Journal of Ethnopharmacology 133 (2011) 1008–1014

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Anti-inflammatory effect of ethyl acetate extract from *Cissus quadrangularis* Linn may be involved with induction of heme oxygenase-1 and suppression of NF-κB activation

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ARTICLE INFO

Article history:
Received 8 May 2010
Received in revised form
11 November 2010
Accepted 11 November 2010
Available online 19 November 2010

Keywords:
Cissus quadrangularis
Nitric oxide
Heme oxygenase-1
Inducible nitric oxide synthase
Macrophage

ABSTRACT

Aim of the study: Cissus quadrangularis (family: Vitaceae) has been widely used in traditional herbal medicine for the treatment of hemorrhoids, gastric ulcers and bone healing. In the present study, we determined the anti-inflammatory activity and the molecular mechanism of the ethyl acetate extract of *Cissus quadrangularis* stem (CQE) in LPS-stimulated RAW 264.7 macrophage cells.
Materials and methods: The inhibitory effect of CQE on LPS-induced nitric oxide (NO) production was evaluated in conditioned media. Cell viability was monitored by MTT assay. Protein and mRNA expressions were determined by RT-PCR and Western blotting analysis, respectively.
Results: CQE potently inhibited lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 macrophage cells in a dose-dependent manner. The mRNA and protein expressions of inducible nitric oxide synthase (iNOS) were suppressed also by CQE as was p65 NF-κB nuclear translocation. Further study demonstrated that CQE by itself induced heme oxygenase-1 (HO-1) gene expression at the protein and mRNA levels in dose- and time-dependent manner. In addition, the inhibitory effects of CQE on NO production were abrogated by a HO-1 inhibitor, zinc protoporphyrin IX (ZnPP).
Conclusions: Collectively, these results suggest that CQE exerts an anti-inflammatory effect in macrophages, at least in part, through the induction of HO-1 expression. These findings provide the scientific rationale for anti-inflammatory therapeutic use of *Cissus quadrangularis* stem.

1. Introduction

Cissus quadrangularis Linn (Vitaceae), whose Thai name is Pet-Sang-Khaat, is widely used in herbal medicine. The stem of *Cissus quadrangularis* has been used for the treatment of irregular menstruation, asthma and hemorrhoids and healing bone fractures (Potu et al., 2007; Rao et al., 2007; Ponsana, 2007). Several studies have demonstrated that the extracts from *Cissus quadrangularis* have anti-oxidant, anti-bacterial, anti-osteoporotic, gastroprotective, analgesic and anti-inflammatory properties (Murthy et al., 2003; Shirwaikar et al., 2003; Jainu and Devi, 2006; Panthong et al., 2007). The methanolic extract of *Cissus quadrangularis* has been shown to inhibit iNOS activity in damaged gastric mucosa induced by aspirin (Jainu and Devi, 2006). Moreover, Panthong et al. (2007) demonstrated that *Cissus quadrangularis* extract suppressed ear and paw edema in rats induced by ethyl phenylpropiolate and by both carrageenin as well as arachidonic acid, respectively. However, the molecular mechanism of anti-inflammatory activity of *Cissus quadrangularis* remains unclear.

Nitric oxide (NO), a small free radical, is produced by nitric oxide synthase (NOS; EC 1.14.13.39). To date, three NOS isoforms have been characterized in mammalian tissues: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Alderton et al., 2001). iNOS is induced only in response to inflammatory cytokines and bacterial lipopolysaccharides (LPSs) in macrophages, neutrophils, hepatocytes and other cells (Geller et al., 1993; Sunyer et al., 1996; MacMicking et al., 1997; Alderton et al., 2001). Large

Abbreviations: LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NF-κB, nuclear factor-kappaB; IκB, inhibitory protein of NF-κB; ZnPP, zinc protoporphyrin IX; HO-1, heme oxygenase-1; RT-PCR, reverse transcription-polymerase chain reaction; mRNA, messenger ribonucleic acid; CO, carbon monoxide; cDNA, complementary deoxyribonucleic acid; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol.

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doi:10.1016/j.jep.2010.11.029

amounts of NO produced iNOS functions mainly in pathogen killing processes (Coleman, 2001). However, overproduction of NO appears to be involved in pathogenesis of various inflammatory diseases (Wright et al., 1992; Dorheim et al., 1994; Guzik et al., 2003; Latham et al., 2005). Expression of iNOS gene in LPS-stimulated macrophages is mainly dependent on transcription factor nuclear factor-kappaB (NF-κB) (Lowenstein et al., 1993). NF-κB is a homo- or hetero-dimeric transcription factor composed of different combinations of Rel family proteins such as p50, p52, c-Rel, RelA (p65) and RelB. In unstimulated cells, NF-κB is observed in cytoplasm complexed to the inhibitory protein of NF-κB, IκB. After exposure cells to various stimuli including LPS, IκB can be phosphorylated and finally degraded by 26S proteasome, resulting in translocation of NF-κB to the nucleus. Activated NF-κB subunits bind to κB sites of several proinflammatory genes including iNOS and promote target gene expression (Bauerle and Baltimore, 1996; Nishikori, 2005).

