method [13]. The initial rate of superoxide-induced NBT reduction was determined by subtracting the NBT reduction in the presence of superoxide dismutase (100 U/mL). The results are expressed as the percent inhibition of the initial rate on NBT reduction. Under the same condition, the effect of test compounds on the enzymatic activity of xanthine oxidase was determined by spectrophotometrically measuring uric acid formation as previously mentioned.

### 2.4. Cell cultivation

RASMCs were enzymatically isolated from aortic media by the modified explant method described by Yan *et al.* [14]. Briefly, Wistar rats were exsanguinated, and the thoracic aorta was excised and placed in cold oxygen-purged Hank's buffered salt solution. The aortas were enzymatically treated with collagenase type I (0.1%) and trypsin (0.1%) for 10–15 min. The vessels were then rinsed, the ends cut away, and the remaining tissues were cut into 18–22 ring segments (1–1.5 mm). Ring explants were carefully immersed into multiwell plates (24 wells) with Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 20 mM HEPES, 10% heat-deactivated fetal calf serum (FCS), 1% (w/v) penicillin/streptomycin, and 2 mM glutamine at 37° in a humidified atmosphere of 5% $CO_2$. This process is defined as the first stage. The rings were transferred into new culture wells, and the next stage was begun. During the sixth stage, nearly pure RASMCs were obtained, the cells showed the "hills and valleys" pattern, and the expression of α-smooth muscle actin was confirmed. Throughout the experiments, cells were used between passages 3 and 7 from the origin of the preparation (the sixth stage).

RASMCs ($10^5$ cells per well) were seeded in 24-well plates until 80% confluence. Cells were then co-stimulated with increasing concentrations of either LPS or IFN-γ for 24 hr. The ideal co-stimulatory condition was LPS (100 μg/mL)/IFN-γ (100 U/mL) as determined in a preliminary experiment (data not shown). To evaluate the bioactive effect on nitrite formation, octyl caffeate (1–50 μM)

was added 30 min before the co-stimulation of LPS/IFN-γ. The conditioned media were collected, centrifuged, and stored at –70° for less than 2 weeks.

### 2.5. Determination of nitrite concentration

To determine nitric oxide production, nitrite (a stable oxidative endproduct of NO) accumulation in the media of RASMCs was measured using the colorimetric method [15] with minor modification. Twenty-four hours after exposure to the experimental conditions, nitrite accumulation was determined 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 with a microplate reader (MRX microplate reader). Nitrite concentrations were calculated by regression with standard solutions of sodium nitrite prepared in the same culture medium.

### 2.6. Cell viability

RASMC viability after 24 hr of continuous exposure to octyl caffeate (10–150 μM) was measured with a colorimetric assay based on the ability of mitochondria in viable cells to reduce the MTT as described previously [16]. Percentage of cell viability was calculated as the absorbance of treated cells/control cells × 100.

### 2.7. Western blot analysis

Treated RASMC lysates were obtained by treatment with ice-cold lysis buffer (10 mM Tris–HCl, 140 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 1 mM DTT, 2 mM phenylmethylsulfonyl fluoride, 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 the phosphorylated MAPK analysis. The 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 TBST buffer (10 mM Tris-base, 100 mM NaCl, 0.1% Tween 20, pH 7.5), blots were treated with either an anti-iNOS monoclonal antibody (mAb) (1:2000; Transduction Lab), anti-MAPK mAb (1:2000; Transduction Lab) or a rabbit anti-human IκBα antibody (1:3000; Santa Cruz Biotech) in a TBST buffer for 3 hr. They were subsequently washed three times with TBST and incubated with peroxidase-conjugated goat anti-mouse or anti-rabbit antibody (Amersham) for 2 hr. The 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).

### 2.8. Isolation of total RNA and reverse transcription polymerase chain reaction

Total RNA was isolated from RASMCs 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 RT/Taq mixture in one cycle of 30 min at 50° for reverse transcription and one cycle for 94° for 2 min, followed by 35 cycles of 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′-CTGG-CAGCAGCGGCTCCATG-3′ (sense) at base positions 2987–3006 and 5′-GAAAAGACCGCACCGAAGAT-3′ (antisense) at base positions 3389–3409 of rat iNOS cDNA [17]. The GAPDH primers sets were 5′-GCCGCCTGGT-CACCAGGGCTG-3′ (sense) and 5′-ATGGACTGTGG-TCATGAGCCC-3′ (antisense). For visualization and quantification by densitometry of each RT-PCR reaction, a 10-μL aliquot was electrophoresed in 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 mentioned. Preliminary experiments were performed to determine the range of amplification cycles and beginning RNA substrate within the linear phase of the exponential increase of PCR products for each particular primer pair.

