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A Methanol Extract of *Adansonia digitata* L. Leaves Inhibits Pro-Inflammatory iNOS Possibly via the Inhibition of NF-κB Activation

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Abstract

This study examined the total polyphenol content of eight wild edible plants from Ethiopia and their effect on NO production in Raw264.7 cells. Owing to its relatively high polyphenol concentration and inhibition of NO production, the methanol extract of *Adansonia digitata* L. leaf (MEAD) was subjected to detailed evaluation of its antioxidant and anti-inflammatory effects. Antioxidant effects were assessed by measuring free-radical-scavenging activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and oxygen-radical-absorbance capacity (ORAC) assays, while anti-inflammatory effects were assessed by measuring inducible nitric oxide synthase (iNOS) expression in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. In the ORAC assay, MEAD was 10.2 times more potent than vitamin C at eliminating peroxyl radicals. In DPPH assay, MEAD also showed a strong ROS scavenging effect. MEAD significantly inhibited iNOS activity (IC_{50}=28.6 μg/ml) of LPS-stimulated Raw264.7 cells. We also investigated the relationship between iNOS expression and nuclear factor kappa B (NF-κB) activation. MEAD inhibited IκBα degradation and NF-κB translocation from the cytosol to the nucleus in LPS-induced RAW264.7 cells without significant cytotoxic effects, as confirmed by MTT assay. These results suggest that MEAD inhibits anti-inflammatory iNOS expression, which might be related to the elimination of peroxyl radicals and thus the inhibition of IκBα-mediated NF-κB signal transduction.

Key Words: *Adansonia digitata*, Antioxidant, Anti-inflammatory, NF-κB, IκB, iNOS

INTRODUCTION

There is growing evidence that chronic inflammation predisposes individuals to various life-threatening diseases, such as rheumatoid arthritis, atherosclerosis, asthma, sepsis, stroke, and inflammatory bowel disease (Mueller *et al*., 2010). A main cause of inflammation is the production of pro-inflammatory mediators (Wang *et al*., 2009). Nuclear factor-κB (NF-κB) has been suggested to be involved in the same process (Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009; Oeckinghaus *et al*., 2011). We thus evaluated the inhibitory effects of various plant extracts and compounds on iNOS expression and NO production in murine macrophage RAW264.7 cells. When stimulated with lipopolysaccharide (LPS), these cells initiate iNOS transcription and protein synthesis, with a corresponding increase in NO production. Furthermore, LPS stimulation induces IκBα proteolysis and NF-κB nuclear translocation (Xie *et al*., 1994).

In most cell types, the inactive form of NF-κB is sequestered in the cytoplasm via its physical association with inhibitory proteins, including IκBα and related factors. In response to inflammatory stimuli such as LPS, reactive oxygen species (ROS), and tumor necrosis factor alpha (TNF-α), IκBα is phosphorylated by IκB kinase and degraded rapidly by proteasomes after polyubiquitination (Karin and Ben-Neriah, 2000). IκBα degradation leads to the release of NF-κB and allows its translocation into the nucleus and the subsequent up-regulation of inflammation-related genes, such as those for cytokines (IL-1, IL-2, IL-6, IL-8, and TNF), cell adhesion molecules (E-selectin, ICAM-1, and VCAM-1), iNOS, and cy-

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clooxygenase (COX)-2 (Weber, 1996; Gordon *et al.*, 2011; Oh *et al.*, 2012; Wang *et al.*, 2012). This triggers chronic inflammation, which contributes to the pathogenesis of various diseases (Wang *et al.*, 2009).