Heme oxygenase-1 (HO-1; EC 1.14.99.3) is a stress inducible protein, which catalyzes oxidative degradation of heme, eliminating the potentially toxic free-heme, but liberating biliverdin, carbon monoxide (CO), and ferrous iron (Pae and Chung, 2009). There are at least 3 isoforms of HO in most mammals; the oxidative stress or heme-inducible HO-1 and the constitutively expressed non-inducible HO-2 and HO-3. HO-1 expression is enhanced not only by its substrate free-heme but also by various other proinflammatory non-heme stimulants such as NO, LPS, cytokines, heavy metals and other oxidants (Terry et al., 1998; Naughton et al., 2002; Srisook et al., 2004; Srisook and Cha, 2005). In mammalian cells, biliverdin is converted rapidly to bilirubin, a well-known antioxidant scavenging reactive oxygen and reactive nitrogen species (Stocker et al., 1987; Kaur et al., 2003). Moreover, bilirubin reduces NO production and iNOS expression in murine macrophages stimulated with LPS (Wang et al., 2004; Lanone et al., 2005). CO, another product of HO-derived heme degradation, inhibits NO production in macrophages and reduces inflammation. It is suggested that CO binds with heme in NOS and prevents oxidation of arginine to citrulline and NO. Thus, enhancing HO-1 expression and HO activity can negatively modulate iNOS and is involved in decreasing the amount of free radicals (Lanone et al., 2005; Srisook et al., 2006; Ashino et al., 2008).

Cissus quadrangularis in Thailand is mostly prepared as powder in capsules for medical uses and it was believed that the powder contains some active compounds which are released after digestion process. To study bioactive gradients of the plant by cell-based bioassay, the stem of *Cissus quadrangularis* was therefore extracted with various organic solvents and the ethyl acetate extract gave the most potent inhibitory effect on NO production. In the present study, we examined the anti-inflammatory activity elicited by the ethyl acetate extract of *Cissus quadrangularis* (CQE). We also assessed the possible mechanism underlying its anti-inflammatory effect in LPS-induced inflammatory responses. We found that CQE significantly inhibited NO production in LPS-treated murine macrophages RAW 264.7, and that this inhibition was correlated with a decrease in iNOS expressions as well as in NF-κB activation. Induction of heme oxygenase-1 expression might be implicated in the anti-inflammatory effect of CQE. These data help to elucidate the mechanism underlying the potential therapeutic value of *Cissus quadrangularis* extract through its involvement in HO-1 induction.

2. Materials and methods

2.1. Chemicals and reagents

Fetal bovine serum (FBS), Penicillin-Streptomycin, and Dulbecco's modified Eagle's medium (DMEM) were bought from Invitrogen/Gibco (Grans Island, NY, USA). Antibodies for HO-1 and p65 NF-κB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for iNOS was obtained from BD Bioscience (San Jose, CA, USA). Avian myeloblastosis virus reverse transcriptase, Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (H + L) was bought from Promega (Madison, WI, USA). Lipopolysaccharide or LPS (*Escherichia coli* serotype O111:B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and anti-mouse β-actin antibody were all purchased from Sigma Chemical (St. Louis, MO, USA). Oligonucleotide primers were obtained from Operon Biotechnologies (Germany). Zinc protoporphyrin IX (ZnPP) was purchased from Calbiochem (San Diego, CA, USA). Taq DNA polymerase was bought from NEB (UK). BCA™ protein assay kit and Super Signal West Pico Chemiluminescent substrate were bought from Pierce (Rockford, IL, USA)). TRI reagent was bought from Molecular Research Center (Cincinnati, OH, USA).

