SPANDIDOS PUBLICATIONS

Chamomile: An anti-inflammatory agent inhibits inducible nitric oxide synthase expression by blocking RelA/p65 activity

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Abstract. Chamomile has long been used in traditional medicine for the treatment of inflammation-related disorders. In this study we investigated the inhibitory effects of chamomile on nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression, and explored its potential anti-inflammatory mechanisms using RAW 264.7 macrophages. Chamomile treatment inhibited LPS-induced NO production and significantly blocked IL-1ß, IL-6 and TNFα-induced NO levels in RAW 264.7 macrophages. Chamomile caused reduction in LPS-induced iNOS mRNA and protein expression. In RAW 264.7 macrophages, LPS-induced DNA binding activity of RelA/p65 was significantly inhibited by chamomile, an effect that was mediated through the inhibition of IKKß, the upstream kinase regulating NF-κB/Rel activity, and degradation of inhibitory factor-κB. These results demonstrate that chamomile inhibits NO production and iNOS gene expression by inhibiting RelA/p65 activation and supports the utilization of chamomile as an effective anti-inflammatory agent.

Introduction

Chronic inflammation of longstanding duration plays a critical role in the initiation and development of various human diseases including cancer (1). Macrophages play a central role in the inflammatory response and produce excess amounts of pro-inflammatory cytokines and enzymes and inflammatory mediators, the accumulation of which leads to disease development (2,3). Activated macrophages transcriptionally express inducible nitric oxide synthase (iNOS), which catalyzes the oxidative deamination of L-arginine to produce nitric oxide (NO), and is responsible for prolonged and profound production of NO (4). High output of NO by iNOS induces deleterious effects such as inflammation and cancer (5,6).

Molecular cloning and sequencing analysis studies have demonstrated the existence of at least 3 main types of NOS isoforms, endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (7). Expression of the iNOS gene is regulated at the transcriptional level by the NF-κB/Rel family of transcription factors, which are involved in regulation of immune and inflammatory responses. The murine iNOS promoter contains two NF-κB/Rel binding sites located at 55 and 971 base pairs upstream of the TATA box. Moreover, it has been reported that the protein binding to both of these κB sites is necessary for the full induction of the iNOS gene by pro-inflammatory mediators such as lipopolysaccharides (8,9). In unstimulated cells, NF-κB/Rel exists in an inactive state in the cytoplasm, complexed with an inhibitory protein called IκB. Upon activation, IκB undergoes phosphorylation and degradation and the NF-κB/Rel heterodimer is translocated into the nucleus where it binds to DNA and activates transcription (10). A number of inflammatory stimuli and pro-inflammatory cytokines activate immune cells to upregulate inflammation, and consequently are potential targets for exploration of the molecular mechanisms underlying the activation processes, with the aim of developing effective anti-inflammatory drugs to ameliorate their untoward effects.

Chamomile is a herbal plant that has been used for centuries in many human cultures to treat various inflammatory conditions such as eczema, ulcers, gout, neuralgia and rheumatic pains (11,12). Dried flowers of *Matricaria chamomilla* L. are used in the preparation of tea, which is consumed at a rate of more than a million cups per day (13). The beneficial effects of chamomile are related to the presence of several flavonoid constituents and the core structure consists of either flavone mono- (apigenin, luteolin) or flavonol-derivatives (quercetin, patuletin). These occur in various forms such as aglyco- and di-glycosides and/or acyl-derivatives. Other principal components are essential oils such as terpenoids, α-bisabolol

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Abbreviations: EMSA, electrophoretic mobility shift assay; GM-CSF, granulocyte-macrophage colony-stimulating factor; HPLC, high performance liquid chromatography; NO, nitric oxide; NOS, nitric oxide synthase; IL-1ß, interleukin 1ß; TNFα, tumor necrosis factor-α; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB, IKK, IκB kinase

Key words: inflammation, nitric oxide, chamomile, nuclear factor-κB, macrophages, pro-inflammatory cytokines

and its oxides, azulenes including chalmuzene and acetylene derivatives (14).

