Cover letter

1. None of the material in the manuscript entitle “Anti-inflammatory activities of red curry paste extract on lipopolysaccharide-activated murine macrophage cell line” has been published previously or is currently under consideration for publication elsewhere.

2. The authors’ responsibilities were as follows, Siriporn Tuntipopipat, Channarong Muangnoi, Pimjai Chingsuwanrote and Somsri Charoenkiatkul contributed to the design of the study protocol. Channarong Muangnoi and Pimjai Chingsuwanrote conducted the cell culture protocol. Siriporn Tuntipopipat, Channarong Muangnoi, Pranom Chantravisut and Montira Parengam analyzed all biological markers except RT-PCR. Pimjai Chingsuwanrote and Saovaros Svasti analyzed and interpreted data obtained from RT-PCR. Siriporn Tuntipopipat wrote the manuscript. All authors reviewed and provided inputs to the manuscript.

3. Revision version of manuscript-II has 35 pages including 8 figures and no heading.

4. The authors cut some old and irrelevant references out and added 2 new references (number 7-8 on introduction and references part by bold) which were more recent and relevant to the content of the manuscript. However, the authors could add only one reference (number 7) which was suggested by the chief editor.
submit manuscript to J Food Sci

From: siriporn tuntipopipat (stuntipopipat@hotmail.com)
Sent: Saturday, January 16, 2010 3:36:03 AM
To: lee@aesop.rutgers.edu

Dear Dr. Lee

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To: stuntipopipat@hotmail.com
Cc: akferguson@ift.org; tungching.lee@Verizon.net; LEE@AESOP.Rutgers.edu

Dear Professor stuntipopipat:

Thank for your inquiry.

I have looked through your abstract.

My major concern is whether there is sufficient data on the chemical identification, characterization and standardization for the bioactive components in the ethanol extract from Thai red curry paste, consisting of red chili pepper, garlic, shallot, kaffer lime peel, lemon grass, galangal and pepper as main ingredients used in your study were determined qualitatively and quantitatively. Dose/response data of your bioactivity assay also are required. JFS will not accept paper for publication without such data.

If you have further question, please let me know.
Best regards.

Tung-Ching
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Cc: Journal of Nutritional Biochemistry (jnb@uky.edu)
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Thank you and kind regards,

Dr. Daniela Oberreuther-Moschner
Editorial Office

Prof. Gerhard Rechkemmer
Editor-in-chief
European Journal of Nutrition

submit manuscript

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Abstract

Spices and herbs are rich in phytochemicals capable of suppressing production of pro-inflammatory mediators. However, these biological functions of a mixture of spices and herbs present in an actual diet have not been investigated previously. The anti-inflammatory activities of an ethanolic extract from Thai red curry paste, consisting of red chili pepper, garlic, shallot, kaffer lime peel, lemon grass, galangal and pepper as main ingredients, were assessed in lipopolysaccharide-activated mouse RAW264.7 macrophages. Cells treated with 65-260 µg/ml of red curry paste extract significantly suppressed LPS-induced NO, iNOS, COX-2, TNF-α and IL-6 protein and mRNA expression in a dose-dependent manner without cytotoxic effect. The inhibitory effect was mediated by inhibited phosphorylation and degradation of IκB-α. It also suppressed activation of mitogen-activated protein kinases (MAPKs) including p38, c-jun NH₂-terminal kinases (JNK) and extracellular signal-regulated kinases (Erk1/2). Furthermore the extract blocked intracellular accumulation of reactive oxygen species in RAW264.7 cells stimulated with LPS. These results indicated that the anti-inflammatory property of Thai red curry paste stems from bioactive phytochemicals present in the spice and herb constituents. The health benefits of Thai red curry paste warrants further investigations in vivo.

Thank you for taking time to take a look my abstract. Best regard.

Siriporn Tuntipopipat, Ph.D.
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Institute of Nutrition at Mahidol University
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Rescinded decision for your submission NUT-D-10-00036

Dear Dr. Tuntipopipat,

The latest decision on your submission "Anti-inflammatory activities of red curry paste extract on lipopolysaccharide-activated murine macrophage cell line" was made in error and has been rescinded (that is, the decision has been cancelled and the manuscript has been restored to its previous status). We would like to send your manuscript out for review, if you still wish us to do so, and you have not submitted it to another journal. Please confirm.

With kind regards,

Michael M. Meguid, MD, PhD
Editor-in-Chief
Nutrition
Dear Dr. Tuntipopipat,

Your article Anti-inflammatory activities of red curry paste extract on lipopolysaccharide-activated murine macrophage cell line has been returned by our reviewers. **In its present form, it cannot be accepted for publication in Nutrition.**

However, Nutrition would be willing to reconsider it for possible publication, if you feel you can fully address the reviewers' comments. Please remember that we would have to send your revised manuscript back to the original referees and keep this time frame in mind when you respond to the critiques.

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We look forward to hearing from you.

Yours sincerely,

Michael M. Meguid, MD, PhD
Editor-in-Chief
Nutrition
Anti-inflammatory activities of red curry paste extract on lipopolysaccharide-activated murine macrophage cell line

Siriporn Tuntipopipat, Ph.D.,*, Channarong Muangnoi, M.Sc., Pimjai Chingsuwanrote, M.Sc., Pranom Chantravisut, B.Sc., Somsri Charoenkiatkul, D. Sc. and Saovaros Svasti, Ph.D.,

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Abstract

Objective: This study investigated anti-inflammatory and antioxidant activities of an ethanol extract from Thai red curry paste.

Methods: RAW264.7 murine macrophage cell line was incubated with the extract (65-260 µg/ml) with or without lipopolysaccharide (LPS). The anti-inflammatory activities of the extract were examined by measuring: inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor alpha (TNF-α) and interleukine-6 (IL-6) mRNA and protein level by RT-PCR, western blotting and ELISA, respectively. The nitric oxide (NO) production and intracellular reactive oxygen species generation were determined by Griess method and fluorescence intensity. The activation of mitogen activated protein kinases (MAPKs) and inhibitor kappa B (IκB) were determined by western blot.

Results: Exposure of cells with the extract significantly suppressed LPS-induced NO production and iNOS, COX-2, TNF-α and IL-6 expression ($P < 0.05$) by dose-dependent without cytotoxic effect. Intracellular reactive oxygen species significantly decreased ($P < 0.05$) in LPS-induced RAW264.7 cells. The inhibitory effect was mediated partly by inhibiting activation of IκB-α and MAPKs.

Conclusion: These results suggest that the anti-inflammatory and antioxidant properties of Thai red curry paste stems from bioactive compounds present in the spice and herb constituents. The health benefits of Thai red curry paste warrants further investigations in vivo.

Keywords: COX-2; iNOS; TNF-α; IL-6; Inhibitor kappa B; Mitogen-activated protein kinase; RAW264.7 cells
Introduction

Macrophages are professional immune cells that play an important role during inflammatory responses against various stimuli including lipopolysaccharide (LPS) from gram-negative bacterial cell wall. LPS binds to TLR4 and MD2 proteins on macrophage plasma membrane resulting in the activation of several intracellular signaling pathways, involving IKK-NF-κB and three mitogen-activated protein kinases (MAPKs), namely, p-38, c-Jun N-terminal kinases (JNK) and extracellular signal-regulated kinases (Erk1/2) [1]. These signaling pathways subsequently activate transcription factors, viz. NF-κB and AP-1, that regulate expression of several genes associated with functional responses [1-2], including increased expression of pro-inflammatory cytokines (IL-1, IL-6, TNF-α), adhesion molecules (ICAM-1, VCAM-1) and pro-inflammatory enzymes (iNOS and COX-2) [3]. Overproduction of these inflammatory mediators can cause of patho-physiological conditions associated with many chronic diseases, including cardiovascular, gastrointestinal, cancer, diabetes, rheumatoid arthritis and neurodegenerative disorders [4].

As nonsteroidal anti-inflammatory drugs can manifest adverse side effects [5], the efforts to identify natural products for prevention and treatment of inflammatory disorders continue. In particular, the use of medicinal plants including spices and herbs in traditional remedies are of interest as reliable and safe alternatives. There is increasing evidence that various phytochemicals may prevent or attenuate inflammation-associated chronic diseases [6]. For example, in vitro and in vivo studies have shown that phytochemicals in habitual diets, such as curcumin, diallyl sulfide, capsaicin, eugenol and gingerol, suppress the expression of pro-inflammatory gene products, including cytokines, chemokines, adhesion factors and enzymes [7-9]. The mechanism of suppression generally is modulated through inhibiting phosphorylation of
signaling proteins in pathways that activate NF-κB-mediated transcription of pro-inflammatory genes [4, 10]. Also, pre-treatment of neutrophils with anti-oxidants, N-acetyl cysteine (NAC) or \( \alpha \)-tocopherol inhibits LPS-induced NF-κB activation and suppresses production of pro-inflammatory cytokines [11]. LPS-induced reactive oxygen species (ROS) generation and NF-κB activation are mediated by direct interaction of TLR4 with NADPH oxidase4 (Nox4), an enzyme related to phagocytic cell NADPH oxidase 2 (Nox2) [12]. In addition, ROS production in response to LPS mediates interactions between TRAF6 and redox-sensitive ASK1, which subsequently activates p38, a downstream target of the LPS signaling pathway [13].

In tropical regions including Asia, habitual diets generally contain various spices and herbs for flavoring, deodorizing, pungency, coloring and enhancing taste [14]. Habitual Thai diets are similar to other Asian cuisines that often include spices and herbs in their recipes [15]. Although phytochemicals in several individual spices and herbs have been demonstrated to suppress pro-inflammatory mediator expression [4, 7-8], these functional activities have not been investigated in a dietary matrix to our knowledge. Red curry paste, the most common ingredient in Thai curry dishes [15], is composed of 5 to 7 distinct spices and herbs. Here, we used LPS-induced RAW264.7 murine macrophage cell line to demonstrate the anti-inflammatory including the underlying mechanism of an ethanol extract from the seven spices and herbs present in Thai red curry paste. The results demonstrate the possible health benefits of red curry paste and support the need for further investigations in animal models and human subjects afflicted with inflammatory-associated diseases.