2.2. Preparation of extract

Cissus quadrangularis was obtained and authenticated by the Eastern botanical garden section, Khao Hin Son Royal Development Study Center, Chachoengsao Province, Thailand. A voucher specimen (KS-SCBUU-0011) was preserved at Faculty of Science, Burapha University. Stems of *Cissus quadrangularis* were cleaned with tap water, dried and finally powdered. The powder (246.8 g) was sequently extracted with n-hexane, dichloromethane, ethyl acetate and methanol (1 l 3× for each extract). Solutions were filtered, removed solvents *in vacuo* and ultimately yielded hexane, dichloromethane, ethyl acetate and methanol crude extracts, as 7.68 g (3.11%, w/w), 4.73 g (1.92%, w/w), 2.71 g (1.09%, w/w) and 14.14 g (5.73%, w/w), respectively. Dried powder of ethyl acetate extract was dissolved in dimethyl sulfoxide (DMSO), and subsequently passed through a 0.22 μM sterile filter.

2.3. Cell culture

Murine macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml of penicillin, 100 μg/ml of streptomycin, 4 mM L-glutamine, 25 mM D-glucose, 1 mM sodium pyruvate and 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in humidified air containing 5% CO_2. Cells were subcultured by scrapping. Cell used in all experiments were from the passage 6th to 9th.

2.4. Cell viability test using MTT assay

Cell viability was evaluated on the basis of their ability of mitochondria to reduce tetrazolium salt (MTT) into MTT-formazan crystals, mainly by mitochondrial dehydrogenases. Formazan formation is proportional to the number of functional cell mitochondria. This method was performed as described by Srisook and Cha (2005). Briefly, RAW 264.7 macrophages (1.5×10^5 cells) were plated into each well of 24-well plates and exposed to various chemicals for 20 h and MTT solution (5 mg/ml in PBS) was added to each well for 4 h. DMSO was then added into each well to solubilize the blue formazan crystal product. Absorbance of solubilized formazan solution was measured at 550 nm. Percentage of cell viability is expressed as: (absorbance of treated well/absorbance of control well) × 100.

2.5. Nitrite determination (NO production)

Nitrite determination by Griess reaction has been widely utilized in indirect evaluation of NO production. Nitrite, a stable degradation product of NO, present in the conditioned media

Table 1
Primer sequences used in RT-PCR.

Target	Primer sequences	Accession no.
iNOS	5′-CTAAGAGTCACCAAAATGGCTCCC-3′ (sense) 5′-ACCAGAGGCAGCACATCAAAGC-3′ (antisense)	NM010927.2
HO-1	5′-TGAAGGAGGCCACCAAGGAGG-3′ (sense) 5′-AGAGGTCACCCAGGTAGCGGG-3′ (antisense)	NM010442.1
β-Actin	5′-ATGGTGGGAATGGGTCAGAAGGAC-3′ (sense) 5′-CTCTTTGATGTCACGCACGATTTC-3′ (antisense)	NM007393.2

was determined by spectrophotometry as described previously by Srisook et al. (2006). Macrophage cells (5×10^4 cells/well) were cultured in a 96-well plate overnight. Cells were exposed with various chemicals for 24 h. One hundred μl of the conditioned media was incubated with the same volume of Griess reagent [0.1% (w/v) *N*-(1-naphthyl)-ethylenediamine and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid] for 10 min at room temperature. Absorbance at 546 nm was measured using a microplate reader and the nitrite concentration determined by using a standard curve of sodium nitrite made up in DMEM free of phenol red.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Cell numbers for mRNA expressions were 1×10^6 in 3 ml of medium in 60-mm plates. Isolation of total RNA was carried out using TRI reagent according to manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed in a 30 μl reaction volume to make cDNA. The reaction mixture contained 3 units of AMV reverse transcriptase, 0.08 mM of oligo $(dT)_{15}$ primer, 1× RT buffer (50 mM Tris–HCl pH 8.8, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM spermidine and 1 mM DTT), 0.66 mM of each dNTPs and 20 units of RNase inhibitor. The reaction mixture was incubated at 42 °C for 45 min to reverse transcribe, and finally at 99 °C for 5 min to inactivate the AMV reverse transcriptase and provide cDNA that was used in PCR. The primer sequences of the target gene are described in Table 1. The PCR reaction mixture contained 200 μM each of the dNTPs, 1 unit Taq polymerase, 1× NEB buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl, 2 mM $MgSO_4$ and 0.1% Triton X-100 pH 8.8), 3 μl of cDNA, 0.25 μM of the sense and antisense primers for iNOS, 0.15 μM for HO-1 and 0.02 μM for β-actin and sterile distilled water to make up 30 μl. The following PCR conditions were applied: iNOS, 32 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; HO-1, 25 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s; and (-actin, 27 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The amplified products were separated in a 1.5% agarose gel in TBE buffer (89 mM Tris, 89 mM borate, 2 mM EDTA [pH 8.0]) and stained with ethidium bromide solution. Quantification of RT-PCR products was performed by densitometry of each band using BIOPROFIL Bio-1D version 11.9 (Vilber Lourmat Biotechnology, France). The image densities of PCR products for iNOS and HO-1 were normalized with the density of β-actin.