Since time immemorial, many plants have been used medicinally to inhibit inflammation-related diseases, since they contain thousands of phytochemicals (Manach *et al.*, 2004). *Adansonia digitata* L. (Bombacaceae) (baobab) is popular in many African countries due to its nutritional and medicinal value. The leaves of the plant are the main source of food and folk medicine for many populations in Africa and are eaten fresh or dried. Besco *et al.* (2007) reported on the antioxidant properties of *Adansonia digitata* fruit parts. Particularly, the integral antioxidant activity (IAC) of baobab red fiber (part of the fruit) was 66 times higher than that of orange pulp. Baobab fruit pulp, which is traditionally used to treat many diseases, has also been recognized as a botanical remedy due to its antioxidant effect (Chadare *et al.*, 2009). However, the effect of *Adansonia digitata* on iNOS has to our knowledge not been determined.

In this study, we evaluated the antioxidant activity of MEAD and its pro-inflammatory effects on iNOS expression. We also determined whether NF-κB activity is involved in regulation of the production of pro-inflammatory mediators.

MATERIALS AND METHODS

Reagents

The following reagents were used: Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were purchased from Gibco Laboratories (Detroit, USA). Lipopolysaccharide (LPS), bovine serum albumin (BSA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid (APPH), TritonX-100 and vitamin C were purchased from Sigma Chemical Co (St. Louis, MO, USA). Rabbit anti-NF-κB antibody and rabbit anti-IκBα were purchased from Santa Cruz Biotech, Inc. (CA, USA). Goat anti-rabbit IgG-Alexa488 was purchased from Molecular Probes (Eugene, USA). Normal goat serum was purchased from Vector Laboratories (CA, USA), and Hoechst 33342 was purchased from Pierce Biotech (IL, USA).

Plant materials, extraction and analysis of MEAD

For screening purposes, eight Ethiopian wild edible plants were collected and identified by comparison with herbarium specimens using published volumes on the flora of Ethiopia and confirmed with the assistance of taxonomists. Specimens were deposited in the Food Science and Nutrition Program, Addis Ababa University. The parts of the plants were air dried, ground, and extracted three times with methanol at room temperature and the solvent was evaporated under reduced pressure.

An *Adansonia digitata* L. (Bombacaceae) specimen was collected from the Zequala district, Amhara region, northern Ethiopia, in September, 2011 by one of the authors (Yihunie Ayele) and identified by Mr. Melaku Wondafrash, National Herbarium of Addis Ababa, Ethiopia. The plant specimen was deposited in the Food Science and Nutrition Program, Addis Ababa University, under accession number YA-03-2011. Samples were prepared for analysis. Briefly, *Adansonia digitata* leaves were shade-dried completely and extracted three times with methanol at room temperature. The extracts were pooled and passed through Whatman No. 5A filter paper. The methanol extract of *Adansonia digitata* L. leaf (MEAD) was evaporated under reduced pressure at 45°C using an EYELA N-1200A rotary evaporator (Rikakikai, Japan). The MEAD was then weighed to calculate the yield (14.5%) and analyzed by HPLC (RP-18, 5 μm, 250×4.6 mm). A mobile phase system (0.1% formic acid in water, and 50% acetonitrile in methanol) was applied at a flow rate of 0.4 ml/min for 30 min. The MEAD HPLC profile was quantified using its integrated area (Fig. 1), and was confirmed using GCMS-QP2010 Ultra (Shimadzu, Japan). Epicatechin, and procyanidin b2 were used as standards.

Cell culture

Murine macrophage RAW264.7 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The Raw264.7 cells were cultured in DMEM supplemented with 10% (w/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in an incubator at 37°C with a humidified atmosphere of 95% air and 5% CO_2.

Total phenol extraction and determination

One gram of powder from each sample was soaked in 40 ml of methanol (50%) for 18 h with occasional shaking. The extract was centrifuged at 3,000 rpm for 20 min. The residue was then extracted with 40 ml of acetone/water (4:1) agitated and centrifuged. The two supernatants were pooled and passed through Whatman No. 5A filter paper. The total phenolic content of each sample was determined using the Folin–Ciocalteu colorimetric method, as described previously, with modifications (Inglett *et al.*, 2011). Briefly, 9 ml of de-ionized water and 1 ml of Folin-Ciocalteu (Roura *et al.*, 2006) reagent (F9252, Sigma Aldrich, St. Louis, MO, USA) were added to 100 μl of extract, and mixed in a vortex mixer. After 15 min, 1.5 ml of Na_2CO_3 (1.85 M) were added and incubated for 120 min at room temperature. Then, 200 μl of each sample and standard solution were pipetted into 96-well plates. The absorbance at 734 nm was then measured using a microplate absorbance reader (Tecan Group, Austria). The results are expressed as mg tannic acid equivalents g^{-1}.