### 2.9. 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* (Washington, DC: National Academy Press, 1996). Octyl caffeate (10 mg/kg) was dissolved in cosolvent as cremophor EL/ethanol (1:1) and diluted (1:10) with normal saline before injection. Control rats were injected with an equivalent volume of the vehicle solution (cosolvent).

Rats were anesthetized by 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. The PE-50 tube was inserted into

the right femoral artery for the 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. Rats were then assigned to receive LPS (*E. coli* LPS, serotype 0127:B8, 7.5 mg/kg, i.v.) or a placebo (normal saline). To examine the effects of octyl caffeate on the hemodynamic parameters during endotoxemia, octyl caffeate (10 mg/kg) was administered 30 min before LPS treatment, and animals were continuously monitored for 4 hr.

### 2.10. Statistical analysis

The experimental results are expressed as the means ± SEM and are accompanied by the number of observations. Data were assessed by the Student's unpaired *t*-test method. The ANOVA followed by a multiple comparison test (Scheffe's test) was used to determine the 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 octyl caffeate on lipid peroxidation and superoxide anion-quenching action

Octyl caffeate (0.1–1.0 μM) exerted a concentration-dependent inhibition of iron-catalyzed lipid peroxidation (TBARS formation) in rat brain homogenates (Fig. 2A). In addition, at a higher concentration (1.0 μM), octyl caffeate also inhibited spontaneous lipid peroxidation by about 95% (data not shown). The $IC_{50}$ of octyl caffeate for inhibition of TBARS was about 0.45 ± 0.06 μM. Furthermore, octyl caffeate (1.0 μM) alone did not significantly interfere with the absorption at 532 nm when added to rat brain homogenates that were either intact or already oxidative modified (data not shown).

With the spectrophotometric method, octyl caffeate (20, 50, and 100 μM) concentration-dependently diminished the initial rate of superoxide-induced NBT reduction by about 13.1 ± 1.7, 31.6 ± 0.6, and 69.0 ± 2.0% (Fig. 2B). Tiron, a direct scavenger of superoxide anions [13], exerted 20.4 ± 2.1% inhibition at 100 μM. Therefore, octyl caffeate was more potent than Tiron at inhibiting superoxide production on a molar basis. In the presence of SOD (10 units/mL), NBT reduction was abolished by more than 87% (data not shown). In addition, under a similar superoxide generation system, the sensitive short-term lucigenin-enhanced chemiluminescence was also inhibited by octyl caffeate in a concentration-dependent manner (50 and 100 μM), and its inhibitory biphasic tracing was similar to that of the combination of Tiron with allopurinol (data not shown).

Furthermore, octyl caffeate (20, 50, and 100 μM) concentration-dependently attenuated the enzymatic activity of xanthine oxidase by about 16.0 ± 2.7, 28.0 ± 5.7, and 52.5 ± 2.8% (Fig. 2B). Allopurinol (100 μM), as a positive control, exerted inhibition of 80.0 ± 3.8% (N = 4) (data not shown). These results reveal that octyl caffeate possesses both activities of superoxide anion scavenger and xanthine oxidase inhibitor.

### 3.2. Effect of octyl caffeate on nitrite production and iNOS expression in LPS/IFN-γ-induced RASMCs

According to our preliminary tests, activation of primary RASMCs by LPS (100 μg/mL)/IFN-γ (100 U/mL) induced a significant and submaximal increase in nitrite formation, a stable oxidative endproduct of NO. Therefore, we used these concentrations of LPS (100 μg/mL) and IFN-γ (100 U/mL) for the following experiments. In this study, concentration of nitrite production in the cell supernatant was elevated from 2.8 ± 0.4 to 24.9 ± 2.0 mg/mL at 24 hr after addition of LPS/IFN-γ (Fig. 3A). Octyl caffeate (10 and 50 μM) significantly inhibited nitrite production about 64 and 93% stimulated by LPS/IFN-γ, respectively (Fig. 3A). Octyl caffeate neither interfered with the Griess reaction nor reacted with native NO. These results demonstrate that octyl caffeate markedly suppresses NO formation stimulated by LPS/IFN-γ in RASMCs.