2.7. Western blot analysis

Cells (3×10^6) plated in 10 ml of medium in 100-mm plates were used for the preparation of proteins to be analyzed by Western-blot assays. Cells were scraped in the presence of ice-cold lysis buffer containing 50 mM HEPES (pH 7.5), 2 mM EDTA, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 0.3% Triton X-100 and a mixture of protease inhibitors (Complete mini, Roche, Germany). The cell lysate was sonicated twice on ice for 1 min with at 1 s interval with a Vibracell ultrasonic processor set at 2 W. Protein concentrations were quantified with the BCA protein assay kit. Equal amounts of proteins obtained from cells were subjected to electrophoresis using 10% SDS-polyacrylamide gels. Separated proteins were transferred onto PVDF membrane and non-specific bindings were blocked with TBS-T buffer (10 mM Tris–HCl, pH 7.4, 100 mM NaCl and 0.1% (v/v) Tween 20) containing 5% nonfat dried milk for 1 h at room temperature. The membrane was then incubated further with primary antibodies of HO-1 (1:1000 dilution), iNOS (1:1000 dilution) and β-actin (1:5000 dilution) dissolved in 5% bovine serum albumin solution at room temperature. Subsequently, the membrane was incubated with goat anti-mouse or goat anti-rabbit IgG:horseradish peroxidase secondary antibodies for 1 h at room temperature. The specific protein bands on the PVDF membrane were visualized on X-ray film activated by chemiluminescence using SuperSignal West Pico Chemiluminescent substrate. The intensities of each band signal were determined by densitometry using BIOPROFIL Bio-1D version 11.9 (Vilber Lourmat, France). The image densities of specific protein bands were normalized with the density of β-actin band as the internal control to compare amounts of specific protein accumulated in each sample.

2.8. Nuclear protein extraction

RAW 264.7 macrophages (3×10^6cells/plate) were plated in 100-mm plates. Then, cells were treated with 1 μg/ml of LPS in the presence or absence of a tested compound for 2 h. Treated cells were scraped in cold PBS on ice, and centrifuged at 9500 × *g* for 5 min at 4 °C. Each cell pellet was resuspended in cold lysis buffer 1 (25 mM HEPES, pH 7.9, 0.5 mM $MgCl_2$, 5 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF). Cell suspensions were incubated on ice for 20 min and mixed by vortex stirring every 5 min. After incubation, cell suspensions were added with 200 μl of buffer 2 (25 mM Hepes, pH 7.9, 0.5 mM $MgCl_2$, 5 mM KCl, 0.5 mM DTT, 0.5 mM PMSF and 5% nonidet P-40) and rotated at 4 °C for 15 min prior to centrifugation at 13,700 × *g* for 6 min at 4 °C. Pellet nuclei were washed with cold lysis buffer 3 (1:1 mixture of buffer 1 and buffer 2), the wash removed by pipetting and pellets nuclei resuspended in buffer 4 (25 mM HEPES, pH 7.9, 20% glycerol, 420 mM NaCl, 1.5 $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF). Suspensions were incubated for 40 min on ice with vigorous mixing every 5 min. Afterwards, suspensions were centrifuged again at 13,700 g for 20 min at 4 °C and supernatants collected in new tubes as nuclear protein extracts. Protein concentration was determined by Bradford method (Bio-Rad protein assay). Levels of NF-κB p65 subunit in nuclear protein extract were determined by Western blotting analysis as described above.

2.9. Statistical analysis

Statistical analyses were performed using two-tailed student's *t*-test. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Inhibition of NO by CQE in LPS-stimulated macrophage cells

Lipopolysaccharide (LPS) is a bacterial endotoxin that promotes the secretion of proinflammatory cytokines and related molecules, including NO in many cell types. To investigate the effect of CQE on LPS-induced NO production, macrophages were exposed with LPS (1 μg/ml) and various concentrations of CQE (50, 100, 200 and 400 μg/ml) for 24 h. Nitrite, a stable degradation product of NO, was determined by Griess reaction. Nitrite content was significantly elevated in the macrophages after treatment with LPS (Fig. 1A). Treatment with CQE at 50–400 μg/ml concentration-dependently diminished LPS-stimulated NO production in the macrophages with an IC_{50} of 53.88 μg/ml. The number of viable activated cells