Materials and methods

Chemicals
Dulbecco’s modified Eagle’s medium (DMEM), LPS (E. coli O111:B4), anti-β-actin-HRP and species-specific HRP-conjugated secondary polyclonal antibodies were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from PAA Laboratories (Haidmannweg, Austria). Penicillin and streptomycin were obtained from Invitrogen (Grand Island, NY, USA). Primary antibodies against iNOS, COX-2, phospho-p38, total p38, phospho-JNK, total JNK, phospho-Erk1/2, total Erk1/2 were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemical and reagents were of analytical grade.

Preparation of ethanol fraction from red curry paste

The curry paste consisted of a mixture of the following materials: dried chili pepper (Capsicum annuum) 22.5%, garlic bulb (Allium vineale) 20%, fresh lemon grass (Cymbopogon citrates) 17%, shallot bulb (Allium cepa) 11%, fresh galangal (Alpinia galangal) 6%, dried kafferlime peel (Citrus hystrix) 2.5%, dried pepper seed (Piper nigrum) 0.5% and shrimp paste 4.5% with 16% salt. The paste was freeze dried, packed in aluminum foil in vacuo and stored at -20°C until use. The dry paste powder was extracted twice with 90% ethanol (0.1g sample/3 ml solvent). Samples were centrifuged at 4,500 g for 10 min at room temperature and the combined supernatants evaporated under vacuum at 45-50 °C to dryness. The dry extract was solubilized in DMSO: ethanol (1:1) to 0.2% final concentration and diluted to designated concentrations with serum-free, phenol red-free medium prior to addition to cell cultures.

Growth and activation of cells

Murine macrophage RAW 264.7 cells (TIB71) obtained from ATCC (Bethesda, MD, USA) were grown in DMEM supplemented with 10% fetal bovine serum, 15 mM HEPES, 100 units/ml
penicillin and 100 μg/ml streptomycin at 37 °C in humified atmosphere of 5% CO₂/95% air.

Cells were used at 80% confluency between passages 8 and 20. Cells (7.5 x 10⁵ cells/ml) were seeded for 24 h to allow attachment. The attached cells were incubated with 65-260 μg/ml extract for 1 h prior to stimulate with 5 ng/ml LPS in phenol red and serum-free medium for 24 h.

Cell viability

Viability of treated cells in washed monolayer was assessed using sulforhodamine B (SRB) assay [16]. In brief, cells pre-treated with or without extract and subsequently stimulated with LPS were washed with phosphate-buffered saline (PBS) and fixed with 50% cold TCA at 4 °C for 2 h. Cells were stained with 0.1% w/v Sulforhodamine B in 1% acetic acid for 20 min and solubilized with 10 mM Tris-hydromethylaminomethane, pH 10. Absorbance at 500 nm was measured, with absorbance of vehicle control defined as 100% viability.

Measurement of nitric oxide

Nitrite concentration was used as an assessment of NO production. Cells were plated and treated with test extract in the presence or absence of LPS. After incubation for 24 h, spent media were collected to measure nitrite concentrations using the Griess reaction [17] by adding 100 μl of Griess reagent (0.1% naphthylethlenediamide dihydrochloride in H₂O and 1% sulphanilamide in 5% concentrated phosphoric acid) to 100 μl of sample. Nitrite concentration was calculated by comparison with sodium nitrite.

Enzyme-linked immunosorbent assay (ELISA) of TNF-α and IL-6
TNF-α and IL-6 in cell-treated culture medium were determined by quantitative “sandwich” ELISA using paired antibodies purchased from Endogen Inc. (Rockford, IL, USA) and eBioscience Inc. (San Diego, CA, USA), respectively. In brief, high-binding plates (NUNC, Denmark) were coated with capture antibody for mouse TNF-α and IL-6. After overnight incubation at 25 °C, plates were washed and blocked for 2 h with 1% bovine serum albumin (BSA) in PBS. Culture medium or various concentrations of recombinant mouse TNF-α and IL-6 protein (standards) were incubated at 4 °C overnight prior to adding biotinylated antibodies to the wells. After 2 h at 25 °C, immune complex was detected using streptavidin horseradish peroxidase (HRP)-tetra methyl benzidine detection system (Pierce, Rockford, IL, USA). Reactions were terminated with 2M H₂SO₄ and absorbance at 450 nm was determined using a microtiter plate reader (TECAN, GmbH, Austria). Concentrations of TNF-α and IL-6 in samples were calculated by comparing absorbance with the standard curve.

Western blot analysis
Treated cells were harvested, washed twice with ice-cold PBS and resuspended in ice cold lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% SDS, 50 mL sodium fluoride, 10 mM sodium pyrophosphate and 0.2% protease inhibitor cocktail (Sigma) for 30 min at 4 °C. Cell lysate was collected after centrifugation at 13,500 g at 4 °C for 5 min. Protein content was determined by BCA (Endogen) method using BSA as a standard. Samples (40 µg of protein/well) were separated on 8% (for detection of iNOS and COX-2 protein) or 10% (MAPKs and NF-κB protein) SDS-PAGE and transferred onto nitrocellulose membranes (Whatman, GmbH, Germany). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h and incubated with specific primary
antibody in TBST containing 5% BSA overnight at 4 °C. After washing three times, membranes were reacted with HRP-conjugated secondary antibody for 1 h. Following five washes, membranes were incubated with Super Signal solution (Endogen) for 2 min and exposed to X-ray film. The membranes were then stripped of bound antibody and re-probed with anti-β actin or anti-total MAPKs protein to assess quantity of protein loaded. The density of target bands was quantified by Image J program. Results are expressed as relative ratio of band density between protein of interest and β-actin.

RNA extraction and RT-PCR

Total RNA was extracted with TRI REAGENT® (Molecular Research Center, Cincinnati, OH, USA) as described in manufacture. One µg of total RNA was reversed transcribed at 50 °C for 1 h using 200 U of reverse transcriptase (SuperScript™ III Reverse Transcriptase, Invitrogen, USA) with oligo-dT18 primer. Reactions were terminated by heating at 70 °C for 15 min. The oligonucleotide primer pairs (Bio Basic, Ontario, Canada) used to amplify interested cDNA was the following: COX-2, sense: 5′-AGGTCATTGGTGAGAGGTG-3′; antisense: 5′GAGTCCAT GTTCCAGGAGA-3′; iNOS, sense: 5′-CACCTTGAGTGTCACCCAGT-3′; antisense: 5′-TG GTCACATTCTGCTTCTGG-3′; TNF-α, sense: 5′-TCGTAGCAAACCACCAAGTG-3′; anti- sense: 5′-CGGACTCCGCAAAGTCTAAG-3′; IL-6, sense: 5′-GCAAGAGACTTCCATCCAG TTG-3′; antisense: 5′-ACTCCAGGTAGCTATGGTACTCCA-3′; β-actin, sense: 5′-GGCACCA CACCTTCTACAATG-3′; antisense: 5′-GGTCTCAAACATGATCTGGGTC-3′. Amount of cDNA template for PCR amplification were optimized for each target gene in order to obtain a clear PCR product. Amplification was performed in a MyCycler thermal cycler (BioRad, Hercules, CA, USA) as follows: denaturation at 95 °C for 15 min for the first cycle; 94 °C for
30 s; annealing at either 62 °C (COX-2 and IL-6), 58.1 °C (iNOS) or 55 °C (TNF-α) for 30 s; and, extension at 72 °C (iNOS) and 68 °C (COX-2, TNF-α and IL-6) for 45 s for either 25 repetitive cycles (COX-2, iNOS and IL-6) or 23 repetitive cycles (TNF-α). Final extension was performed at 72 °C for 10 min. All target genes were performed duplex PCR with β-actin as an internal control. The PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide. The density of target bands was quantified by Image J program. Results were expressed as relative ratio of band intensity between mRNA of interest and β-actin mRNA.

Measurement of intracellular ROS

The level of intracellular ROS in treated RAW264.7 cells was measured as previously described [18]. In brief, treated cells were washed with warm PBS (37 °C) and incubated with 5 µM 2, 7-dichlorofluorescein diacetate (Sigma,) in basal medium at 37 °C for 30 min. Then cells were washed with 3 times with warm PBS (37 °C) and subsequently lysed with 0.5% TritonX-100 in PBS. Fluorescence in supernatant obtained by centrifugation of cell lysate at 12,000 g for 5 min was measured with excitation at 485 nm and emission at 530 nm by Luminescence Spectrometer LS55 (Perkin Elmer Instruments LCC, Shelton, CT, USA).

Statistical Analysis

Statistical analysis was performed using SPSS (version 14.0, SPSS Inc., Chicago, Illinois). Data are presented as means ± SE. The mean values were calculated from at least three separated experiments conducting on separate days. Statistically significance differences were determined using the independent Student’s t test. Significance was set at $P < 0.05$. 
Results

Red curry paste extract decreases NO production and iNOS expression

Exposure of RAW264.7 macrophages to 5 ng LPS/ml significantly increased the concentration of nitrite, a stable oxidized product of NO, in medium, whereas there was no significant change in cultures exposed to either vehicle or red curry extract alone (Fig. 1A). Pre-treatment of cells with red curry paste extract (65-260 µg/ml) significantly suppressed (P<0.05) LPS-induced NO production in a dose-dependent manner without exerting cytotoxicity (data not shown). To determine the cause of reduction in NO production by the extract, the expression of iNOS was examined by Western blot and RT-PCR analysis. As expected, the expression of iNOS was increased significantly after exposure to LPS for 24 h (Fig. 1B and C). Pre-treatment with curry paste extract prior to stimulation with LPS showed a concentration dependent inhibition of LPS-induced iNOS protein and mRNA expression. These results indicated that red curry paste extract decreased the NO production by suppression of iNOS mRNA expression

Red curry paste extract attenuates LPS-mediated increases in expression of COX-2, TNF-α and IL-6.