Chamomile has been approved by the German Commission E for oral consumption in the management of various inflammatory diseases of the gastrointestinal tract, and for topical application in the treatment of various skin and inflammatory disorders of certain mucosal surfaces, such as the oral cavity and ano-genital areas (15). Recent studies have demonstrated its antioxidant, hypocholesterolemic, anti-parasitic, anti-aging, and anticancer properties, supporting its longstanding traditional use for treating various human ailments (16-18). In previous investigations, we have demonstrated that chamomile is a selective COX-2 inhibitor with anti-inflammatory activities (19). In the current study, we investigated the effects of chamomile on NO synthesis in LPS activated macrophages and analyzed the underlying mechanisms of action using RAW 264.7 murine macrophages.

Materials and methods

Materials. Dry chamomile flower of Egyptian origin was purchased from Bec's Tea Nirvana, Cleveland, OH. Cell culture medium, DMEM, fetal bovine serum, penicillin-streptomycin cocktail and phosphate buffered saline were purchased from Cellgro Mediatech, Inc. (Herndon, VA). Lipopolysaccharide (LPS, *E. coli*), and apigenin 7-O-glucoside (>95% pure) were purchased from Sigma (St. Louis, MO). Mouse rTNF-α, mouse rIL-6, and mouse rIL-1ß were purchased from R&D Systems (Minneapolis, MN). L-NMMA NG-monomethyl-L-arginine, monoacetate salt was purchased from Calbiochem (Brookfield, WI). All reagents used in the experiments when applicable, were of analytical reagent grade or HPLC grade.

Preparation of extracts. Dry chamomile flowers were weighed and crushed to powder with a marble pestle and mortar and a 5% w/v suspension was prepared in a flask by adding hot boiled water. The flask was then placed on a shaker (200 rpm) for 4 h and the temperature was maintained at 37˚C. After shaking, the flask was brought to room temperature and the suspension was filtered through a series of Whatman filters and finally passed through 0.22 micron filter (Millipore, Billerica, MA). The filtered aqueous extract was freeze-dried and stored at -20˚C until use. For cell culture studies, the dried material from aqueous extract was weighed and dissolved in culture medium to achieve desired concentration.

Cell culture. Murine RAW 264.7 macrophages were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified essential medium in appropriate culture conditions. Cell stimulation was performed with 1 μg/ml of LPS, mouse rTNF-α, mouse rIL-6, and mouse rIL-1ß.

Cell viability assay. Cell respiration, an indicator of cell viability, was determined by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan. After the supernatants were removed for nitrite assay, cells were incubated at 37˚C with MTT (0.5 mg/ml) for 45 min. The medium was aspirated and cells were solubilized in dimethyl sulfoxide (250 μl) for at least 2 h in the dark. The extent of reduction of MTT was quantified by optical density measurement at 550 nm.

Nitrite estimation. RAW 264.7 macrophages were cultured in 6-well plates. After incubation with LPS and other cytokines and various doses of chamomile for 12-24 h, nitric oxide synthesis was determined by assaying the culture medium for nitrite, which is the stable reaction product of nitric oxide with molecular oxygen, using Griess reagent kit obtained from Biotium (Hayward, CA).