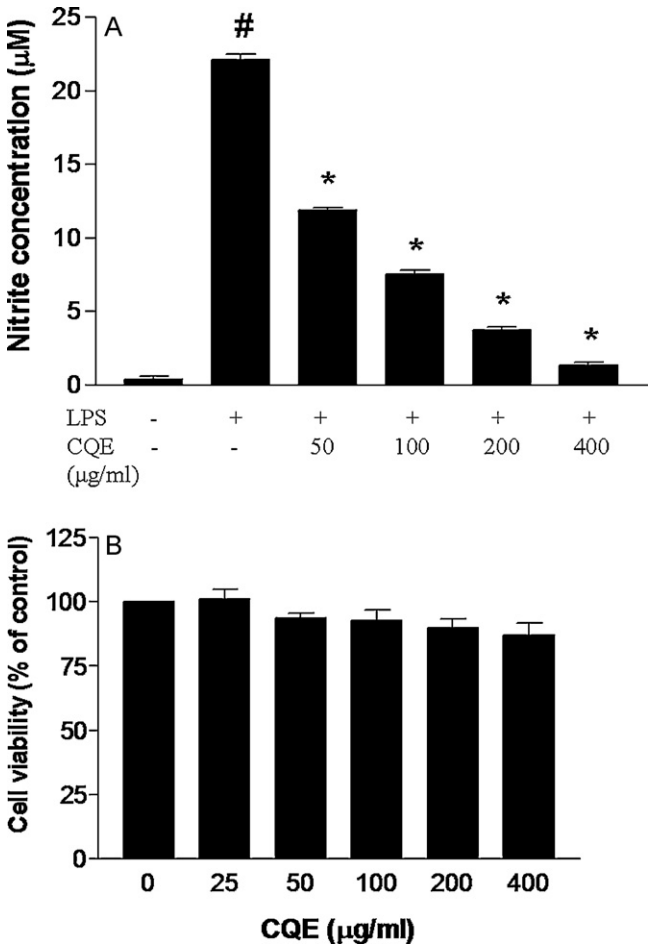

Fig. 1. Inhibition of nitrite production by CQE in LPS-stimulated RAW 264.7 macrophages. (A) Cells were co-incubated with the indicated concentrations of CQE (50–400 μg/ml) and LPS (1 μg/ml) for 24 h. The culture supernatants were subsequently isolated and analyzed for nitrite production. Each column shows the mean ± SD of three independent experiments with triplicate samples. $^{\#}P < 0.001$ vs. control and $^{*}P < 0.001$ vs. LPS alone. (B) Viability of cells harvested at 24 h after the treatment with CQE (25, 50, 100, 200 and 400 μg/ml) was determined using the MTT test as described in Section 2. Each column shows the mean ± SD of three independent experiments with triplicate samples.

was not significantly altered by CQE (Fig. 1B), indicating nitrite inhibition was not attributable to cell death.

3.2. Effect of CQE on iNOS expression in LPS-stimulated macrophage cells

Western blot and RT-PCR analyses were also performed to determine whether the inhibitory effects of CQE on NO were related to the modulation of iNOS expression. As shown in Fig. 2, unstimulated cells and CQE alone did not express detectable iNOS mRNA and protein. iNOS protein and mRNA expressions increased precipitously after treating macrophages with LPS. In contrast these expressions decreased significantly in a concentration-dependent manner in cells co-treated with LPS and CQE. These results indicate that CQE suppresses LPS-stimulated iNOS expression and NO production in a sequential manner.

As NF-κB plays a critical role in the LPS-induced expression of iNOS in RAW 264.7 macrophage cells, we examined the effects of CQE on LPS-induced nuclear translocation of p65 NF-κB, the major component of NF-κB, by immunoblotting. LPS induced an increase in p65 in the nuclear fraction (Fig. 3). The LPS-induced increase in p65 NF-κB level in the nucleus was inhibited directly by CQE concentration. This suggests CQE may inhibit the expression of iNOS at transcription levels.

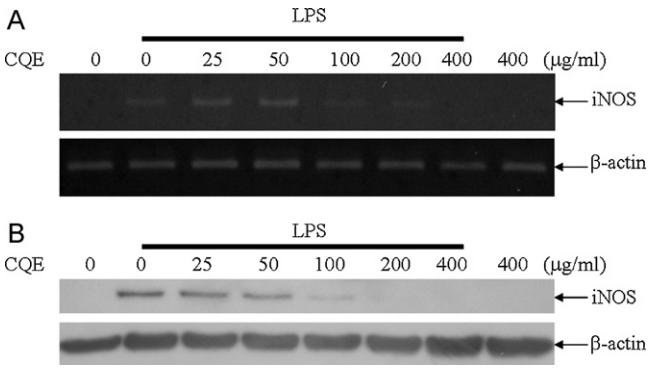

Fig. 2. Effect of CQE on iNOS expression in LPS-stimulated RAW 264.7 macrophages. (A) Cells were incubated in CQE (25–400 μg/ml) and LPS (1 μg/ml) for 12 h. Total RNA were isolated, iNOS mRNA levels were determined by RT-PCR. β-Actin expression was used as an internal control for RT-PCR. (B) Cells were incubated with various concentrations of CQE and LPS (1 μg/ml) for 24 h. Cells were lysed, and iNOS and β-actin protein levels were determined by Western blot analysis.