COX-2 can be induced by several stimuli such as LPS, cytokines and growth factors during active inflammatory episodes [19-20]. Exposure of RAW264.7 cells to LPS for 24 h markedly induced COX-2 protein and mRNA expression (Fig. 2A and 2B). Pre-treating RAW264.7 cells with red curry paste extract at 65-260 µg/ml significantly suppressed COX-2 protein and mRNA expression in a dose dependent manner. Likewise, exposure of RAW 264.7 cells to LPS for 24 h significantly enhanced TNF-α secretion in culture medium from a basal level of 3.3 ng/ml to more than 100 ng/ml (Fig 3A). Pre-treating cells with the extract significantly decreased TNF-α
secretion (Fig 3A) and mRNA content (Fig 3B) in a dose dependent manner. LPS also significantly induced IL-6 secretion (Fig 4A). As expected, IL-6 mRNA was increased in cells exposed with LPS (Fig 4A). Pre-treatment with the extract significantly decreased both IL-6 secretion (Fig 4A) and mRNA (Fig 4B) dose dependently.

Red curry paste extract suppresses MAPKs signaling pathways activation

LPS is a well-known activator of mitogen activated protein kinases (MAPKs), viz. Erk1/2, p38 and JNK [21] in macrophages and many other cell types. The effects of red curry paste extract on LPS-induced phosphorylation of Erk1/2, p38 and JNK were examined by Western blot. Exposure of RAW264.7 cells to LPS activated phosphorylation of Erk1/2, p38 and JNK (Fig 5A-C). Phosphorylation of the three MAPKs was inhibited in a dose-dependent manner in cells pre-treated with the extract (Fig 5A-C). Thus, the extract suppressed LPS-induced pro-inflammatory mediator production in part by blocking phosphorylation of the three MAPKs proteins.

Red curry paste extract inhibits phosphorylation and degradation of IκB-α

In resting status, NF-κB, a dimeric transcription factor, is sequestered in the cytoplasm in an inactive form by associated with IκB-α. Upon exposure of macrophages to LPS, NF-κB signaling cascade is activated and results in phosphorylation- induced proteolytic degradation of IκB-α. Free NF-κB translocates to the nucleus where it induces transcription of a variety of genes including pro-inflammatory mediators (iNOS, COX-2, TNF-α and IL-6) [3]. We investigated whether the red curry paste extract mediated its inhibitory effect on iNOS, COX-2, TNF-α and IL-6 expression by inactivation of NF-κB. LPS markedly induced phosphorylation of IκB-α and degradation of IκB-α in RAW264.7 cells (Fig 6A and 6B). Pre-treatment of RAW264.7 cells
with the extract significantly inhibited LPS-induced phosphorylation and degradation of IkB-α in a dose-dependent manner for up to 24 h (Fig 6A and 6B).

Red curry paste extract suppresses reactive oxygen species (ROS) production

LPS-stimulated macrophage generates reactive oxygen species (ROS) via the activation of a membrane-bound NADPH oxidase [22], leading to activation of MAPKs and NF-κB nuclear translocation and results in expression of pro-inflammatory mediators [23]. Pre-treating RAW264.7 cells with red curry paste extract significantly reduced LPS-induced ROS production in a dose dependent manner (Fig 7). These results suggest that red curry paste extract possesses antioxidant activity that suppresses LPS-induced iNOS, COX-2, TNF-α and IL-6 expression by attenuating ROS accumulation that in turn blocks activation of MAPKs and NF-κB.

DISCUSSION

The present study clearly demonstrates that an ethanolic extract of Thai red curry paste decreases the production of inflammatory mediators by LPS-activated RAW264.7 macrophages. We demonstrated that phytochemicals present in red curry paste extract significantly inhibited production of NO by suppressing the expression of iNOS, as well COX-2, TNF-α and IL-6 in a dose-dependent manner. This activity appears to be mediated by attenuating the phosphorylation of MAPK Erk1/2, p38 and JNK and thus suppressing NF-κB activation via scavenging intracellular ROS in LPS-treated macrophages.

Thai red curry paste generally contains dried chili pepper, garlic bulb, fresh lemon grass, shallot bulb, fresh galangal, dried kaffer lime peel, dried pepper seed and shrimp paste as its major ingredients. The main ingredients are similar among products producing by home-made,
small scale commercial products and industrial export products. The amount of red curry paste in
typical Thai recipes is 15 g per serving [24]. Previous studies have demonstrated that aqueous
and organic extracts from a number of individual spices and herbs exhibit a variety of health-
promoting activities including antioxidant and anti-inflammatory activities in vitro and in vivo [4,
8, 25-27]. Compounds that appear to contribute to such activities have been identified in some
components of Thai red curry paste.

Chili pepper is a widely consumed spice throughout the world and is a common spice in
many Thai recipes. It is the main ingredient in the curry paste (22.5%). We previously reported
that pre-treatment of RAW264.7 cells with ethanolic extract of chili fruit significantly suppressed
NO and TNF-α production in a dose dependent manner [28]. Capsaicin and its derivatives are
primary bioactive compounds in chili pepper. Capsaicin exhibits anti-inflammatory activity in
vitro and in vivo [29-33]. In particular, it inhibits COX-2 and iNOS expression in LPS-stimulated
peritoneal mouse macrophage [32] and suppresses inflammatory responses of infiltrate
macrophages around the adipose tissue [33]. In addition, red chili pepper contains other
carotenoids, including β-carotene, lutein and β-cryptoxanthin [33-34]. β-carotene inhibits
expression of inflammatory genes in LPS-stimulated mouse macrophages by suppressing redox-
based NF-κB activation [23, 34, 36] and lutein decreases NO production and iNOS protein and
mRNA expression in mouse macrophage [36]. A previous study showed that red pepper
(Capsicum baccatum) juice exhibits anti-inflammation against carrageenan- and antigen-induced
inflammation in rats [37]. The suppressive effects of ethanolic extract of red curry paste on pro-
inflammatory mediators and ROS generation in LPS-activated RAW 264.7 cells in the present
study are likely due to the combination of such compounds from red chili present in the paste.
The peel of kaffer lime or leech lime fruit (*Citrus hystrix*) is a common spice in every Thai curry paste including that in this study. Flavonoids present in the peel of various citrus fruits have been shown to suppress LPS-activated NO production and iNOS expression in RAW264.7 cells (38, 39). Others have observed that the flavonoids myricetin [40], isorhamnetin, quercetin [41], cyanidin [42], luteolin [43] and apigenin [44] derived from citrus plants exert anti-inflammatory effects by suppressing expression of iNOS and COX-2, and reducing production of NO and PGE\(_2\) in LPS-activated macrophages. Furthermore, citrus fruit flavonoids such as naringin, naringenin and hesperidin attenuate ROS production by phorbol-myristate acetate stimulated neutrophils [45]. Such compounds in kaffer lime peel in the curry paste extract may contribute to the attenuation of iNOS, COX-2 expression and ROS production in LPS activated RAW264.7 cells in the present study.

A diarylheptanoid isolated from galangal (*Alpinia galangal*) exhibits a suppressive effect on LPS-induced production of NO, IL-1\(\beta\) and TNF-\(\alpha\) and inhibits iNOS and COX-2 gene expression by blocking phosphorylation of Erk1/2 [46]. Seven diarylheptanoids from the methanol extract of the rhizomes *Alpinia officinarum* showed strong anti-inflammatory effects induced by 12-\(\text{O}\)-tetradecanoylphorbol-13-acetate on mice ear [47]. Here we have shown the extract from red chili paste inhibited phosphorylation of p38 and JNK as well as Erk1/2 in LPS-treated RAW264.7 cells indicating a general suppression of MAPKs signaling activity.

Lemon grass (*Cymbopogon citratus*) is a common spice seasoning in sour spicy soup of the Thai popular dish, “tom yum”. It contributes up to 17% raw materials of red curry paste. Lemon grass possesses several essential oils including citral. Recently, it has been demonstrated that citral (3–12 \(\mu\)g/mL) suppressed LPS-induced nitric oxide [48] production, iNOS expression, nuclear translocation of NF-\(\kappa\)B, and pro-inflammatory gene expression. The suppression of iNOS
expression and NF-κB inactivation in LPS activated RAW264.7 cells in the present study partly contributes by the citral and essential oils in lemon grass in the red curry paste.

The curry paste contained 20% garlic. Its bulb has several sulfur compounds (e.g., allicin) with high potential health benefits [49]. Allicin markedly suppresses spontaneous and TNF-α-induced secretion of inflammatory cytokines and chemokines in intestinal HT-29 and Caco-2 epithelium cell lines [50]. Diallyl disulfide in garlic also can ameliorate inflammatory responses of adipose tissue in obesity by inhibiting inflammatory actions of infiltrating macrophages [51]. More recently, diallyl sulfide has been shown to suppress IL-1β and monosodium urate crystal-induced COX-2 expression in synovial cells and chondrocytes and to ameliorate urate crystal-induced synovitis through a mechanism involving NF-κB [52]. The inhibitory effect of the curry paste extract on IκB-α activation in the present study likely results from the combined activities of such compounds from garlic present in the paste. Shallot also likely contributes to the anti-inflammatory activity of red curry paste extract as it contains a relatively high concentration of quercetin, which exhibits anti-inflammatory activity both in vitro and in vivo [53-54].