Western blot analysis. Macrophages, grown in 6-well plates to confluence, were incubated with or without LPS in the absence or presence of the test agents for 16 h. Cells were washed with ice-cold PBS and stored at -70˚ until further analysis. Frozen plates were put on ice and cells were lysed in 1% Triton X-100, 0.15 M NaCl, and 10 mM Tris-HCl pH 7.4 for 30 min. Lysates were homogenized through a 22 G needle and centrifuged at 10,000 x g for 10 min at 4˚C. The supernatants were collected and protein was measured by the method according to Bradford (20). Cell lysates, containing equal amounts of protein, were boiled in SDS sample buffer for 5 min before running on a 10% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene fluoride membranes (Invitrogen, Carlsbad, CA). Membranes were blocked with 5% fat-free dry milk in TBS-T pH 8.0 [Tris-buffered saline (50 mM Tris, pH 8.0, 150 mM NaCl) with 0.1% Tween-20] and then incubated with antibodies *viz*. anti-iNOS (SC-7271), anti-IκBα (SC-1643), anti-NF-κB/RelA (SC-8008) anti-ß-actin (SC-47778) obtained from Santa Cruz (Santa Cruz, CA), anti-p-IKKα/IKKß (Ser180/181; Cat#2681), anti-p-IκBα (Ser32/36; Cat#9246) obtained from Cell Signaling Technology (Beverly, MA) with appropriate dilutions and incubated overnight at 4˚C. After washing 3 times with TBS-T, and bands were visualized by using appropriate IgG, horseradish peroxidase conjugate and the enhanced chemiluminescence system (ECL™, Amersham Pharmacia Biotech). Signal intensities were evaluated by densitometric analysis (Kodak Digital Science™ Image Station 2000R Life Science Products).

Reverse transcriptase (RT)-PCR analysis. RAW 264.7 cells ($5x10^6$ cells/100-mm dish) were incubated for 8 h with or without various concentrations of chamomile and LPS (1 μg/ml). After washing with PBS twice, total RNA was isolated from the cell pellet using RNA isolation kit (Invitrogen, CA). The total amount of RNA was determined by absorbance at 260 nm. RNA (1 μg) was reverse transcribed into cDNA using avian myeloblastosis virus (AMV) reverse transcriptase and oligo$(dT)_{15}$ primer (Promega Co., Madison, WI, USA). The PCR samples contained 50 μl of the reaction mixture, comprised of 50 mM KCl, 5 mM $MgCl_2$, 0.16 mM dNTP, 5.0 units of Taq DNA polymerase (Qiagen, Valencia, CA, USA), and 20 pmol of sense and antisense primers in 10 mM Tris-HCl (pH 8.3). The primer for iNOS was 5'-CAGTTCTGCG CCTTTGCTCAT-3' (sense) and 5'-GGTGGTGCGGCTGG ACTTT-3' (antisense), and for GAPDH 5'-AGGCCGGTGCT GAGTATGTC-3' (sense) and 5'-TGCCTGCTTCACCACCT TCT-3' (antisense). The PCR amplification was performed under the following conditions, 30 cycles of denaturation at 98˚C for 30 sec, annealing at 60˚C for 30 sec and extension