As shown in Fig. 3B, LPS/IFN-γ-induced iNOS expression was markedly detectable as compared with that of the control group after a 24-hr treatment (Fig. 3B, lanes 1 and 2). Pretreatment with various concentrations of octyl caffeate for 30 min before LPS/IFN-γ administration revealed that octyl caffeate (10 and 50 μM) markedly inhibited the expression of iNOS protein by about 17 and 83%, respectively (Fig. 3B, lanes 4 and 5).

To further demonstrate whether octyl caffeate inhibits iNOS expression in RASMCs through cytotoxic effects, cells were preincubated with various concentrations of octyl caffeate for 24 hr. We found that octyl caffeate (10 and 50 μM) had no significant cytotoxicity to RASMCs according to the MTT assay, even at a higher concentration (150 μM) (data not shown).

### 3.3. Effect of octyl caffeate on LPS/IFN-γ-induced expression of iNOS mRNA in RASMCs

As shown in Fig. 4, LPS/IFN-γ markedly stimulated a 65-fold increase in iNOS mRNA in RASMCs as compared with the resting control (lanes 1 and 2). Pretreatment with octyl caffeate (10 and 50 μM) for 30 min reduced the expression of iNOS mRNA by about 32 and 84%, respectively (Fig. 4, lanes 3 and 4). These results reveal that octyl