In summary, we have shown that an ethanolic extract of Thai red curry paste containing a complex mixture of several spices and herbs suppresses the production of pro-inflammatory mediators in murine macrophage-like cells exposed to LPS. The presence of a combination of bioactive compounds in the spice and herbal constituents’ Thai red curry is most likely responsible for the observed activities. Our results strongly support a previous hypothesis suggesting that the mixture of phytochemicals present in whole diets has a synergistic effect to enhance their health benefit functions including antioxidants and anti-inflammation [55-56]. The challenge now is to delineate in vivo actions of the extract and identify interactions among the
various constituents to provide a better understanding of the health promoting effects of a complex ingredient consumed in a relatively large region of the world.

Acknowledgements

This work was supported by the research grant from Mahidol University, Thailand. We would like to thank Dr. Prapon Wilairat from Mahidol University, Thailand and Dr. Mark Failla from The Ohio State University, USA for their kindly review and comment during manuscript preparations. We also appreciate the assistance of Dr. Sitima Jittinandana and Dr. Aikkarach Kettawan for red curry paste preparation.

References


[24] Thai Food and Drug Administration (Thai FDA), In Food labeling. Ministry of Public Health 1998; Notification no. 182 B.E.


[52] Lee HS, Lee CH, Tsai HC, Salter DM. Inhibition of cyclooxygenase 2 expression by diallyl sulfide on joint inflammation induced by urate crystal and IL-1[beta]. Osteoarthr Cartil 2009;17:91-9.


Legends

Fig.1. Red curry paste extract decreases NO production and iNOS expression in LPS-activated macrophage. Cells were pre-treated with 65-260 μg/ml of the extract for 1 h prior to incubation with 5 ng/ml LPS for 24 h. Unstimulated cells were treated identically except that vehicle alone was added to medium. Culture media were collected to determine NO concentration (A). Lysates from treated cells were analyzed by immunoblotting using antibodies against iNOS and β-actin. Results are expressed as ratio of band density of iNOS to β-actin protein (B). (C) After 24 h incubation with LPS, total RNA was extracted and relative amounts of iNOS and β-actin mRNAs determined by RT-PCR. Results are expressed as relative ratio of band density of iNOS to β-actin amplicons. Data are means ± SEM of 3 replicate experiments. *P<0.05 vs. incubation with LPS alone.

Fig.2. Red curry paste extract attenuates increases in COX-2 expression. Cells were treated as described in Figure 1. (A) Cell lysates were analyzed by immunoblotting using antibodies against COX-2 and β-actin. Results are expressed as ratio of band density of COX-2 to β-actin protein. (B) COX-2 mRNA level normalized relative to β-actin in treated cells was determined by RT-PCR with results expressed as relative ratio of band density of COX-2 to β-actin amplicons. Data are mean ± SEM of 3 replicate experiments. *P<0.05 vs. incubation with LPS alone.

Fig.3. Red curry paste extract inhibits TNF-α mRNA expression and secretion. Cells were treated as described in Figure 1. (A) TNF-α concentration in culture medium was measured by ELISA. (B) TNF-α mRNA normalized relative to β-actin in treated cells was determined by RT-PCR and
results are expressed as relative ratio of band density of TNF-α to β-actin amplicons. Data are mean ± SEM of 3 replicate experiments. *P<0.05 vs. incubation with LPS alone.

Fig.4. Red curry paste extract decreases IL-6 mRNA expression and production. Cells were treated as described in Figure 1. (A) IL-6 concentration in culture medium was measured by ELISA. (B) IL-6 mRNA normalized relative to β-actin in treated cells was determined by RT-PCR with results expressed as relative ratio of band density of IL-6 to β-actin amplicons. Data are mean ± SEM of 3 replicate experiments. *P<0.05 vs. incubation with LPS alone.

Fig.5. Red curry paste extract suppresses activation of MAPKs signaling pathways. Cells were treated as described in Figure 1. Western blot analysis was conducted using 30 µg cell lysate protein/lane and reacted with antibodies against (A) phospho-p38 and p38, (B) phospho-JNK and JNK, and (C) phospho-Erk1/2 and Erk1/2. Results are expressed as relative ratio of band density of phosphorylated forms of p-38, p-JNK and p-Erk1/2 to the respective total proteins. Data are mean ± SEM of 3 replicate experiments. *P<0.05 vs. incubation with LPS alone.

Fig.6. Red curry paste extract inhibits phosphorylation and degradation of IκB-α. Cells were treated as described in Figure 1. (A) Cell lysates were analyzed by immunoblotting by reacting with antibodies against (A) phospho-IκB-α and (B) IκB-α compared to β actin. Results are expressed as ratio of band density of p-IκB-α to β-actin and IκB-α to β-actin. Data are mean ± SEM of 3 replicate experiments. *P<0.05 vs. incubation with LPS alone.
Fig.7. Red curry paste extract suppresses intracellular reactive oxygen species (ROS) production. After 24 h incubation with LPS, cells were incubated with 2, 7-dichlorofluorescein diacetate at 37 °C for 30 min and washed with PBS and subsequently lysed with 0.5% TritonX-100. ROS formation expressed as an arbitrary unit (AU) was determined in the supernatant of cell lysate. Data are means ± SEM of 3 replicate experiments. *$P<0.05$ vs. incubation with LPS alone.
Response to reviewer’s comment

Anti-inflammatory activities of red curry paste extract on lipopolysaccharide-activated murine macrophage cell line

Reviewer #1: highlight in yellow

1) Introduction section. Please delete "professional"

Reply

Page 3 line # 2: delete "professional"

2) Introduction section. Delete "safe alternatives" as there are some natural products that are not safe. For instance, marihuana is a natural product and it is not a safe alternative.

Reply

Page 3 line # 21: delete "safe alternatives"

3) Introduction section. Insert "effect" 4 lines before the end of introduction section.

Reply

Page 4 line # 19: add effect

4) Several abbreviations are not defined: iNOS, COX-2, JNK, ERK1/2, DMSO, HEPES, TAC, BCA, BSA, TBST, etc.

Reply

Define abbreviations: in parenthesis and high light in yellow

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activated protein 1</td>
</tr>
<tr>
<td>BCA</td>
<td>bicininc acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>Erk1/2</td>
<td>extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-Hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
</tr>
</tbody>
</table>
iNOS  inducible nitric oxide synthase
IkB  inhibitor kappa B
IKK-NF-κB  inhibitor kappaB kinase-nuclear factor kappaB
IL-1  interleukine-1
IL-6  interleukine-6
JNK  c-Jun N-terminal kinases
LPS  ipopolysaccharide
TLR4  Toll-like receptor 4
MD-2  myeloid differentiation protein-2
MAPKs  mitogen-activated protein kinases
NOX4  NADPH oxidase4
NAC  N-acetyl cysteine
NO  nitric oxide
NF-κB  nuclear factor kappaB
PBS  phosphate-buffered saline
p-p38  phospho-p38
p-IkB  phospho-inhibitor kappa B
p-JNK  phospho c-Jun N-terminal kinases
p-Erk1/2  phospho- extracellular signal-regulated kinases
ROS  reactive oxygen species
SRB  sulforhodamine B
SDS-PAGE  sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBST  Tris-buffered saline containing 0.1% Tween 20
TCA  trichloroacetic acid
TNF-α  tumor necrosis factor alpha
TRAF6  tumor necrosis factor receptor associated factor 6
VCAM-1  vascular cell adhesion molecule-1

5) Page 10, Line 2: Delete "LPS also significantly induced IL-6 secretion (Fig. 4A)."

Reply

Page 12, line #17: Delete "LPS also significantly induced IL-6 secretion (Fig. 4A)."

6) Insert periods at the end of Fig legends 1 and 8.

Reply

Page 26: add 24 h at line # 11 and 18
Page 27: add 24 h at line # 2, 8, 15 and 21
Page 28: add 24 h at line # 6 and 15

7) Align figs

Reply
Align figures on page 29-36

8) Delete heading of all figures

Reply

Delete heading on page 29-36

Reviewer #3: high light in green

1 - Please, justify the used in vitro concentrations (65-260 µg/ml) from an in vivo (physiological) point of view. Are these levels found in the blood/tissues of humans that ingest red curry past? Please, add more on that.

Reply

The statement is added on page 18 at line # 13-16.

However, these in vitro concentrations can’t be used to justify the level in blood or tissue because we don’t know the bioavailability of the bioactive compounds presence in the red curry paste. Most plant phytochemicals are transformed by phase I and II enzymes to other physiological forms by enterocytes during absorption prior to transport in blood and deliver to target tissue.

2 - Statistically significance differences were determined using the independent Student’s t test. This is not the accurate test to compare results derived from studies with 3 or more groups. In fact, the present data should be analyzed by one-way ANOVA. Please, revise it.

Reply

Add the following statement on Page 11, line # 13-15

Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test for multiple comparison of group means.

3

3.1- The authors incubated the cells just 1 h with the extract and then added LPS for 24 h. Was the medium changed before LPS addition? Please, inform it.

Reply

Add the following statement on Page 6, line # 10-12
The attached cells were incubated with 65-260 µg/ml extract for 1 h prior to co-incubated with 5 ng/ml LPS in phenol red and serum-free medium for another 24 h without changing the culture medium.

3.2 An interesting question to be solved is whether the beneficial effects of the extract are due to increased expression of the endogenous antioxidant molecules or just due to the own antioxidant properties of the extract. Please, add more on that. Alternatively, additional experiments with protein synthesis inhibitors, such as cycloheximide, could solve this question.

Reply

We did an additional experiment with cycloheximide to answer your question. We found that the beneficial effects of the extract are due to its own antioxidant properties as shown in figure 8 in the manuscript. If the curry paste extract enhance the expression of the endogenous antioxidant molecules, the ROS formation in LPS + extract group should significantly lower than those producing by cells treated with cycloheximide + LPS + extract group. However, there was no significantly different between these two treatment groups.