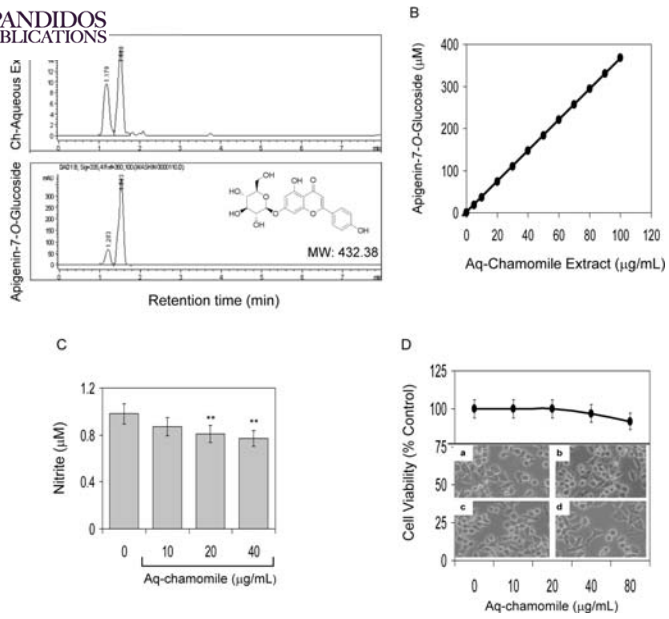

Figure 1. (A) HPLC chromatogram of aqueous chamomile extract demonstrates apigenin 7-O-glucoside as major constituent. (B) Standardization of chamomile extract with apigenin 7-O-glucoside concentration. (C) Effect of chamomile on endogenous nitrite levels in culture medium of RAW 264.7 macrophages. Bars represent mean ± SEM of at least 3 independent experiments each performed in triplicate **P<0.05 (ANOVA). (D) Effect of chamomile on cell viability as determined by MTT assay and lower panel shows photograph of macrophages (a-d) after treatment with 0, 10, 20 and 40 μg/ml chamomile extract. Details are described in Materials and methods.

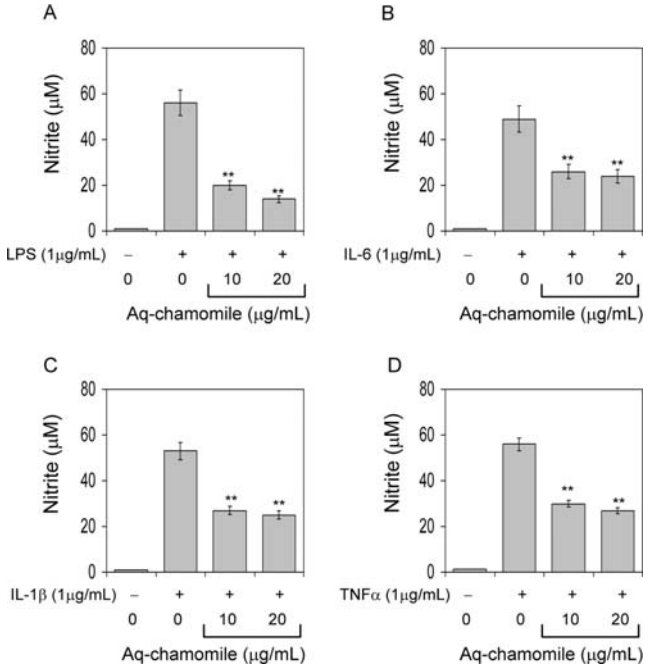

Figure 2. Effect of chamomile on NO production in culture medium of RAW 264.7 cells (A). RAW 264.7 cells activated with 1 μg/ml of each LPS (B) IL-6, (C) IL-1ß, and (D) TNFα in the absence and presence of chamomile (10 and 20 μg/ml) for 16 h. Bars represent mean ± SEM of at least 3 independent experiment each performed in triplicate, **P<0.001 (ANOVA), compared to LPS-challenge group. Details are described in Materials and methods.

at 74˚C for 1 min, using a thermal cycler (Px2, Thermo Electron Corporation). The amplified PCR products were run on a 2% agarose gel and visualized by SYBR Gold staining.

Electrophoretic mobility shift assay (EMSA). EMSA for NF-κB was performed in the nuclear fraction of RAW 264.7 macrophages incubated for 16 h with or without various concentrations of chamomile and LPS (1 μg/ml) using Lightshift™ Chemiluminiscent EMSA kit (Pierce Biotechnology, Rockford, IL) following manufacturer's protocol as previously described (21).

Statistical analysis. Nitrite estimation and cell viability were performed in triplicate. All experiments were repeated at least twice. Results are expressed as mean values ± SEM. Statistical comparisons were made by ANOVA followed by a Dunnett's multiple comparison test. P-values <0.05 were considered significant.

Results

First, we performed HPLC analysis on aqueous chamomile extract with reference to apigenin 7-O-glucoside, the major constituent of chamomile. As shown in Fig. 1A, HPLC analysis demonstrated two major peaks with retention times of 1.179 min (27.7%) and 1.520 min (63.3%) and 5 other minor peaks which together constitute 90% of the total flavonoids. The two major peaks in the aqueous chamomile extract correspond to apigenin 7-O-glucoside (63.3%) and apigenin 7-O-neohesperidoside (27.7%), which was further confirmed by LC-MS analysis as previously demonstrated (12).