MEAD antioxidant capacity

The antioxidant properties of MEAD were evaluated by means of measurement of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity by the method of Hong *et al.* (2008), with some modification. Briefly, DPPH (0.2 mM) was prepared by dissolving 3.94 mg of DPPH in 50 ml of pure methanol (HPLC grade). MEAD and standards (ascorbic acid) were prepared at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 μg/ml. Then, 100 μl of each concentration was incubated with 100 μl of 0.2 mM DPPH solution at room temperature for 40 min in a 96-well plate. The absorbance at 540 nm of the resulting solution was monitored after 40 min using a Tecan Infinite F200 microplate reader (Tecan Group, Australia). All determinations were performed in triplicate. The radical scavenging activity of the extract was determined and compared with that of the vehicle-treated control group.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was performed according to the method of Choi *et al.* (2010). First, 60 μM of 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid (APPH) (60 mM) and 50 nM

fluorescein were used as a peroxyl radical generator and fluorescent probe, respectively. In the ORAC assay, based on the hydrogen atom transfer (HAT) reaction, the antioxidant reacts with the peroxyl radical competitively with fluorescein. All reagents were prepared with 75 mM phosphate buffer (pH 7.4). The wells of a 96-well plate were filled sequentially with fluorescein solution (66 nM, 190 μl) and 30 μl of serially diluted MEAD and standards. Then, 30 μl APPH (500 mM) was added to each well quickly using a multi-channel pipette. The decreasing fluorescence was measured every 5 min for 5 h. The analysis was performed on a fluorescence microplate reader at 37°C and excitation and emission wavelengths of 485 and 530 nm, respectively, using a Tecan Infinite F200 microplate reader. ORAC values were calculated as described by Cao and Prior (1999). The area under the curve (AUC) and net AUC of the standards and sample were determined using the equation $AUC = (0.5 + f_5/f_0) + f_{10}/f_0 + f_{15}/f_0 + \ldots + f_{300}/f_0) \times 5$, where f_0 is the initial fluorescence reading on initiating the reaction. The Net $AUC = AUC_{sample} - AUC_{blank}$.

Nitric oxide assay

The inhibitory activity of MEAD on the production of nitric oxide by LPS-treated Raw264.7 cells was evaluated as described by Yoon *et al.* (2009). Briefly, Raw264.7 cells in 10% FBS-DMEM without phenol red were plated in 24-well plates (1×10^5 cells/well/500 μl), and incubated for 12 h. The cells were incubated with 1 μg/ml of LPS in the presence of MEAD at different concentrations for 24 h. The quantity of nitrite in the culture medium was measured using Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). Then, 100 μl of Griess reagent mixture was added to the cell culture medium, incubated at room temperature for 10 min, and the absorbance at 540 nm was measured using a Tecan Infinite F200 microplate reader. The inhibitory activity of MEAD was calculated using the equation $\text{Inhibition (\%)} = 100\times[OD_{sample} - OD_{LPS}]/[OD_{LPS} - OD_{DMSO}]$.