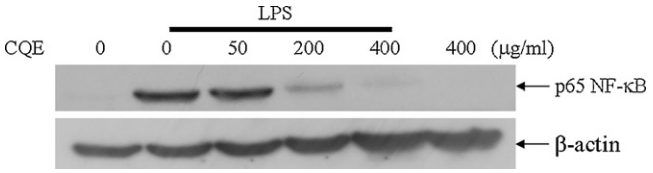

Fig. 3. Effects of CQE on the p65 NF-κB nuclear translocation. RAW 264.7 cells were co-incubated with CQE (50, 200 and 400 μg/ml) and LPS (1 μg/ml) for 2 h. The levels of p65 subunit in nuclear protein were determined by Western blotting analysis.

3.3. HO-1 mediates CQE suppression of LPS-induced NO production

Based on observations that the inhibition of NO production was stronger in cells co-treated with CQE and LPS, we hypothesized that CQE-induced certain molecules to regulate inflammatory responses. Since HO-1 is known to contribute to inflammatory reactions and changes in HO-1 protein levels were examined in CQE-treated cells. Western blot analysis showed that HO-1 protein levels were increased in a dose-dependent manner in RAW 264.7 cells treated with CQE (Fig. 4A). The stimulating action of 400 μg/ml CQE displayed time-dependence occurring after 3 h exposure (Fig. 5A). In order to determine if induction of HO-1 gene expression by CQE appeared at the transcription level, RT-PCR was performed using a specific primer of HO-1 and β-actin. HO-1 mRNA levels increased with dose and exposure time to CQE (Figs. 4B and 5B). These results indicated that CQE is a potent HO-1 inducer, and induction of HO-1 gene expression by CQE was examined at both the transcription and translation levels. CQE enhances HO-1 expression and diminish the formation of NO in RAW 264.7 macrophage cells exposed to LPS. Furthermore, the CQE-induced suppression of NO was inhibited by zinc protoporphyrin IX (ZnPP) ($P < 0.05$) in the presence of HO-1 (Fig. 6). Thus, the activation of HO-1, at least in part, mediates the inhibitory effects of CQE on LPS-induced inflammatory responses.

4. Discussion

Recently, Panthong et al. (2007) demonstrated the anti-inflammatory effect of *Cissus quadrangularis* extract in carrageenin-induced rat paw edema and ethyl phenylpropiolate-induced rat ear edema, which are the useful properties for treating hemorrhoids in

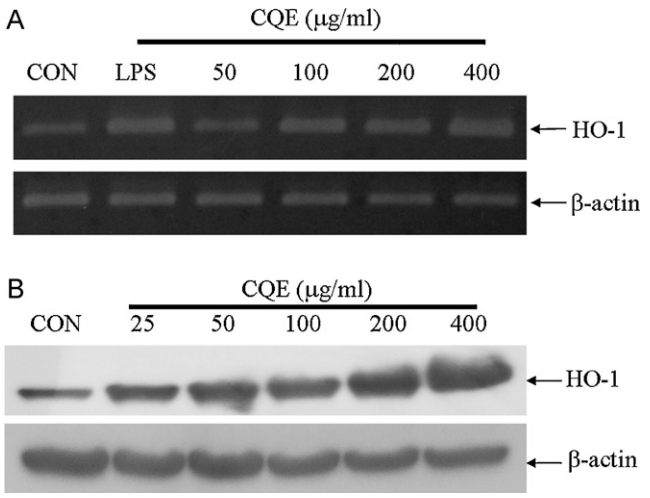

Fig. 4. Induction by CQE of HO-1 protein expression in RAW 264.7 cells. The cells were treated with 50, 100, 200 and 400 μg/ml CQE alone for 12 h. Total RNA were isolated, HO-1 and β-actin mRNA levels were determined by RT-PCR. (B) Cells were incubated with various concentrations of CQE (50–400 μg/ml) for 24 h. Cells were lysed, and HO-1 and β-actin protein levels were determined by Western blot analysis. CON, cells without any treatment.