Next we standardized aqueous chamomile extract with doses equivalent to molar concentration of apigenin 7-O-glucoside. For this study, different concentration of apigenin 7-O-glucoside were prepared in methanol and subjected to HPLC (Fig. 1A). The peak area (retention time 1.5-1.7 min) was calculated and plotted to obtain a standard curve, which corresponded to the concentration of apigenin 7-O-glucoside in the aqueous extract on the basis of peak area (Fig. 1B).

Following this, we determined the effect of aqueous chamomile extract on inhibition of constitutive NOS expression in RAW 264.7 cells by measuring the levels of total nitrite. The amount of nitrite, a stable metabolite of NO in the cell culture medium was estimated using Griess reagent as an index for nitric oxide. RAW 264.7 macrophages in the unstimulated state produced 0.98±0.01 μM nitrite in the medium. Treatment of macrophages with chamomile cause a modest decrease in the endogenous NO levels in RAW 264.7 cells which was more pronounced at 20- and 40-μg/ml doses of chamomile (Fig. 1C). Chamomile exposure did not affect cell viability at the test concentration up to 40 μg/ml. At 80 μg/ml chamomile, a modest decrease in cell viability was observed.

Since IL-1ß, IL-6 and TNFα are known pro-inflammatory cytokines and LPS causes NO production, we examined whether chamomile is capable of inhibiting NO levels after being challenge with these pro-inflammatory mediators. As shown in Fig. 2A-D, chamomile at 10- and 20-μg/ml doses significantly blocked NO production after challenge of RAW 264.7 cells with these molecules up to 40-60%. Since the NO production was highest with LPS challenge, therefore for further experiments we used LPS (1 μg/ml).

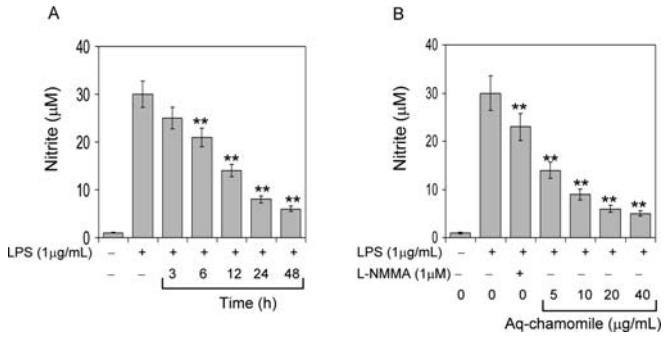

Figure 3. Effect of chamomile on NO production in the culture medium of RAW 264.7 macrophages. (A) Time-dependent effect using 1 μg/ml LPS in the absence and presence of 10 μg/ml chamomile for indicated times. (B) Dose-dependent effect using 1 μg/ml LPS for 16 h in the presence and absence of chamomile at indicated doses. L-NMMA (1 mM), a non-selective inhibitor of all NOS isoforms was used as positive control. Bars represent mean ± SEM of at least 3 independent experiments each performed in triplicate. **P<0.001 (ANOVA) compared to LPS challenge group. Details are described in Materials and methods.

Next we performed time course experiments to determine the decrease in levels of NO by chamomile. As shown in Fig. 3A, treatment of RAW 264.7 cells with 10 μg/ml for 3-48 h caused a significant decrease in NO production in the culture medium by 16.6% starting as early as 3 h and 79.8% at 48 h, respectively. In a dose-dependent assay, treatment of RAW 264.7 macrophages with LPS for 24 h, the nitrite concentration in the culture medium increased to ~30-fold. Treatment with chamomile caused a significant decrease in NO production with 53.3% at 5 μg/ml to 83.3% at the highest dose of 40 μg/ml, respectively. However, a decrease of 23.3% in NO production was achieved after treatment of RAW 264.7 macrophages with L-NMMA, a non-selective inhibitor of all NOS isoforms (Fig. 4B).