Western blot

RAW264.7 cells (5×10^5 cells/2 ml) seeded in six-well plates for 12 h were incubated with LPS with or without samples for 24 h. The Raw264.7 cells were lysed for 10 min at 4°C in lysis buffer containing 25 mM HEPES-NaOH (pH 7.4), 2 mM ethyleneglycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), 1% Triton X-100, 10% glycerol, 1 mM Na_3VO_4, 5 mM NaF, 1 mM 4-(2-aminoethyl)benzene sulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/ml leupeptin. The lysate was centrifuged at 3,000 × *g* for 10 min, and the protein concentration of the resulting supernatant was measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, USA). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the lysate was diluted 1:5 with 5× sample buffer [10% SDS, 12.5% β-mercaptoethanol, 300 mM Tris (pH 6.8), 0.05% bromophenol blue, and 50% glycerol] and heated at 95°C for 5 min before resolution on SDS-PAGE gels. The proteins were transferred onto poly (vinylidene fluoride) membranes. Then, the membranes were blocked with 5% non-fat milk in TBS/T [25 mM Tris (pH 8.0), 150 mM NaCl, and 0.01% Tween 20] and incubated at 4°C overnight with a 1:1,000 dilution of rabbit anti-β-actin (NeoMarkers, Fremont, CA, USA), or a 1:1,000 dilution of rabbit anti-iNOS (Cell Signaling Technology, MA, USA) in 5% milk in TBS/T. Reactive proteins were visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham, England) and chemiluminescence using Western Lightning ECL (Amersham, England).

Nuclear localization of p65 NF-κB and IκBα by western blot and immunocytochemistry

The cell cultures were pretreated for 2 h with final concentrations of 200, 40, 8, and 1.6 μg/ml MEAD. After 2 h, 1 μg/ml LPS was added and fixed with methanol after 30 min or the nucleus and cytoplasm were lysed separately, and stored at freezer for later analysis using western blot. The nuclear translocation of the p65 subunit of NF-κB was examined using an immunocytochemical method. The methanol-fixed cells were permeabilized with 0.2% TritonX-100. After washing in phosphate-buffered saline (PBS), the slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal anti-p65 or IκBα antibodies at 1:100 dilution. After overnight incubation at 4°C, the slides were washed, incubated with goat anti-rabbit IgG-Alexa 488 (Molecular Probes, Eugene, OR, USA) at 1:100 dilution for 1 h, and counterstained for nuclei with Hoechst 33342 (50 ng/ml) for 15 min. The stained slides were mounted with Dako mounting medium (Dako North America, CA, USA) and visualized by laser-scanning confocal microscopy (Olympus Optica, Tokyo, Japan). To analyze the effect of MEAD against NF-κB activa-

Table 1. The yield, effect on iNOS activity in Raw264.7 cells, total polyphenol content, and cytotoxicity of methanol extracts of various parts of eight food plants

Scientific name/family name	Part	MeOH (Yield, %)	Polyphenol equal to tannic acid (μg)	iNOS activity IC_{50}* (μg/ml)	MTT assay CC_{50}** (μg/ml)
Adansonia digitata/Bobacaceae	Leaf	12.4	990.5	28.6	>100
Amaranthus caudatus/Amaranthaceae	Seed	4.4	71.4	>100	>100
Ficus sur/Moraceae	Fruit	59.8	516.2	>100	>100
Prosopis juliflora/Fabaceae	Pulp	38	249.8	>100	>100
Mimusops kummel/Sapotaceae	Pulp	58.2	14.3	>100	>100
Moringa stenopetala/Moringaceae	Leaf	22.7	670.5	>100	>100
Solanum nigrum/Solanaceae	Leaf	14.4	737.1	>100	>100
Urtica simensis/Urticaceae	Leat	17.5	301.0	>100	>100

*IC_{50}: 50% Inhibition Concentration; **CC_{50}: 50% cytotoxic concentration.

tion, cytosol and nucleus were used to analyze IκBα and NF-κB, respectively. Western blot analysis was conducted at least three times using the same antibodies.