Add the following statement on Page 13, line #23 and Page 14, line # 1-4

The beneficial effects of the curry paste extract were due to its own antioxidant properties not due to enhancing expression of endogenous antioxidant molecules (Fig 8). Because ROS level between pre-treatment the cells with or without cycloheximide prior to incubate with the extract and LPS didn’t show a significant change.

4 - In the Discussion Section, it is mentioned that the suppressive effects of the ethanolic extract of red curry paste on pro-inflammatory mediators and ROS generation in LPS-activated RAW 264.7 cells are likely due to the combination of several compounds present in the paste. Are the anti-inflammatory effects of the curry paste extract (present study) higher than that of chili fruit extract (reference 28)? Are the anti-inflammatory effects of the curry paste extract higher than that of citral (reference 48)? Please, discuss it from a comparative point of view. This comparative analysis could give important information about potential synergistic events, which were discussed in the last paragraph.

Reply

It is hard to compare the suppressive effect of the plant extract between studies. Because plant materials between studies came from different sources and in turn we didn’t know the amount of bioactive compounds which postulated to exhibit the suppressive effects on pro-inflammatory mediators and ROS generation between 2 studies. 

Note: the author changed the issue in the discussion part.
Minor points:
- The authors state that "The dry paste powder was extracted twice with 90% ethanol (0.1g sample/3 ml solvent)". Please, inform the interval of the extraction and temperature

Add the following statement on Page 5, line # 18-21

The dry paste powder 0.1g was extracted with 3 ml of 90% ethanol by vigorously mixing for 2 min and sonicated in ultrasonic bath for 2 min at room temperature. The supernatant was collected after centrifuged at 4,500 g for 10 min at room temperature. The extraction procedure was repeated a second time as above.

Revise the following sentence:

- Overproduction of these inflammatory mediators can cause OF patho-physiological conditions.

Add the revised sentence on page 3, line # 14-17

Overproduction of these inflammatory mediators is postulated to be risk factors of patho-physiological conditions associated with many chronic diseases, including cardiovascular, neurodegenerative and gastrointestinal disorders, cancer, diabetes and rheumatoid arthritis [4].

Reviewer #2: in blue letters

The paper "Anti-inflammatory activities of red curry paste extract on lipopolysaccharide-activated murine macrophage cell line" deals about the effect of an ethanol extract from Thai red curry paste. This paste is a mixture of various spices and herbs and is a common constituent of the habitual Thai diets, and in other Asian cities. The immunological determinations to demonstrate the anti-inflammatory activity on a cell culture of macrophage are well performed, as well as the analysis of the results.

1. One important point is that all determinations about the anti-inflammatory activity are demonstrated by in vitro assay. It is important to consider that the same effect is not only observed in vivo assay due to the complexity of the different ecosystems, specially the gut, and of the inflammatory immune response by itself.
We agree with you that the results from in vitro experiment could not extrapolate to in vivo finding. However, in vitro experiment provides a good preliminary step to investigate the anti-inflammatory and antioxidant activities of spices and herbs extracts.

We had added this sentence to discussion, line # 17-19 page 18
“The further challenge now is to delineate in vivo actions of the extract and identify interactions among the various constituents to provide a better understanding of the health promoting effects of a complex ingredient consumed in a relatively large region of the world.”

2. The authors point the presence of bioactive compounds in the ethanol extract; however there is no demonstration of such compounds. No analysis of the extract was performed. The discussion is a review of the different papers with the individual spices and herbs showing anti-inflammatory activities. The authors extend these findings to the ethanol extract curry paste without to know if in the extract, the bioactive compounds are still present to speculate, for example, that the reduction in the level of pro-inflammatory cytokine is due to those bioactive compounds.

This study would be a good contribution if the identification of some bioactive compound had been demonstrated. Otherwise the anti-inflammatory properties in the whole curry paste were just demonstrated.

Reply

2.1 We have identified some of the bioactive compounds, specifically flavonoids and carotenoids in the red curry paste extract. The discussion has been revised to emphasize previous reports about the anti-inflammatory and anti-oxidant activities of these compounds with cultures of immune cells. These studies provide the basis for our suggestion that the mixture of such compounds in the extract we used likely contributed to the outcomes we report in this manuscript.

Add the methods and the identified compounds found in the extract into the materials and methods part on page 6: line # 14-23 and page 7: line # 1-19)

2.2 We changed the discussion content to emphasize on the anti-inflammatory and anti-oxidant properties of the identified compounds by HPLC.

See the discussion content of the re-submit version in the blue letters.
Anti-inflammatory activities of red curry paste extract on lipopolysaccharide-activated murine macrophage cell line

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Abstract

Objective: This study investigated anti-inflammatory and antioxidant activities of an ethanol extract from Thai red curry paste.

Methods: RAW264.7 murine macrophage cell line was incubated with the extract (65-260 µg/ml) with or without lipopolysaccharide (LPS). The anti-inflammatory activities of the extract were examined by measuring: inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor alpha (TNF-α) and interleukine-6 (IL-6) mRNA and protein level by RT-PCR, western blotting and ELISA, respectively. The nitric oxide (NO) production and intracellular reactive oxygen species generation were determined by Griess method and fluorescence intensity. The activation of mitogen activated protein kinases (MAPKs) and inhibitor kappa B (IκB) were determined by western blot.

Results: Exposure of cells with the extract significantly suppressed LPS-induced NO production and iNOS, COX-2, TNF-α and IL-6 expression (P < 0.05) by dose-dependent without cytotoxic effect. Intracellular reactive oxygen species significantly decreased (P < 0.05) in LPS-induced RAW264.7 cells. The inhibitory effect was mediated partly by inhibiting activation of IκB-α and MAPKs.

Conclusion: These results suggest that the anti-inflammatory and antioxidant properties of Thai red curry paste stems from bioactive compounds present in the spice and herb constituents. The health benefits of Thai red curry paste warrants further investigations in vivo.

Keywords: COX-2; iNOS; TNF-α; IL-6; Inhibitor kappa B; Mitogen-activated protein kinase; RAW264.7 cells
Introduction

Macrophages are immune cells that play an important role during inflammatory responses against various stimuli including lipopolysaccharide (LPS) from gram-negative bacterial cell wall. LPS binds to Toll-like receptor 4 (TLR4) and myeloid differentiation protein-2 (MD2) on macrophage plasma membrane resulting in the activation of several intracellular signaling pathways, involving inhibitor kappaB kinase-nuclear factor kappaB (IKK-NF-κB) and three mitogen-activated protein kinases (MAPKs), namely, p-38, c-Jun N-terminal kinases (JNK) and extracellular signal-regulated kinases (Erk1/2) [1]. These signaling pathways subsequently activate transcription factors, viz. nuclear factor kappaB (NF-κB) and activated protein 1 (AP-1), that regulate expression of several genes associated with functional responses [1-2]. LPS-induced gene products are pro-inflammatory cytokines such as interleukine-1 (IL-1), interleukine-6 (IL-6) and tumor necrosis factor alpha (TNF-α), adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [3]. Overproduction of these inflammatory mediators is postulated to be risk factors of patho-physiology associated with many chronic diseases, including cardiovascular, neurodegenerative and gastrointestinal disorders, cancer, diabetes and rheumatoid arthritis [4].

Since nonsteroidal anti-inflammatory drugs can manifest adverse side effects [5], one of the efforts has been to identify natural products for prevention and treatment of inflammatory disorders. In particular, the use of medicinal plants including spices and herbs in traditional remedies are of interest. There is increasing evidence that various phytochemicals may prevent or attenuate inflammation-associated chronic diseases [6]. For example, in vitro and in vivo studies have shown that phytochemicals in spices, such as curcumin, diallyl sulfide, capsaicin, eugenol
and gingerol, suppress the expression of pro-inflammatory gene products, including cytokines, chemokines, adhesion factors and enzymes [7-9]. The mechanism of suppression was found to be modulated through inhibiting phosphorylation of signaling proteins in the pathways that activate NF-κB-mediated transcription of pro-inflammatory genes [4, 10]. In addition, pre-treatment of neutrophils with anti-oxidants, N-acetyl cysteine (NAC) or α-tocopherol inhibits LPS-induced NF-κB activation and suppresses production of pro-inflammatory cytokines [11]. LPS-induced reactive oxygen species (ROS) generation and NF-κB activation are mediated by direct interaction of TLR4 with NADPH oxidase4 (Nox4), an enzyme related to phagocytic cell NADPH oxidase 2 (Nox2) [12]. In addition, ROS production in response to LPS mediates interactions between tumor necrosis factor receptor associated factor 6 (TRAF6) and redox-sensitive ASK1, which subsequently activates p38, a downstream target of the LPS signaling pathway [13].

In tropical regions including Asia, habitual diets contain several spices and herbs used for flavoring, deodorizing, pungency, coloring and enhancing taste [14]. Habitual Thai diets are similar to other Asian cuisines [15]. Although phytochemicals in individual spices and herbs have been demonstrated to suppress pro-inflammatory mediator expression [4, 7-8], these functional activities have not been investigated in a food matrix. Red curry paste, most commonly used in Thai curry dishes [15], is composed of 5 to 7 distinct spices and herbs. Here, we used LPS-induced RAW264.7 murine macrophage cell line to demonstrate the anti-inflammatory effect and the underlying mechanism of an ethanol extract of Thai red curry paste. The results demonstrate the possible health benefits of red curry paste and support the need for further investigations in animal models and human subjects afflicted with inflammatory-associated diseases.
Materials and methods

Chemicals

Dulbecco’s modified Eagle’s medium (DMEM), LPS (*E. coli* O11:B4), anti-β-actin conjugate horse radish peroxidase (HRP) and species-specific HRP-conjugated secondary polyclonal antibodies were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from PAA Laboratories (Haidmannweg, Austria). Penicillin and streptomycin were obtained from Invitrogen (Grand Island, NY, USA). Primary antibodies against iNOS, COX-2, phospho-p38 (*p*-p38), total p38, phospho-JNK (*p*-JNK), total JNK, phospho-Erk1/2 (*p*-Erk1/2), total Erk1/2, phospho-inhibitor kappa B (*p*-IκB), total IκB were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemical and reagents were of analytical grade.