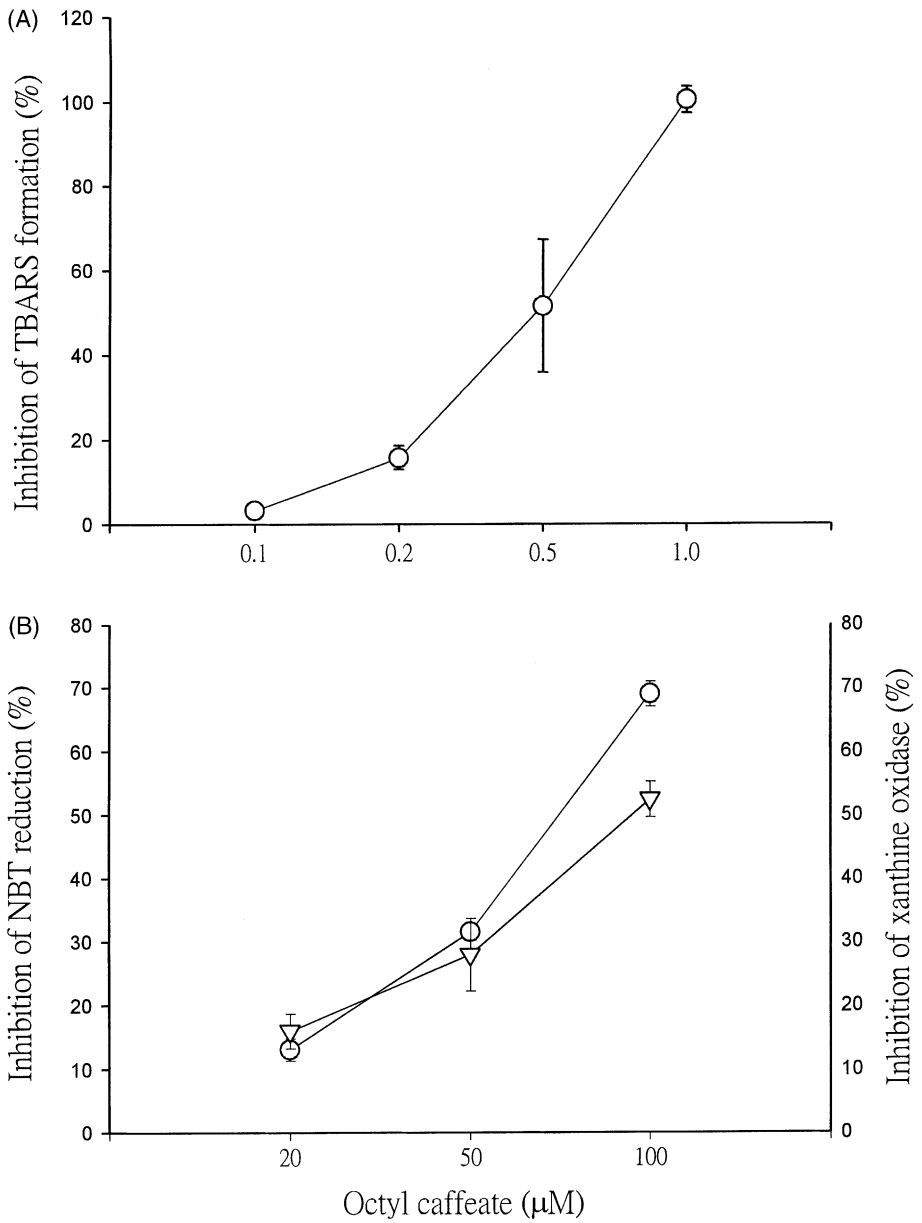


Fig. 2. Antioxidant effects of octyl caffeate. (A) Effects of octyl caffeate on lipid peroxidation. Brain homogenates were perincubated with DMSO (0.1% as the control group) or various concentrations of octyl caffeate (0.1–1.0 μM) for 30 min following by the addition of 200 μM $Fe^{2+}$. In the control group, lipid peroxidation resulted in the formation of 10.1 ± 0.5 nmol MDA/mg protein in brain homogenates. The results are represented as the percent inhibition of TBARS formation vs. the control group. Data are presented as the means ± SEM (N = 4). (B) Inhibition of octyl caffeate on superoxide radical formation (○) and xanthine oxidase activity (▽). Detections of superoxide radical formation and enzymatic activity were subjected to xanthine plus xanthine oxidase in 50 mM phosphate buffer as described in Section 2. Data are presented as the means ± SEM (N = 4).

caffeate significantly inhibits the expression of iNOS mRNA in RASMCs stimulated by LPS/IFN-γ.

### *3.4. Effects of octyl caffeate on NF-κB and MAPK activations*

To further investigate the inhibitory mechanisms of octyl caffeate on reduction of iNOS expression in LPS/IFN-γ-stimulated RASMCs, we detected several signaling molecules including IκBα and MAPKs, which we refer to as p42/44 MAPK (ERK 1/2) and p46 MAPK (JNK). The immunoblotting analysis shown in Fig. 5 reveals that treatment with LPS/IFN-γ caused a rapid and time-dependent disappearance in the immunoreactive bands of IκBα (lanes 2 and 4). IκBα protein was slightly detectable within a period of 7–15 min after LPS/IFN-γ administration, and returned to basal levels after 30 min (lane 7). However, pretreatment with octyl caffeate (50 μM) did not significantly attenuate the disappearance of the immunoreactive bands of IκBα (lanes 3, 5, and 7). In addition, Fig. 6 showed that LPS/IFN-γ significantly induced phosphorylation of 42-, 44 (ERK 1/2 MAPK)- and 46 (JNK/SPAK)-kDa protein