MTT cell viability assay

The cytotoxicity profile of MEAD was assayed using tetrazole [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)]. Raw264.7 cells (4×10^4 cells/well/200 μl) were seeded on 96-well plates, grown for 24 h, and treated with LPS (1 μg/ml) in the presence of MEAD (200, 40, 8, and 1.6 μg/ml). After a 24-h incubation, spent medium was pipetted out and replaced with new medium (200 μl). Then, 10 μl of MTT solution was added to the medium and incubated for an additional 4 h at 37°C and 5% CO_2. Next, the supernatant was removed and the formazan crystals were dissolved in 100 μl DMSO. The absorbance at a wavelength of 540 nm was read using a microplate reader.

RESULTS

Total polyphenol content

The total polyphenol contents measured using the Folin-Ciocalteu method and expressed as tannic acid equivalents are shown in Table 1. The radical-scavenging capacities of the methanol extracts of the plants might be related mostly to their phenolic hydroxyl groups. MEAD exhibited the highest radical-scavenging activity (Fig. 2) and inhibition of iNOS activity (Fig. 3), probably due to its high polyphenol content.

Antioxidant activities of MEAD

In general, anti-oxidant capacities can be determined by using different types of chemical reactions: hydrogen atom transfer-based assay (i.e. ORAC assay) and single electron transfer based assay (i.e. DPPH assay). To evaluate the radical-scavenging activity of MEAD, we used two different assay systems: hydrogen-atom-transfer-based (ORAC) and single-electron-transfer-based (DPPH) assays. Our ORAC assays indicated that MEAD significantly delayed the decay rate of APPH-induced fluorescein fluorescence (Fig. 2A). MEAD was more than 10-times as potent as ascorbic acid, a known antioxidant.

We also evaluated the antioxidant potential of MEAD using DPPH, which stably generates free radicals in ethanol solution. As shown in Fig. 2B, MEAD exhibited dose-dependent radical scavenging activity.

Effects of MEAD on iNOS expression

To investigate the anti-inflammatory activities of plants, the inhibitory effects on iNOS expression in LPS-stimulated RAW264.7 cells were used as a model. Cell viability upon exposure to the plant extracts was not altered at concentrations <100 μg/ml, as determined by MTT assay with CC50 (Fig. 3C and Table 1).

Of the plant methanol extracts, only MEAD, with an IC_{50} of 28.6 μg/ml, strongly inhibited NO production (Table 1). The inhibitory activity was dose-dependent, with high potency at concentrations of 50-100 μg/ml (Fig. 3B), whereas the other extracts did not show inhibitory activity. Whether MEAD inhib-

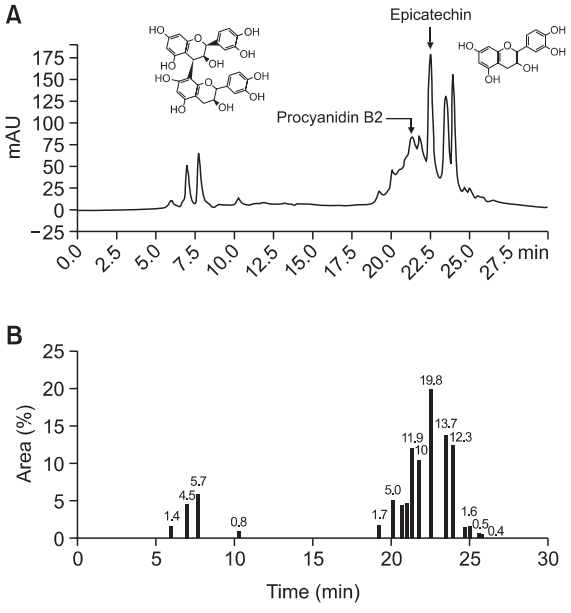

Fig. 1. (A) HPLC chromatogram of MEAD. (B) The relative constituents of MEAD and the major compounds, epicatechin (19.8%) and procyanidin B2 (11.9%)