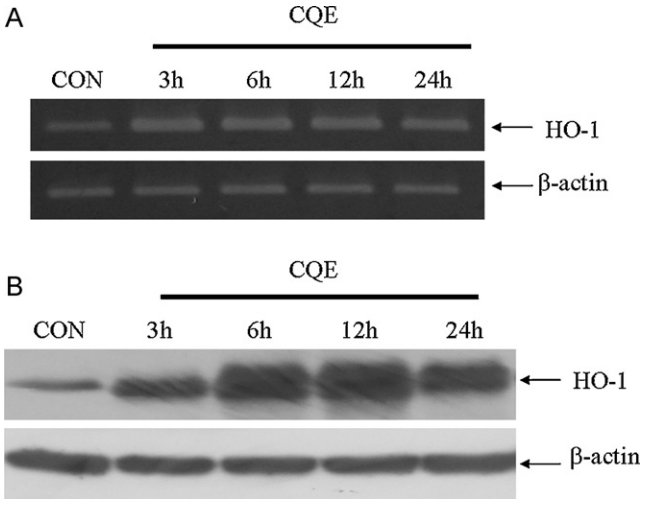

Fig. 5. Induction by CQE of HO-1 protein expression in RAW 264.7 cells. Cells were treated with 400 μg/ml CQE for 3, 6, 12 and 24 h. (A) Levels of HO-1 mRNA were determined by RT-PCR analysis.(B) Levels of HO-1 protein were determined by Western blotting analysis. β-Actin expression was used as an internal control. CON, cells without any treatment.

humans. However, the precise physiological mechanism by which *Cissus quadrangularis* suppresses inflammation still unknown. Thus, we assessed the possible molecular mechanism underlying its anti-inflammatory effect in LPS-induced inflammatory responses. In the present study, we examined the effects of the ethyl acetate extract of *Cissus quadrangularis* (CQE) on the LPS-induced inflammation in RAW 264.7 macrophage model. We found that CQE can down-regulate iNOS expression, and NO production as well as NF-κB p65 nuclear translocation in LPS-stimulated macrophages. CQE also induced HO-1 expression in RAW 264.7 murine macrophages. Inhibition of HO-1 activity by ZnPP reversed these effects which suggest that activation of HO-1 is, at least partly, involved in the inhibition of LPS-induced inflammatory responses by CQE.

Although NO can mediate regulatory physiological functions such as neurotransmission, vasodilation and host defense mechanisms (Moncada et al., 1991). The excessive amounts of NO produced by iNOS in response to bacterial LPS or cytokines play

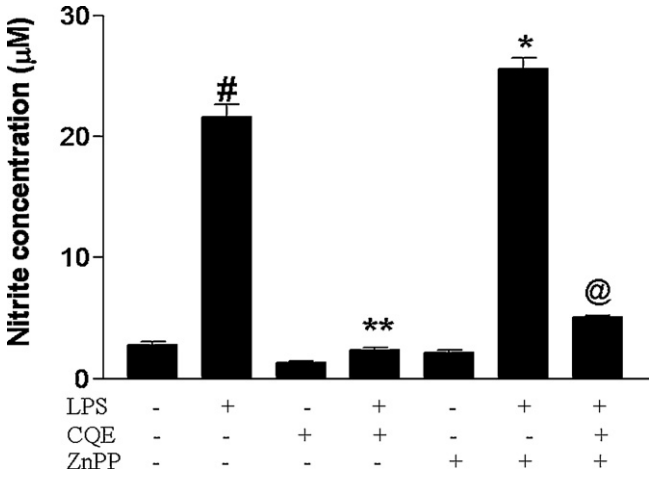

Fig. 6. HO-1 mediates the suppression of LPS-induced NO production in RAW 264.7 cells treated with CQE. Cells were pre-incubated with 10 μM ZnPP for 1 h before exposure to 400 μg/ml CQE and LPS (1 μg/ml) for 24 h. Each column shows the mean ± SD of three independent experiments with triplicate samples. $^{\#}P < 0.001$ vs. control, $^{*}P < 0.01$ and $^{**}P < 0.001$ vs. LPS alone and $^{@}P < 0.001$ vs. LPS + CQE.

an important role in the inflammatory condition (Wright et al., 1992; Dorheim et al., 1994; Guzik et al., 2003; Latham et al., 2005). The production of NO is a crucial part of the immune response to some inflammatory stimuli. For example, large amounts of this mediator have been detected in humans suffering from septic shock, endotoxemia, rheumatoid arthritis, and atherosclerosis (Vallance and Leiper, 2002). Thus, suppression of NO may be an effective therapeutic strategy for prevention and treatment of a variety of inflammatory and infectious diseases (Murakami, 2009). In supporting these finding, we also demonstrated that both NO production and iNOS expression were diminished in the presence of CQE in LPS-induced inflammation in macrophage model. In accord with the results of our study, aspirin-stimulate iNOS activity decreased in the gastric mucosa of rats pretreated with methanolic extract of *Cissus quadrangularis* (Jainu and Devi, 2006). These results suggest that mechanism of the inhibitory activity on NO production of the extract of *Cissus quadrangularis* might associate with the regulation of iNOS gene expression.