Preparation of ethanol fraction from red curry paste

The curry paste consisted of a mixture of the following materials: dried chili pepper (*Capsicum annum*) 22.5%, garlic bulb (*Allium vineale*) 20%, fresh lemon grass (*Cymbopogon citratus*) 17%, shallot bulb (*Allium cepa*) 11%, fresh galangal (*Alpinia galangal*) 6%, dried kafferlime peel (*Citrus hystrix*) 2.5%, dried pepper seed (*Piper nigrum*) 0.5% and shrimp paste 4.5% with 16% salt. The paste was freeze dried, packed in aluminum foil in vacuo and stored at -20°C until use. The dry paste powder 0.1g was extracted with 3 ml of 90% ethanol by vigorously mixing for 2 min and sonicated in ultrasonic bath for 2 min at room temperature. The supernatant was collected after centrifuged at 4,500 g for 10 min at room temperature. The extraction procedure was repeated a second time as above. The combined supernatants were evaporated under vacuum at 45-50 °C to dryness. The dry extract was solubilized in dimethyl sulfoxide (DMSO): ethanol
(1:1) to 0.2% final concentration and diluted to designated concentrations with serum-free, phenol red-free medium prior to addition to cell cultures.

**Growth and activation of cells**

Murine macrophage RAW 264.7 cells (TIB71) obtained from ATCC (Bethesda, MD, USA) were grown in DMEM supplemented with 10% fetal bovine serum, 15 mM N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C in humidified atmosphere of 5% CO₂/95% air. Cells were used at 80% confluency between passages 8 and 20. Cells (7.5 x 10⁵ cells/ml) were seeded for 24 h to allow attachment. The attached cells were incubated with 65-260 µg/ml extract for 1 h prior to being incubated with 5 ng/ml LPS in phenol red and serum-free medium for another 24 h without changing the culture medium.

**HPLC analysis for carotenoids**

The film of the dried extract was resolubilized in 1 mL of mobile phase and analyzed by HPLC using a Waters 600 Controller module with photodiode array detector (PDA) (Agilent 1100 series, USA). Carotenoids were separated by C18 reversed-phase column (250 mm x 4.6 mm, internal diameter: 5 µm: GRACE VYDAC 201TP54, USA) preceded by a security guard cartridge with the same packing (Phenomenex KJO-4282, USA). The mobile phase consisted of mixed solvent of acetonitrile : methanol : dichloromethane (80:11:9 v/v/v) containing 0.1% (v/v) triethylamine and 0.1% (w/v) ammonium acetate. Carotenoids were eluted isocratically at a flow rate of 0.7 mL/min with an injection volume 20 µL. Absorbance was monitored at 450 nm and individual carotenoids were identified by retention time and spectral characteristics compared to
known standards. Carotenoids were quantified by comparison of peak area against standard curves prepared with known concentrations of pure carotenoids. The extract contained 5.71 ± 0.36, 2.76 ± 0.06 and 1.34 ± 0.11 µg/g dry weight of lutein, β-carotene and α-carotene, respectively.

HPLC analysis for flavonoids
Dried extract was reconstituted with 2 mL of acid methanol (62.5% methanol: 6M HCl, 4:1 v/v) and shaken at 70 °C for 2 h [16]. Flavonoids were analyzed by HPLC (Agilent Technologies 1100 series coupled with photodiode array detector) equipped with Zorbax Eclipse XDB-C18 column (4.6 mm x 150 mm, 5 µm, Agilent Technologies, U.S.A.) preceded by a cartridge guard column with same packing. The mobile phase consisted of 100% water containing 0.5% (w/w) trifluoroacetic acid (TFA) (solvent A), 100% methanol containing 0.5% (w/w) TFA (solvent B), and 100% acetonitrile containing 0.5% (w/w) TFA (solvent C). Column temperature was controlled at 30°C. The flavonoids were separated by gradient elution programs as previously described [16] and identified by comparison of retention time and spectra with pure standards. Content was quantified at 338 nm by comparing peak area with calibration curves. Total flavonoid content in the extract was 129.7 ± 10.5 µg/g dry weight, consisting of 4 major peaks including quercetin, kaempferol, luteolin and apigenin accounting for 74.4 ± 5.5 %, 13.1 ± 1.2%, 7.7 ± 0.7% and 4.8 ± 0.4%, respectively.

Cell viability
Viability of treated cells in washed monolayer was assessed using sulforhodamine B (SRB) assay [17]. In brief, cells pre-treated with or without extract and subsequently stimulated with LPS
were washed with phosphate-buffered saline (PBS) and fixed with 50% cold trichloroacetic acid (TCA) at 4°C for 2 h. Cells were stained with 0.1% w/v Sulforhodamine B in 1% acetic acid for 20 min and solubilized with 10 mM Tris-hydromethylaminomethane, pH 10. Absorbance at 500 nm was measured, with absorbance of vehicle control defined as 100% viability.

**Measurement of nitric oxide (NO)**

Nitrite concentration was used as an assessment of NO production. Cells were plated and treated with test extract in the presence or absence of LPS. After incubation for 24 h, spent media were collected to measure nitrite concentrations using the Griess reaction [18] by adding 100 µl of Griess reagent (0.1% naphthylethylenediamide dihydrochloride in H₂O and 1% sulphanilamide in 5% concentrated phosphoric acid) to 100 µl of sample. Nitrite concentration was calculated by comparison with sodium nitrite.

**Enzyme-linked immunosorbent assay (ELISA) of TNF-α and IL-6**

TNF-α and IL-6 in cell-treated culture medium were determined by quantitative “sandwich” ELISA using paired antibodies purchased from Endogen Inc. (Rockford, IL, USA) and eBioscience Inc. (San Diego, CA, USA), respectively. In brief, high-binding plates (NUNC, Denmark) were coated with capture antibody for mouse TNF-α and IL-6. After overnight incubation at 25 ºC, plates were washed and blocked for 2 h with 1% bovine serum albumin (BSA) in PBS. Culture medium or various concentrations of recombinant mouse TNF-α and IL-6 protein (standards) were incubated at 4 ºC overnight prior to adding biotinylated antibodies to the wells. After 2 h at 25 ºC, immune complex was detected using streptavidin horseradish peroxidase (HRP)-tetra methyl benzidine detection system (Pierce, Rockford, IL, USA).
Reactions were terminated with 2M H$_2$SO$_4$ and absorbance at 450 nm was determined using a microtiter plate reader (TECAN, GmbH, Austria). Concentrations of TNF-α and IL-6 in samples were calculated by comparing absorbance with the standard curve.

*Western blot analysis*

Treated cells were harvested, washed twice with ice-cold PBS and resuspended in ice cold lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% SDS, 50 mL sodium fluoride, 10 mM sodium pyrophosphate and 0.2% protease inhibitor cocktail (Sigma) for 30 min at 4 °C. Cell lysate was collected after centrifugation at 13,500 g at 4 °C for 5 min. Protein content was determined by bicinic acid (BCA; Endogen USA) method using bovine serum albumin (BSA) as a standard. Samples (40 µg of protein/well) were separated on 8% (for detection of iNOS and COX-2 protein) or 10% (MAPKs and NF-κB protein) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Whatman, GmbH, Germany). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h and incubated with specific primary antibody in TBST containing 5% BSA overnight at 4 °C. After washing three times, membranes were reacted with HRP-conjugated secondary antibody for 1 h. Following five washes, membranes were incubated with Super Signal solution (Endogen) for 2 min and exposed to X-ray film. The membranes were then stripped of bound antibody and re-probed with anti-β actin or anti-total MAPKs protein to assess quantity of protein loaded. The density of target bands was quantified by Image J program. Results are expressed as relative ratio of band density between protein of interest and β-actin.
RNA extraction and RT-PCR

Total RNA was extracted with TRI REAGENT® (Molecular Research Center, Cincinnati, OH, USA) as described in manufacture. One µg of total RNA was reversed transcribed at 50°C for 1 h using 200 U of reverse transcriptase (SuperScript™ III Reverse Transcriptase, Invitrogen, USA) with oligo-dT18 primer. Reactions were terminated by heating at 70°C for 15 min. The oligonucleotide primer pairs (Bio Basic, Ontario, Canada) used to amplify interested cDNA was the following: COX-2, sense: 5′-AGGTCATTGGGTGAGGAGGTG-3′; antisense: 5′GAGTCCAT GTTCCAGGAGGA-3′; iNOS, sense: 5′-CACCTGGAGTTACCCAGT-3′; antisense: 5′-TG GTCACATTCTGCTTCTGG-3′; TNF-α, sense: 5′-TCGTAAGCAAACACCAAGTG-3′; anti-sense: 5′-CGGACTCGCAATCTAAGG-3′; IL-6, sense: 5′-GCAAGAGACTTCCATAGT-3′; antisense: 5′-ACTCCAGGTAGCTATGGTACTCCA-3′; β-actin, sense: 5′-GGCACCA CACCTTCTACAATG-3′; antisense: 5′-GGTCTCAAAAATGACCTGGTGC-3′. Amount of cDNA template for PCR amplification were optimized for each target gene in order to obtain a clear PCR product. Amplification was performed in a MyCycler thermal cycler (BioRad, Hercules, CA, USA) as follows: denaturation at 95°C for 15 min for the first cycle; 94°C for 30 s; annealing at either 62°C (COX-2 and IL-6), 58.1°C (iNOS) or 55°C (TNF-α) for 30 s; and, extension at 72°C (iNOS) and 68°C (COX-2, TNF-α and IL-6) for 45 s for either 25 repetitive cycles (COX-2, iNOS and IL-6) or 23 repetitive cycles (TNF-α). Final extension was performed at 72°C for 10 min. All target genes were performed duplex PCR with β-actin as an internal control. The PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide. The density of target bands was quantified by Image J program. Results were expressed as relative ratio of band intensity between mRNA of interest and β-actin mRNA.
Measurement of intracellular ROS

The level of intracellular ROS in treated RAW264.7 cells was measured as previously described [19]. In brief, treated cells were washed with warm PBS (37 °C) and incubated with 5 µM 2,7-dichlorofluorescein diacetate (Sigma,) in basal medium at 37 °C for 30 min. Then cells were washed with 3 times with warm PBS (37 °C) and subsequently lysed with 0.5% TritonX-100 in PBS. Fluorescence in supernatant obtained by centrifugation of cell lysate at 12,000 g for 5 min was measured with excitation at 485 nm and emission at 530 nm by Luminescence Spectrometer LS55 (Perkin Elmer Instruments LCC, Shelton, CT, USA).