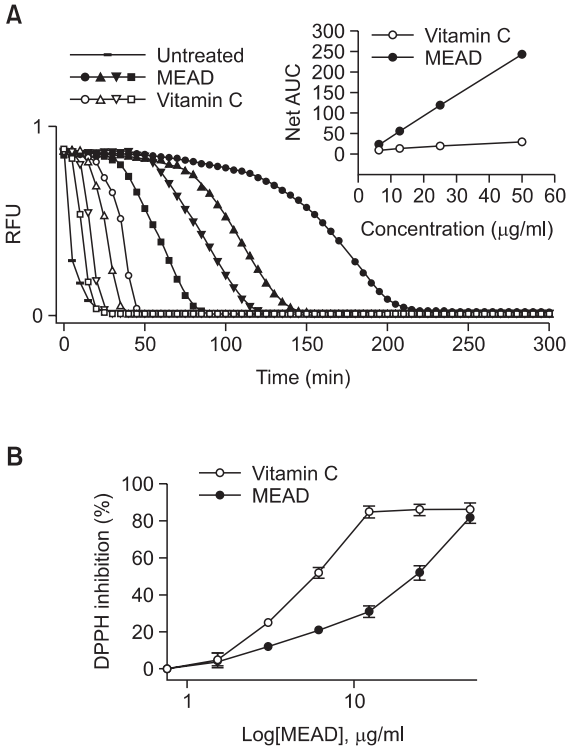

Fig. 2. Scavenging effect of MEAD on free radicals. (A) ORAC assay showing the APPH-induced fluorescence decay curve in the presence of MEAD or vitamin C at various concentrations (■, □ =6.25 μg/ml; ▼, ▽=12.5 μg/ml; ▲, △=25 μg/ml; ●, ○=50 μg/ml). Plots are representatives of three separate experiments. Insert presents best-fit lines between net AUC and different concentrations of MEAD or vitamin C. The Net $AUC=AUC_{sample} - AUC_{blank}$. The linear coefficients (r2) for MEAD and vitamin C are 0.992 and 0.993, respectively. Data represent mean ± S.D., n=3. (B) DPPH reduction assay. Each bar represents mean ± SD of three independent experiments.

its nitrite production via the suppression of iNOS expression at the protein level was determined. As shown in Fig. 3A, in the presence of LPS iNOS protein levels in Raw264.7 cells were increased, but were suppressed by MEAD in a concentration-dependent manner.

Effect of MEAD on NF-κB activation

One of the cellular triggers of chronic inflammation is the translocation of NF-κB into the nucleus and the subsequent up-regulation of inflammation-related genes. Therefore, we investigated the relationship between iNOS activity and NF-κB activation. To determine the duration of exposure required for NF-κB activation, cells were exposed to LPS for various times without treatment of MEAD (Fig. 5A). After 30 min, NF-κB translocated from the cytosol to the nucleus. However, MEAD suppressed LPS-induced NF-κB activation in a dose-dependent manner (Fig. 4A and Fig. 5B). MEAD also inhibited IκBα degradation (Fig. 4B and Fig. 5C). These results indicate that MEAD inhibits IκBα degradation and consequently suppresses NF-κB translocation from the cytosol to the nucleus in LPS-induced RAW264.7 cells.

DISCUSSION

Inflammation is believed to cause several degenerative diseases (Mueller *et al.*, 2010). The widespread nature and severity of the diseases caused by inflammation have stimulated much interest in development of methods of inhibiting this process. Many plant compounds alter inflammation-mediated disease. These comprise various classes of secondary metabolites that act by regulating pro-inflammatory mediators in a variety of biological systems (Reddy *et al.*, 2006; Chen *et al.*, 2008; Choi *et al.*, 2009).

In our study, MEAD significantly inhibited lipopolysaccharide (LPS)-induced iNOS expression in RAW264.7 cells. The plant extract did not alter cell viability, as indicated by MTT assay, indicating that inhibition of NO synthesis by MEAD was not due simply to toxicity.