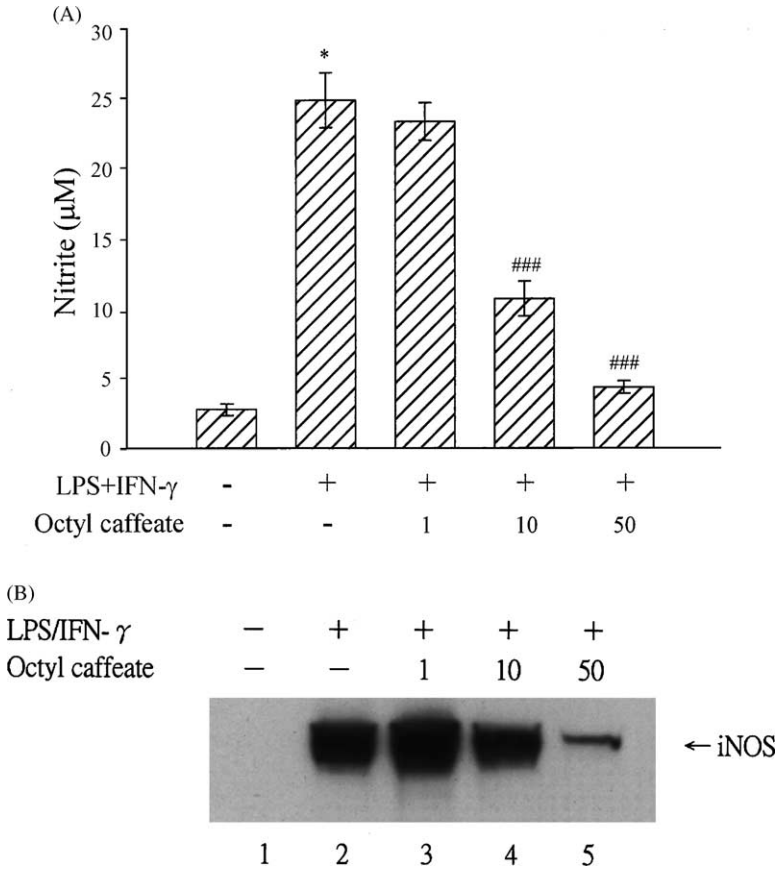


Fig. 3. Effect of octyl caffeate on LPS/IFN-γ-induced (A) nitrite production and (B) iNOS expression in RASMCs. RASMCs ($2 \times 10^6$ cells per dish) were treated with solvent control (0.1% DMSO) or various concentrations of octyl caffeate (1, 10, and 50 μM) for 30 min followed by the addition of LPS (100 μg/mL)/IFN-γ (100 U/mL) for 24 hr. Cell-free supernatants were assayed for nitrite production and cellular lysates were analyzed for iNOS expression as described in Section 2. (A) Data are presented as the means ± SEM (N = 4). $^{*}P < 0.001$ as compared with the resting group; $^{\#\#\#}P < 0.001$ as compared with the LPS/IFN-γ group. (B) Lane 1, cells treated with normal saline only (resting group) and cells pretreated with solvent control (lane 2, 0.1% DMSO) or octyl caffeate (lane 3, 1 μM; lane 4, 10 μM; and lane 5, 50 μM) followed by the addition of LPS/IFN-γ. The results are representative examples of four similar experiments.

bands in RASMCs after 15 min (Fig. 6A and B, lane 2). After being pretreated with octyl caffeate (10 and 50 μM) for 30 min, phosphorylated ERK and JNK stimulated by LPS/IFN-γ were markedly inhibited in a concentration-dependent manner (Fig. 6A and B, lanes 3 and 4). At a higher concentration of 50 μM, octyl caffeate inhibited the expression of phosphorylated p42, p44, and p46 proteins by about 87, 80, and 94%, respectively (Fig. 6A and B, lane 4).

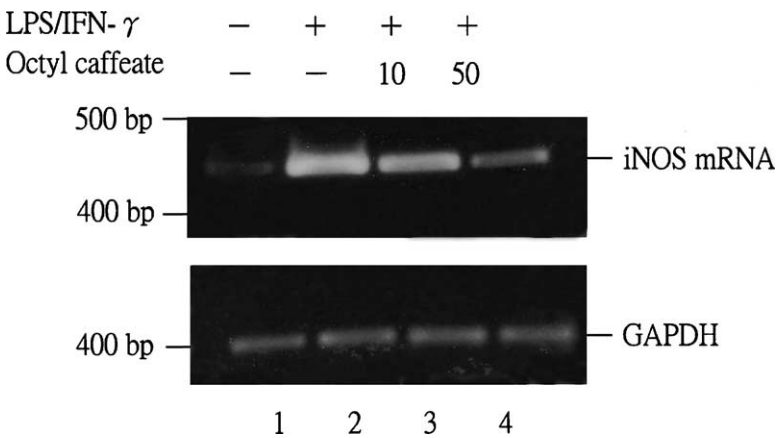


Fig. 4. Effect of octyl caffeate on LPS/IFN-γ-induced iNOS mRNA expression in RASMCs. Cells were pretreated with octyl caffeate (10 and 50 μM) followed by the addition of LPS (100 μg/mL)/IFN-γ (100 U/mL) as described in Section 2. The GADPH levels were used to normalize the amount of the cDNA template used in each PCR reaction. Lane 1, cells treated with normal saline only (resting group) and cells pretreated with solvent control (lane 2, 0.1% DMSO) or octyl caffeate (lane 3, 10 μM and lane 4, 50 μM) followed by the addition of LPS/IFN-γ. The results are representative examples of four similar experiments.