Statistical Analysis

Statistical analysis was performed using SPSS (version 14.0, SPSS Inc., Chicago, Illinois). Data are presented as means ± SE. The mean values were calculated from at least three separated experiments conducting on separate days. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test for multiple comparison of group means. Significance was set at $P < 0.05$.

Results

Red curry paste extract decreases NO production and iNOS expression

Exposure of RAW264.7 macrophages to 5 ng LPS /ml significantly increased the concentration of nitrite, a stable oxidized product of NO, in medium, whereas there was no significant change in cultures exposed to either vehicle or red curry paste extract alone (Fig.1A). Pre-treatment of cells with red curry paste extract (65-260 µg/ml) significantly suppressed (P<0.05) LPS-induced NO production in a dose-dependent manner without exerting cytotoxicity (data not shown).
determine the cause of reduction in NO production by the extract, the expression of iNOS was
examined by Western blot and RT-PCR analysis. As expected, the expression of iNOS was
increased significantly after the exposure to LPS for 24 h (Fig. 1B and C). Pre-treatment with
curry paste extract prior to stimulation with LPS showed a concentration dependent inhibition of
LPS-induced iNOS protein and mRNA expression. These results indicated that red curry paste
extract decreased the NO production by suppression of iNOS mRNA expression.

Red curry paste extract attenuates LPS-mediated increases in expression of COX-2, TNF-α and
IL-6.

COX-2 can be induced by several stimuli such as LPS, cytokines and growth factors during
active inflammatory episodes [20-21]. Exposure of RAW264.7 cells to LPS for 24 h markedly
induced COX-2 protein and mRNA expression (Fig. 2A and 2B). Pre-treating RAW264.7 cells
with red curry paste extract at 65-260 µg/ml significantly suppressed COX-2 protein and mRNA
expression in a dose dependent manner. Likewise, exposure of RAW 264.7 cells to LPS for 24 h
significantly enhanced TNF-α secretion in culture medium from a basal level of 3.3 ng/ml to
more than 100 ng/ml (Fig 3A). Pre-treating cells with the extract significantly decreased TNF-α
secretion (Fig 3A) and mRNA content (Fig 3B) in a dose dependent manner. As expected, IL-6
mRNA was increased in cells exposed with LPS (Fig 4A). Pre-treatment with the extract
significantly decreased both IL-6 secretion (Fig 4A) and mRNA (Fig 4B) dose dependently.

Red curry paste extract suppresses MAPKs signaling pathways activation

LPS is a well-known activator of mitogen activated protein kinases (MAPKs), viz. Erk1/2, p38
and JNK [22] in macrophages and many other cell types. The effects of red curry paste extract on
LPS-induced phosphorylation of Erk1/2, p38 and JNK were examined by Western blot. Exposure of RAW264.7 cells to LPS activated phosphorylation of Erk1/2, p38 and JNK (Fig 5A-C). Phosphorylation of the three MAPKs was inhibited in a dose-dependent manner in cells pre-treated with the extract (Fig 5A-C). Thus, the extract suppressed LPS-induced pro-inflammatory mediator production in part by blocking phosphorylation of the three MAPKs proteins.

Red curry paste extract inhibits phosphorylation and degradation of IκB-α

In resting status, NF-κB, a dimeric transcription factor, is sequestered in the cytoplasm in an inactive form by associated with IκB-α. Upon exposure of macrophages to LPS, NF-κB signaling cascade is activated and results in phosphorylation- induced proteolytic degradation of IκB-α. Free NF-κB translocates to the nucleus where it induces transcription of a variety of genes including pro-inflammatory mediators (iNOS, COX-2, TNF-α and IL-6) [3]. We investigated whether the red curry paste extract mediated its inhibitory effect on iNOS, COX-2, TNF-α and IL-6 expression by inactivation of NF-κB. LPS markedly induced phosphorylation of IκB-α and degradation of IκB-α in RAW264.7 cells (Fig 6A and 6B). Pre-treatment of RAW264.7 cells with the extract significantly inhibited LPS-induced phosphorylation and degradation of IκB-α in a dose-dependent manner for up to 24 h (Fig 6A and 6B).

Red curry paste extract suppresses ROS production

LPS-stimulated macrophage generates ROS via the activation of a membrane-bound NADPH oxidase [23], leading to activation of MAPKs and NF-κB nuclear translocation and results in expression of pro-inflammatory mediators [24]. Pre-treating RAW264.7 cells with red curry paste extract significantly reduced LPS-induced ROS production in a dose dependent manner (Fig 7A).
The beneficial effects of the curry paste extract were due to its own antioxidant properties not due to enhancing expression of endogenous antioxidant molecules (Fig. 8). Because ROS level between pre-treatment of the cells with or without cycloheximide prior to incubate with the extract and LPS didn’t show a significant change. These results suggest that red curry paste extract possesses antioxidant activity that suppresses LPS-induced iNOS, COX-2, TNF-α and IL-6 expression by attenuating ROS accumulation that in turn blocks activation of MAPKs and NF-κB.

**DISCUSSION**

The present study clearly demonstrates that the ethanolic extract at 65-260 μg/ml of Thai red curry paste decreases the production of inflammatory mediators by LPS-activated RAW264.7 macrophages. We demonstrated that phytochemicals present in the red curry paste extract significantly inhibited production of NO by suppressing the expression of iNOS, as well COX-2, TNF-α and IL-6 in a dose-dependent manner. This activity appears to be mediated by attenuating the phosphorylation of MAPK Erk1/2, p38 and JNK, thus suppressing NF-κB activation via scavenging intracellular ROS in LPS-treated macrophages.

Thai red curry paste generally contains dried chili pepper, garlic bulb, fresh lemon grass, shallot bulb, fresh galangal, dried kaffer lime peel, dried pepper seed and shrimp paste as its major ingredients. The main ingredients are similar among the home-made, small scale commercial and industrial export products. The amount of red curry paste used in typical Thai recipes is 15 g per serving [25]. Previous studies have demonstrated that aqueous and organic extracts from a number of the individual spices and herbs present in red curry paste exhibit a variety of health-promoting characteristics including antioxidant and anti-inflammatory activities.
in vitro and in vivo [4, 8, 26-28]. We identified and quantified four flavonoids and three carotenoids in the red curry paste extract used in this study and assume that these compounds contributed to the observed anti-inflammatory activity.

Quercetin is the most common flavonoid present in plants and was the predominant flavonoid in the curry paste extract. Quercetin exhibits anti-inflammatory activity in vitro and in vivo [29-34]. A previous study reported that pre-treatment of quercetin inhibited NO production, iNOS, TNF-α, IL-1β and IL-6 expression in LPS-activated RAW264.7 cells by inhibiting phosphorylation of ERK and p-38 MAP kinases by stabilizing the NF-κB/ΙκB complex [29]. In addition, quercetin suppressed LPS-induced NO and prostaglandin E2 productions, iNOS and COX-2 gene expressions in this cell line [30]. Anti-oxidant activity of quercetin in wine polyphenol was also reported for LPS-stimulated RAW264.7 [35]. Our results are consistent with these previous studies, and suggest that quercetin contributed to the suppressive effects of the curry paste extract on pro-inflammatory mediator expression and intracellular ROS generation.

Kaempferol represented about 13% of the identified flavonoids in the curry paste extract. Its anti-inflammatory property has been reported in several studies [36-39]. Kaempferol (30 μM) significantly decreased TNF-α and IL-β gene expression in LPS-stimulated J774.2 macrophage [36]. Other investigators reported that kaempferol inhibited NO production, iNOS protein and mRNA expression in LPS-activated J774 macrophage cell in a dose dependent manner by inhibiting NF-κB and STAT-1 [37]. Others have reported that kaempferol inhibited LPS/IFN-γ induced synthesis of NO, TNF-α and IL-12 in murine peritoneal macrophage [38], and suppressed iNOS and COX-2 gene expression in LPS-activated RAW264.7 cells [39]. These data support the likelihood that kaempferol in the curry paste extract contributed to attenuation of iNOS, COX-2 expression in the LPS-activated RAW264.7 cells in the present study. It also has
been shown that pre-treatment of hepatocytes with kaempferol significantly inhibited production of peroxides, superoxide anion and nitric oxide in response to a pro-inflammatory mixture of cytokines [40]. Thus, kaempferol probably contributed to the suppressive effects of the curry paste extract on ROS accumulation in LPS-activated RAW 264.7 cells in the present study.