Although NF-κB is a multifunctional transcription factor, it is an important target for controlling inflammation as the transcription of many pro-inflammatory molecules depends on its activation. Attenuation of NF-κB activation is associated with reduced production of inflammatory mediators by activated cells (Wang *et al.*, 2012). The NF-κB signaling system is a promising target for chemoprevention. Paur *et al.* (2010) demonstrated that several dietary plants inhibit NF-κB activation *in vitro*. In our study, in addition to inhibiting the production of NO and expression of iNOS in LPS-activated Raw264.7 cells, MEAD was also inhibited NF-κB activation, suggesting that its

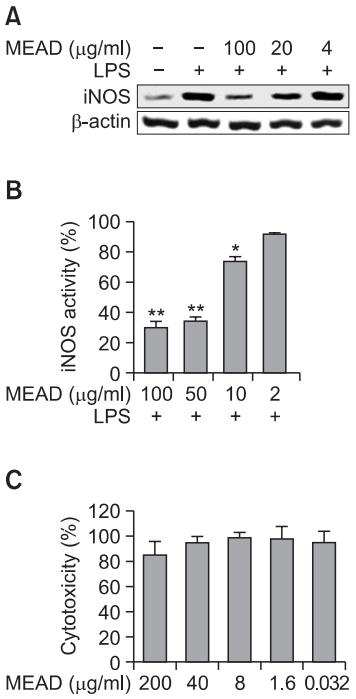

Fig. 3. Inhibitory effect of MEAD against iNOS expression. (A) Western blot analysis of iNOS expression in Raw264.7 cell lysates. (B) Application of various MEAD concentrations (μg/ml) resulted in dose-dependent inhibition of iNOS activity in culture medium of LPS-treated RAW264.7 cells. (C) MTT assay of MEAD. Results as shown as mean ± S.D., n=3. ***p*<0.001 and **p*<0.01 compared with the LPS-induced group.

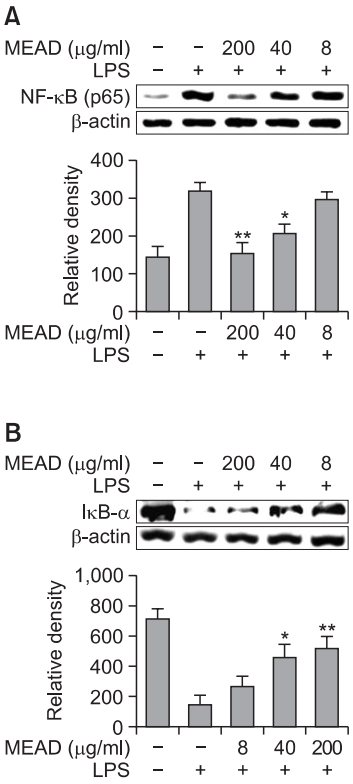

Fig. 4. Effects of MEAD on the activation of NF-κB in LPS-stimulated Raw264.7 cells. (A) Western blot analysis of NF-κB from the nuclei of Raw264.7 cells stimulated with LPS for 30 min. LPS dramatically increased the amount of NF-κB in the nucleus, and this amount was dose-dependently decreased by MEAD. (B) Western blot analysis of IκBα from the cytosolic extracts. The degradation of cytosolic IκBα upon LPS stimulation was suppressed by MEAD, in a dose-dependent manner. Cytosolic extracts of the same cells were used as a control (β-actin). All experiments were repeated at least three times. **p*<0.05, ***p*<0.01, compared with the LPS-induced group.