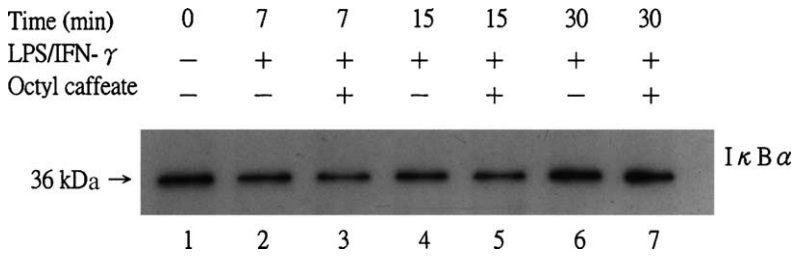


Fig. 5. Effect of octyl caffeate on the degradation of immunoreactive IκBα in RASMCs. RASMCs ($3 \times 10^6$ cells per dish) were pretreated with or without octyl caffeate (50 μM) for 30 min followed by the addition of LPS (100 μg/mL)/IFN-γ (100 U/mL) for the indicated times as described in Section 2. The results are representative examples of four similar experiments.

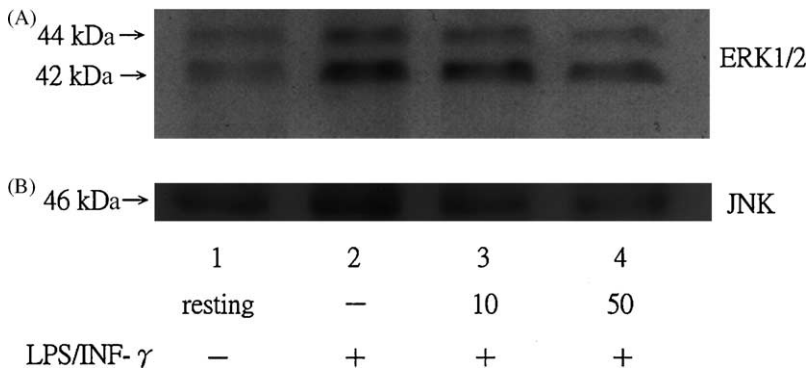


Fig. 6. Effect of octyl caffeate on LPS/IFN-γ-induced activation of (A) ERK 1/2 and (B) JNK/SAPK in RASMCs. ERK 1/2 and JNK/SAPK activations were determined by Western blotting with a monoclonal antibody which recognizes only phosphorylated ERK 1/2 (p42/p44) or JNK/SAPK (p46). Cells were pretreated with either a solvent control (0.1% DMSO) (lane 2) or octyl caffeate (lane 3, 10 μM and lane 4, 50 μM) for 30 min before stimulation with LPS/IFN-γ for 15 min. Cells treated with normal saline only (lane 1) served as the resting group. The results are representative examples of four similar experiments.