The curry paste extract also contained luteolin. This flavone is generally present in plants at a lower concentration than kaempferol and quercetin, although some spices such as thyme, parsley and sage contain considerable amounts [41]. The anti-oxidant, anti-inflammatory and anti-allergic properties of luteolin have been extensively review [42]. One previous study reported that pretreatment of RAW 264.7 with luteolin inhibited LPS-stimulated TNF-α and IL-6 secretion by attenuating phosphorylation of Akt and therefore activation of NF-kB [43]. Luteolin also suppressed the production of inflammatory mediators by inhibiting the phosphorylation of JNK in activated macrophages [44]. Pre-treatment of RAW 264.7 macrophage-like cells with luteolin does-dependently inhibited LPS-induced hydroxyl radical formation and expression of COX-2 protein and PGE2 formation [45]. Luteolin also found to suppress the LPS-elicited inflammatory events in mouse alveolar macrophages by attenuate the LPS-mediated protein kinase B (Akt) and IKK phosphorylation, as well as reactive oxygen species (ROS) production [46]. These data support the possibility that luteolin also may have a role in the suppression of pro-inflammatory mediator expression and ROS accumulation by blocking NF-κB activation in LPS activated RAW264.7 cells in the present study.

Apigenin is another flavonoid commonly found in fruits and vegetables that possesses anti-oxidant and anti-inflammatory activities [36, 39-40, 47]. This flavonoid accounted for 10% of total flavonoids in the curry paste extract. Pretreatment of RAW264.7 cells suppressed LPS-induced expression of iNOS and COX-2 by blocking activation of nuclear NF-kB [39]. Apigenin
also inhibited expression of the inflammatory cytokines IL-8, IL-1β, and TNF in LPS-stimulated primary human monocytes and mouse macrophages by inhibiting IKK kinase activity [48]. Apigenin, like kaempferol, also exhibited similar anti-oxidant activities by decreasing peroxides, superoxide anion and nitric oxide production in cytokine mixture stimulating parenchyma liver cells [40]. These data suggest that apigenin contributed to the suppressive affect of the curry paste extract in our study.

The extract in the present study also contained relatively low concentrations of lutein, β-carotene and α-carotene. Lutein, a non-provitamin A carotenoid present in dark-green leafy vegetables, has been reported to inhibit LPS-stimulated NO production and iNOS gene expression in RAW264.7 cells, as well as scavenge superoxide anion and H₂O₂ and suppress NF-κB-induced expression of inflammatory genes, iNOS, and COX-2 [49, 50]. The suppressive effect was modulated by inhibition of IκB kinase (IKK) activation, IκB degradation, nuclear translocation of NF-κB, and binding of NF-κB to the κB motif of the iNOS promoter. Moreover, lutein attenuated LPS- and H₂O₂-induced increases in phosphatidylinositol 3-kinase (PI3K) activity, NF-κB-inducing kinase (NIK), and Akt phosphorylation, which are all upstream of IKK activation, but did not affect the interaction between Toll-like receptor 4 and MyD88 or the activation of mitogen-activated protein kinases [50]. Thus, lutein may have contributed to the suppressive effects of ethanolic extract of red curry paste on pro-inflammatory mediators and ROS generation in LPS-activated RAW 264.7 cells in the present study.

β-Carotene also possesses anti-inflammatory and anti-oxidant activities and its molecular mechanism has been clearly defined [51]. β-carotene does-dependently suppressed the production and expression of iNOS, COX-2, TNF-α and IL-1β in LPS-induced murine macrophage RAW264.7 cells and primary macrophages by inhibiting IκB phosphorylation and degradation.
and thus blocking nuclear translocation of NF-κB p65 subunit. It also decreased intracellular ROS accumulation in LPS-stimulated RAW264.7 cells. Although the concentration of this carotenoid was low in the extract, it is noteworthy that the combination of low concentration of β-carotene (2µM) and quercetin (20µM) has been shown to synergistically inhibit pro-inflammatory responses and DNA damage ability in PMA-stimulated monocyte/macrophage-like cells (HL-60) [52]. Because of high concentration of quercetin and the relatively lower content of β-carotene in the curry paste extract, an anti-inflammatory interaction may have suppressed LPS-stimulated activation of murine macrophage RAW264.7 cells in the present study.

In summary, we have shown that an ethanolic extract of Thai red curry paste containing a complex mixture of several spices and herbs suppresses the production of pro-inflammatory mediators in murine macrophage-like cells exposed to LPS. The presence of a combination of bioactive compounds in Thai red curry is most likely responsible for the observed activities. However, these in vitro concentrations can’t be used to justify the level in blood or tissue because we don’t know the bioavailability of the bioactive compounds presence in the red curry paste. Most plant phytochemicals are transformed by phase I and II enzymes to other physiological forms by enterocytes during absorption prior to transport in blood and deliver to target tissue. The further challenge now is to delineate in vivo actions of the extract and identify interactions among the various constituents to provide a better understanding of the health promoting effects of a complex ingredient consumed in a relatively large region of the world.

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References


ANTI-INFLAMMATORIES ACTIVITIES OF RED CURRY PASTE


[25] Thai Food and Drug Administration (Thai FDA), In Food labeling. Ministry of Public Health 1998; Notification no. 182 B.E.


ANTI-INFLAMMATORY ACTIVITIES OF RED CURRY PASTE


Legends

Fig.1. Red curry paste extract decreases NO production and iNOS expression in LPS-activated macrophage. Cells were pre-treated with 65-260 μg/ml of the extract for 1 h prior to incubation
with 5 ng/ml LPS for 24 h. Unstimulated cells were treated identically except that vehicle alone
was added to medium. Culture media were collected to determine NO concentration (A). Lysates
from treated cells were analyzed by immunoblotting using antibodies against iNOS and β actin.
Results are expressed as ratio of band density of iNOS to β-actin protein (B). (C) After 24 h
incubation with LPS, total RNA was extracted and relative amounts of iNOS and β-actin mRNAs
determined by RT-PCR. Results are expressed as relative ratio of band density of iNOS to β-actin
amplicons. Data are means ± SEM of 3 replicate experiments. *P<0.05 vs. incubation with LPS
alone for 24 h.

Fig.2. Red curry paste extract attenuates increases in COX-2 expression. Cells were treated as
described in Figure 1. (A) Cell lysates were analyzed by immunoblotting using antibodies against
COX-2 and β-actin. Results are expressed as ratio of band density of COX-2 to β-actin protein.
(B) COX-2 mRNA level normalized relative to β-actin in treated cells was determined by RT-
PCR with results expressed as relative ratio of band density of COX-2 to β-actin amplicons. Data
are mean ± SEM of 3 replicate experiments. *P<0.05 vs. incubation with LPS alone for 24 h.

Fig.3. Red curry paste extract inhibits TNF-α mRNA expression and secretion. Cells were treated
as described in Figure 1. (A) TNF-α concentration in culture medium was measured by ELISA.
(B) TNF-α mRNA normalized relative to β-actin in treated cells was determined by RT-PCR and
results are expressed as relative ratio of band density of TNF-α to β-actin amplicons. Data are
mean ± SEM of 3 replicate experiments. *P<0.05 vs. incubation with LPS alone for 24 h.
Fig. 4. Red curry paste extract decreases IL-6 mRNA expression and production. Cells were treated as described in Figure 1. (A) IL-6 concentration in culture medium was measured by ELISA. (B) IL-6 mRNA normalized relative to β-actin in treated cells was determined by RT-PCR with results expressed as relative ratio of band density of IL-6 to β-actin amplicons. Data are mean ± SEM of 3 replicate experiments. *P<0.05 vs. incubation with LPS alone for 24 h.

Fig. 5. Red curry paste extract suppresses activation of MAPKs signaling pathways. Cells were treated as described in Figure 1. Western blot analysis was conducted using 30 µg cell lysate protein/lane and reacted with antibodies against (A) phospho-p38 and p38, (B) phospho-JNK and JNK, and (C) phospho-Erk1/2 and Erk1/2. Results are expressed as relative ratio of band density of phosphorylated forms of p-38, p-JNK and p-Erk1/2 to the respective total proteins. Data are mean ± SEM of 3 replicate experiments. *P<0.05 vs. incubation with LPS alone for 24 h.

Fig. 6. Red curry paste extract inhibits phosphorylation and degradation of IκB-α. Cells were treated as described in Figure 1. (A) Cell lysates were analyzed by immunoblotting by reacting with antibodies against (A) phospho-IκB-α and (B) IκB-α compared to β-actin. Results are expressed as ratio of band density of p-IκB-α to β-actin and IκB-α to β-actin. Data are mean ± SEM of 3 replicate experiments. *P<0.05 vs. incubation with LPS alone for 24 h.

Fig. 7. Red curry paste extract suppresses intracellular reactive oxygen species (ROS) production. After 24 h incubation with LPS, cells were incubated with 2, 7-dichlorofluorescein diacetate at 37 °C for 30 min and washed with PBS and subsequently lysed with 0.5% TritonX-100. ROS
formation expressed as an arbitrary unit (AU) was determined in the supernatant of the cell lysate. Data are means ± SEM of 3 replicate experiments. *P<0.05 vs. incubation with LPS alone for 24 h.

Fig. 8. Red curry paste extract directly suppresses ROS formation without enhancing expression of endogenous antioxidant molecules. Cells were pre-treated with 1 µg/ml of cycloheximide (CHX) for 30 min and washed out before incubated with 65-130 µg/ml of extract for 1 h prior to co-incubate with LPS for 24 h. Then, cells were incubated with 2, 7-dichlorofluorescein diacetate at 37 °C for 30 min and washed with PBS and subsequently lysed with 0.5% TritonX-100. ROS formation expressed as an arbitrary unit (AU) was determined in the supernatant of the cell lysate. Data are means ± SEM of 3 replicate experiments. *P<0.05 vs. incubation with LPS alone for 24 h.