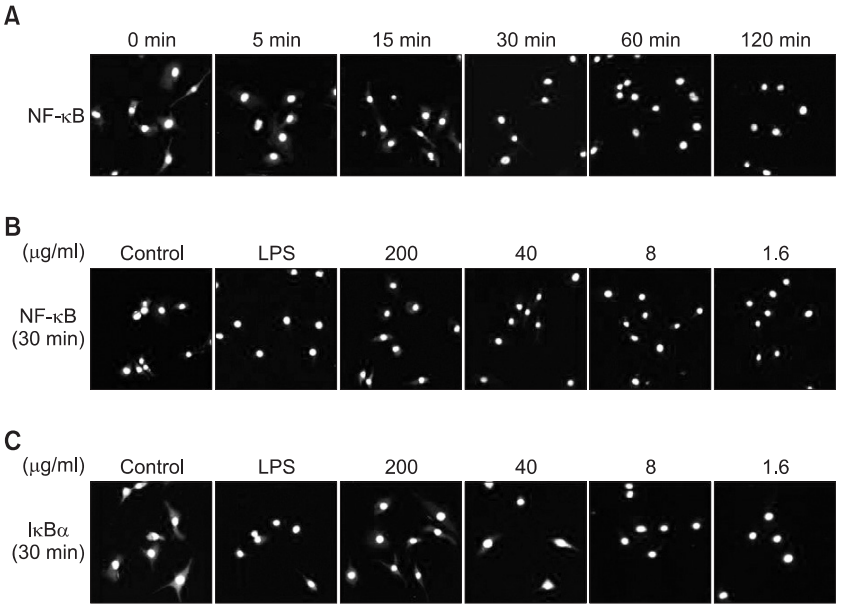

Fig. 5. Dose-dependent effects of MEAD on the translocation of NF-κB (p65) in Raw264.7 cells. (A) Time-dependent translocation of NF-κB was assessed by confocal microscopy. Cytoplasmic NF-κB (p65) protein was completely translocated from the cytosol to the nucleus within 30 min, and this maintained for 120 min after incubation with LPS. (B) Cells were pre-incubated with MEAD for 2 h and then stimulated with LPS (1 μg/ml) for 30 min. MEAD dose dependently inhibited NF-κB translocation in LPS-induced Raw264.7 cells. All experiments were repeated at least three times. (C) MEAD attenuates IκBα degradation 30 min after LPS treatment of Raw264.7 cells. IκBα was stained with rabbit anti-IκBα antibody, and then goat Alexa488-conjugated 2nd antibody. The degradation of of IκBα was evaluated by confocal microscopy. Pretreatment with MEAD dose-dependently inhibited the degradation of IκBα in LPS-induced Raw264.7 cells. Confocal microscope (400× magnification).

anti-inflammatory effect is mediated by the inhibition of NF-κB. Interestingly, MEAD inhibited IκBα degradation, a specific endogenous inhibitor of NF-κB. These results suggest the potential anti-inflammatory effects of MEAD via the inhibition of IκBα degradation.

Similarly, we investigated the radical-scavenging activity of MEAD using two assay systems: hydrogen-atom-transfer-based (ORAC) and single-electron-transfer-based (DPPH) assays (Huang *et al.*, 2005). The ORAC assay indicated that MEAD significantly delayed the decay rate of APPH-induced fluorescein fluorescence and demonstrated its considerable antioxidant capacity compared with a known antioxidant, vitamin C, which was used as the control. As assessed by measuring net AUC in an ORAC assay, MEAD presents 10.2 times more potent anti-oxidative capacity in comparison with those of vitamin C. The DPPH assay also indicated that MEAD has significant antioxidant potential and might prevent malignancies related to inflammation. In addition, concern over synthetic antioxidants has increased, due partly to their possible toxicity. This might explain the growing interest in phytochemicals; indeed, much effort has focused on investigation of plant extracts as sources of inexpensive antioxidants and anti-inflammatory agents. Moreover, the continuing increase in the promotion of antioxidants in foods reflects the hope that these are effective against a wide range of diseases that are believed to be caused or aggravated by 'oxidative stress' (Niki, 2011; Pinchuk *et al.*, 2012). In this regard, dietary antioxidants, substances in food that significantly decrease the adverse effects of reactive oxygen species or reactive nitrogen species during normal physiological processes in humans (Umberto, 2009), may represent a safe remedy for widespread problems.

It appears likely that the beneficial effects of MEAD are due, at least in part, to its inhibition of NF-κB activation, thereby suppressing expression of the pro-inflammatory iNOS gene, resulting in decreased NO production. This is the first report of an anti-inflammatory effect of MEAD.

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