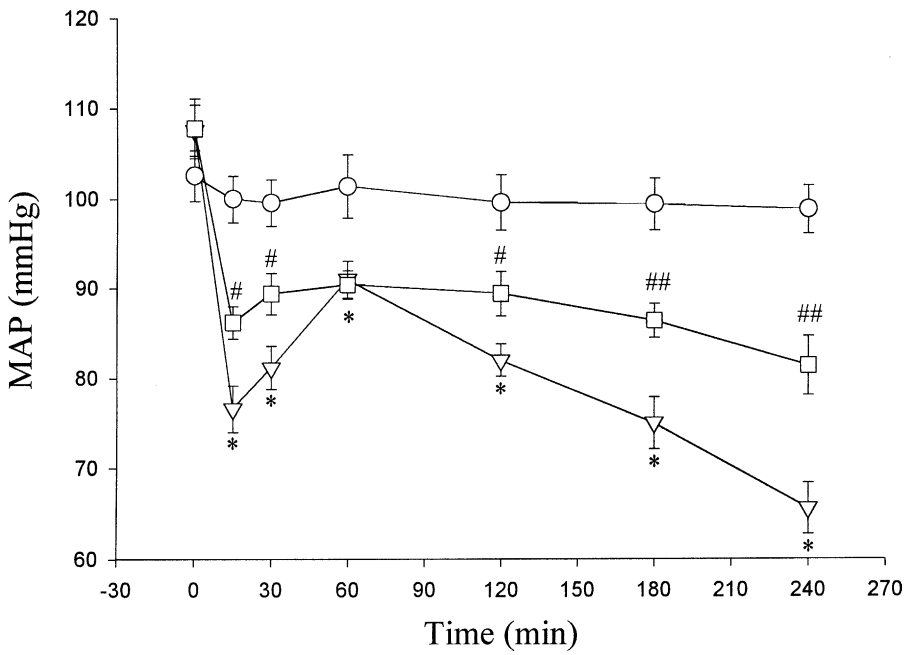


Fig. 7. Effects of octyl caffeate on mean arterial blood pressure (MAP) in rats treated with endotoxin. Depicted are the changes in MAP during the experimental period in different groups of animals that received injections of cosolvent (cremophor EL/ethanol) as the control group (○), cosolvent plus lipopolysaccharide (LPS; 7.5 mg/kg) (▽), or octyl caffeate (10 mg/kg, at 30 min prior to LPS treatment) plus LPS (□). Data are presented as the means ± SEM from five separate animals. $^{*}P < 0.001$ as compared with the control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ as compared with the LPS/IFN-γ group.

### 3.5. Effect of octyl caffeate on LPS-induced hypotension in vivo

As shown in Fig. 7, there was a rapid fall of about 20–30% in mean arterial blood pressure (MAP) within 15–30 min after administration of LPS (7.5 mg/kg, i.v.), and then it was elevated and continuously decreased until 4 hr. At 4 hr after LPS injection, MAP was markedly decreased from 108 ± 2 to 66 ± 3 mmHg. However, in the vehicle-treated group, there was no significant change in MAP during the experimental period. Administration of octyl caffeate (10 mg/kg, i.v.) 30 min before LPS treatment significantly attenuated the early (during 15–30 min) and delayed (e.g. after 120 min) phases of the hypotensive response induced by LPS. The MAP of octyl caffeate-treated groups was elevated by about 23% (from 66 ± 3 to 81 ± 3 mmHg) as compared with vehicle-treated group at 240 min after LPS administration. This result indicates that octyl caffeate significantly improves hypotension in rats with endotoxemia.

## 4. Discussion

iNOS is expressed in RASMCs in response to stimulation by bacterial LPS and/or certain inflammatory cytokines [4,18]. 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 in septic shock [19]. The present experiments demonstrate that octyl caffeate, a novel potent antioxidant, is also a strong inhibitor of iNOS gene expression in RASMCs. Inhibition of iNOS gene expression was evidenced by reductions in inducible nitrite production and iNOS mRNA expression. Octyl caffeate's mechanism appears to involve suppression of signal transductions of *cis*- and *trans*-acting element activation, especially on MAPKs activation. Cell viability was not affected after a continuous 24-hr exposure to octyl caffeate (150 μM). Therefore, the inhibitory effects of octyl caffeate did not occur through cytotoxicity.

It is well known that LPS activation may mediate through triggering receptors expressed on myeloid cell-1 (TREM-1) signaling [20] or those associated with LPS-binding protein (LBP) and by binding to the CD14 receptor; and the LPS is transferred by CD14 to the TLR-4 and MD-2 (accessory protein) complex [21]. TLR-4 signals through MyD88 activate downstream pathways for iNOS expression. Vascular or non-vascular smooth muscle cells have also been shown to express TLR-4, which mediates the action of bacterial products [22]. Either TREM-1 or TLR-4 signals finally trigger various transcription factors, including NF-κB, Elk-1, and AP-1 [23].