The marked reduction in nitrite production with chamomile suggests that it might also affect the iNOS protein expression. Therefore, we examined iNOS protein expression by Western blot analysis. As shown in Fig. 4A, RAW 264.7 macrophages incubated with LPS (1 μg/ml) in the absence and presence of chamomile at 10-40 μg/ml concentration for 16 h and the cell extract was examined for 133-kDa iNOS protein. As shown in Fig. 4A, reduced iNOS protein expression was observed after chamomile treatment compared to LPS-alone challenge, which was dose-dependent. At the message level, chamomile treatment to LPS-challenged RAW 264.7 macrophages resulted in a modest decrease in mRNA expression (Fig. 4B).

Since LPS-mediated activation of NF-κB is associated with prolonged activation of IKK, the upstream kinase, we first sought to determine whether chamomile can alter the level of this kinase. As shown in Fig. 5A, LPS challenge to RAW 264.7 macrophages resulted in a significant increase in phosphorylation of IKKα/ß which was markedly reduced with chamomile treatment in a dose-dependent manner. Activation of IKK leads to the hyperphosphorylation of IκBα as well as its subsequent degradation therefore next we measured the cytosolic levels of IκBα and its phosphorylated forms. Treatment with chamomile resulted in an increase in the total levels of IκBα in the cytosol whereas a significant decrease in the p-IκBα at Serine 32/36 was observed after chamomile treatment which was dose-dependent. Furthermore, treatment of RAW 264.7 cells with chamomile caused a decrease in the nuclear levels of NF-κB/p65 which correlated with a simultaneous increase in the cytosol in a dose-dependent manner (Fig. 5B).

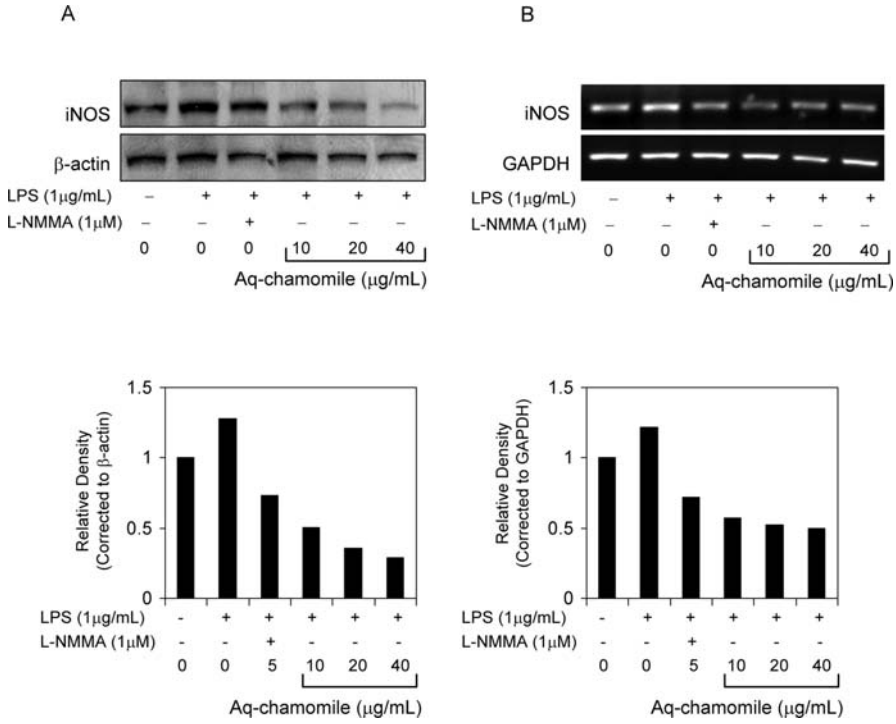

Figure 4. Effect of chamomile on iNOS expression in RAW 264.7 macrophages. (A) Western blot for iNOS protein expression, and (B) mRNA expression of iNOS in RAW 264.7 macrophages stimulated with 1 μg/ml LPS and LPS and chamomile as indicated. Graph represents protein and mRNA levels of iNOS corrected to the corresponding controls. L-NMMA, a non-selective inhibitor of all NOS isoforms was used as positive control. Details are described in Materials and methods.