It is well known that induction of iNOS expression is promoted by activation of NF-κB in cells stimulated with LPS. Activated NF-κB complex translocates from cytoplasm into the nucleus and binds DNA at κB-binding motifs and activate iNOS gene expression (Bauerle and Baltimore, 1996; Nishikori, 2005). In our studies, NF-κB p65 subunit in the nucleus of LPS-stimulated RAW 264.7 macrophages decreased in the presence of CQE, indicating that CQE inhibits the production of proinflammatory mediator, NO, and the expression of iNOS via the reduction of NF-κB p65 nuclear translocation. This is similar to some reports that also demonstrated that extracts of many plants contain anti-inflammatory agents which inhibit NO formation via the inactivation of NF-κB in macrophages treated with LPS (Kim et al., 2006; Jung et al., 2009; Kazłowska et al., 2010).

According to our results, CQE suppresses the production of NO, and decreases iNOS mRNA and protein expression levels in LPS-induced RAW 264.7 cells. We also found that HO-1 could be induced by treatment with CQE. HO-1 seems to be a novel protective protein with potent anti-inflammatory, anti-oxidant, and anti-apoptotic effects (Sarady et al., 2004). Several lines of evidence have demonstrated that HO-1 expression and its activity products are likely to inhibit NO production in many ways. Elevated HO-1 activity would accelerate the degradation of heme leading to impairment of *de novo* synthesis of functional iNOS by limiting the availability of heme into newly synthesized iNOS protein (Xie et al., 1996). Addi-

tionally, treatment of bilirubin can decrease iNOS expression and activity in LPS-treated macrophage cells as well as in hyperbilirubinemia and endotoxemic rats (Wang et al., 2004; Lanone et al., 2005). Another product of HO-1 activity, CO has been reported to bind the heme group in iNOS protein and inhibit NO production (McMillan et al., 1992; Sarady et al., 2004; Srisook et al., 2006; Ashino et al., 2008). Furthermore, low concentrations of CO have been shown to reduced NF-κB activation and DNA binding activity in the LPS-stimulated macrophages (Sarady et al., 2002). The attenuation in NF-κB activation may then cause a decrease in iNOS expression. Recently, Kim et al. (2008) revealed that CO also downregulates iNOS expression associated with the suppression of iNOS dimer formation in IL-1β-stimulated hepatocytes.

A variety of chemical components have been isolated from *Cissus quadrangularis* including quercetin, ascorbic acid, β-sitosterol, α-amyrin, two tetracyclic triterpenoids, quadrangularins A, B, C, resveratol and piceatannol (Adesanya et al., 1999; Attawish et al., 2002; Thiangtham, 2003; Singh et al., 2007; Thakur et al., 2009). Although the exact component responsible for the induction of HO-1 and the anti-inflammatory effects of CQE remain under investigation, quercetin has been demonstrated to inhibit LPS-stimulated NO production in accordance with the induction of HO-1 in mouse macrophage cell lines (Lin et al., 2003). Additionally quercetin, resveratol and piceatannol were identified also as phytochemical inducers of HO-1 expression *in vitro* and *in vivo* (Chen et al., 2005; Wung et al., 2006; Farghali et al., 2009).

5. Conclusions

Our results demonstrated that CQE inhibits LPS-induced NO production and iNOS expression in macrophages. These inhibitory effects, at least in part, might be mediated via induction of HO-1 by CQE, thereby leading to decrease in nuclear level of NF-κB and subsequently reduction in iNOS expression. Therefore, our results provide supporting data that CQE exerts an anti-inflammatory effect and may contain compounds useful in treating inflammatory diseases including hemorrhoids.

Acknowledgements

The work was financially supported by a research program of Faculty of Science, Burapha University and also by the Center for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education, Thailand. The authors would like to express their appreciation to Prof. Dr. Frederick W.H. Beamish, Faculty of Science, Burapha University, for his comments and English correction. We also thank Dr. Rawiwan Watanadilokt, for valuable technical assistance.

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