The iNOS gene is precisely regulated at the level of transcription in mammalian cells [6]. However, the effects of the *trans*-acting element and their corresponding upstream signaling pathways utilized by co-stimulation of LPS/IFN-γ on the induction of iNOS are not fully understood. Recently, it was reported that NF-κB as a transcriptional element mediates LPS induction of iNOS mRNA by binding to its corresponding *cis*-acting element in region I of the iNOS promoter [24]. On the other hand, stabilization of the NF-κB complex plays a crucial role in reduction of iNOS gene expression [25]. In the present study, we found that octyl caffeate did not significantly affect LPS/IFN-γ-mediated degradation of the NF-κB inhibitory protein (IκBα). This result indicates that octyl caffeate possibly does not inhibit iNOS gene expression through stabilizing the association between IκBα and NF-κB. Hecker *et al*. [25] reported that various antioxidants differentially affect NF-κB-mediated iNOS expression, and that transcription factors other than NF-κB may also mediate the induction of iNOS expression. Therefore, in the present study, octyl caffeate did not affecting NF-κB activation point to an alternative action at the MAPK pathways or the post-transcriptional level in RASMCs, presumably involving iNOS mRNA or protein stability [26]. The possible mechanisms of the promoting action through mRNA or protein degradation thus remains to be further elucidated.

The other possible major intracellular signal transduction pathway stimulated by inflammatory mediators such as LPS/IFN-γ is the MAPK. Therefore, our studies further examined the effects of octyl caffeate on MAPK activations. The rationale for these studies stemmed from some recent experimental observations concerning that LPS activated both the NF-κB signaling pathways and MAPK signal transductions such as ERK, JNK, and p38 MAPK. p42/p44 ERK 1/2 is involved in iNOS gene expression in VSMCs [27]. Furthermore, ERK activity is also required for persistent NF-κB activation in iNOS gene expression [28].

Activation of JNK/SAPK kinase is also a key event mediating iNOS induction [29]. It is strongly agreed that expression of the dominant negative mutant of JNK/SAPK and the addition of a JNK/SAPK inhibitor blocked LPS-induced iNOS expression [30]. Furthermore, co-stimulation of iNOS induction by LPS/IFN-γ is also potentially modulated by MAPKs, especially the JNK/SAPK pathway [31]. The inhibitory effect of p38 MAPK appears to be more complex and may be due to the ability of p38 MAPK to inhibit LPS-induced JNK activation [22]. On the other hand, the p38 MAPK cascade represents a crucial signaling mechanism of LPS through TLR, regulating both enhanced L-arginine transport and induced NO synthesis [32,33]. Therefore, MAPKs are important regulators of iNOS expression in RASMCs by co-stimulation of LPS and IFN-γ. The inhibitors of MAPK activation seem to exert a pharmacological effect on prevention of NO-induced pathological events [34]. In the present study, we found that octyl caffeate significantly inhibited the activation of MAPKs including ERK and JNK/SAPK.

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 that leads to multiple organ failure and ultimately death [35]. This systemic inflammatory response is also associated with the production of oxygen-derived free radicals. There is now substantial evidence that impaired endothelium-dependent relaxation, vasocytotoxicity, and even multiple organ failure in endotoxemia is associated with increased generation of superoxide and peroxynitrite, as a toxic oxidant formed from the reaction of NO and superoxide [18,36]. *In vivo* studies have established that vasoresponsiveness and survival of animals improved after treatment with various antioxidants [37]. In the present study, octyl caffeate significantly prevented circulatory failure in rats with endotoxemia. Therefore, we propose that the improvement of septic hypotension by octyl caffeate might not merely be due to its inhibition of iNOS-dependent NO generation, but also to its antioxidant-scavenging activity which reduces concentrations of superoxide and its toxic byproducts.

In conclusion, we demonstrate that octyl caffeate abrogates the iNOS and the overproduction of NO, at least partly, through inhibition of MAPK activations (ERK 1/2 and JNK/SAPK) stimulated by LPS/IFN-γ in RASMCs. Octyl caffeate reduced the circulatory failure in animals with endotoxic shock *in vivo*. This protection by octyl caffeate is associated with suppression of NO production in VSMCs and with the scavenging properties of reactive oxygen species. It will be of interest to further study the anti-inflammatory activities of octyl caffeate in various radical-mediated pathological events, particularly *in vivo*. The toxicity of octyl caffeate, however, must be further assessed.

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