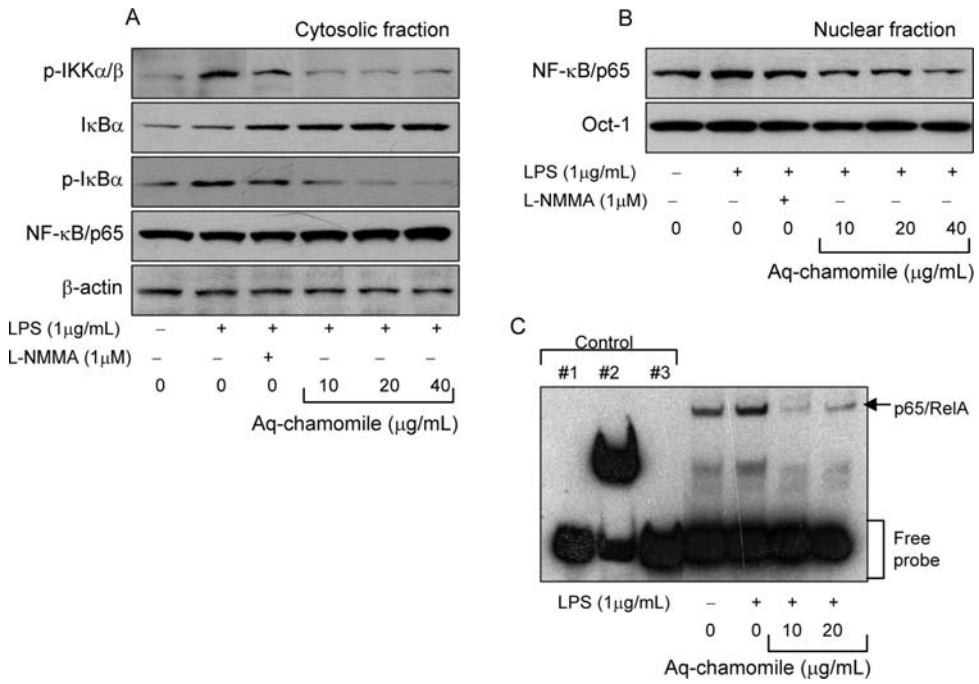

Figure 5. Effect of chamomile on NF-κB activity. (A) Western blot analysis for protein expression of p-IKKα/ß, IκBα and its phosphorylation, and RelA/p65 in the cytosolic fraction of RAW 264.7 macrophages stimulated with 1 μg/ml LPS and LPS and chamomile for indicated doses, (B) RelA/p65 expression in the nuclear fraction, (C) EMSA assay. EMSA was performed to determine the effect of chamomile on the nuclear translocation of NF-κB dimers and their binding to DNA. Controls: 1, Biotin-EBNA control DNA; 2, biotin-EBNA control DNA + EBNA extract; 3, biotin-EBNA control DNA + EBNA extract + 20-fold molar excess of unlabeled EBNA DNA. L-NMMA, a non-selective inhibitor of all NOS isoforms was used as positive control. The details are described in Materials and methods.

To further confirm NF-κB-mediated iNOS gene regulation, an EMSA was performed using an oligonucleotide containing a consensus RelA/p65 binding sequence in the nuclear fractions prepared after LPS challenge and chamomile treatment. A strong NF-κB band was observed in response to LPS-induced activation which was reduced with chamomile treatment (Fig. 5C). These results demonstrate that chamomile blocked NF-κB activation, which might account for the inhibition of iNOS induction in RAW 264.7 macrophages.

Discussion

Chamomile is known to possess anti-inflammatory and anti-oxidant effects. In the present study, we demonstrated that chamomile inhibits NO production and iNOS expression in macrophages, and showed that these effects are mediated through the inhibition of NF-κB/Rel transcription factor. As stated earlier NO plays an important role in the pathogenesis of various inflammatory diseases, including cancer. Therefore, the inhibitory effect of chamomile on iNOS gene expression suggests that this is one of the mechanisms responsible for its anti-inflammatory properties.

Macrophages play a central role in a host's defense against various infections by nature of their phagocytic, cytotoxic, and intracellular killing capacities (22). Stimulation of murine macrophages by LPS, the major component of Gram-negative bacterial cell walls, results in expression of iNOS (4). Increased production of NO plays a critical role in the process of macrophage activation and is associated with acute and chronic inflammations (23). Therefore, the suppression of NO production by inhibition of iNOS expression and/or enzyme activity is potentially a very important therapeutic strategy in the development of therapeutic anti-inflammatory agents. In the current study, we have demonstrated that chamomile inhibits LPS-induced NO production in RAW 264.7 macrophages. The inhibitory effect of chamomile was mediated via a reduction in iNOS both at the protein and message levels.

Macrophages are capable of secreting various mediators such as IL-1, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNFα, which lead to secondary immune responses such as proliferation of T and B cells, activation of macrophages for phagocytosis, and killing of microorganisms. Among these mediators, pro-inflammatory cytokines such as IL-1ß, IL-6 and TNFα can be generated in response to immunological reaction, inflammation and microbial invasion (24). Therefore, we sought to examine whether chamomile could alter NO production after incubation of RAW 264.7 macrophages with these cytokines along with chamomile. Our results demonstrate that chamomile may, in part, exert inhibitory effects on pro-inflammatory cytokines through inhibition of NO production in RAW 264.7 macrophages.

NO production by iNOS is regulated mainly at the transcriptional level, and the expression of the iNOS gene in macrophages is under the control of several transcription factors, which include NF-κB/RelA (8). The presence of *cis*-acting NF-κB element has been demonstrated in the 5' flanking regions of both the COX-2 and iNOS genes (9,25). Recently we demonstrated that chamomile differentially inhibits LPS-induced COX-2 activity and expression in RAW 264.7 macrophages (19). NF-κB is functional as a hetero- or homo-dimeric form of the Rel family proteins, including RelA (p65), RelB, cRel, p50 and p52 (26). In resting cells, cytoplasmic

IκB proteins (IκBα, IκBß, and IκBε) are associated with NF-κB dimers, and some stimuli, such as IL-1ß and LPS, lead to prolonged activation of IKK, the upstream kinase regulating NF-κB (27). Our studies demonstrate that chamomile in a dose-dependent manner suppresses LPS-mediated IKK expression, NF-κB activation and its translocation to the nucleus. These results suggest that chamomile inhibits the expression of iNOS and thus NO production, a process that is mediated through inactivation of NF-κB by reducing IκBα degradation and phosphorylation.

It is well known that RelA/p65 is a critical transactivation subunit for NF-κB (28,29). Our studies performed through EMSA demonstrate that chamomile inhibits RelA/p65 activation. Moreover, recent studies have demonstrated that the transcriptional activity of RelA/p65 subunit is regulated by posttranslational modifications such as phosphorylation and acetylation (30). It has been shown that the phosphorylation status of RelA determines whether it associates with CREB-binding protein/p300, which is a critical regulator of NF-κB (31). In this regard, it is possible that chamomile suppresses transcriptional activity of RelA/p65 by modifying the phosphorylation or acetylation status of the RelA/p65 subunit. Further studies are required to understand how chamomile regulates the transcriptional activity of the RelA/p65 subunit.

In summary, this study demonstrates that chamomile inhibits LPS-induced NO production and iNOS gene expression in macrophages and that these effects are mediated, at least in part, through blockage of NF-κB/Rel transcriptional activation. The fact that NF-κB/Rel is negatively regulated by chamomile is important because this transcription factor plays a critical role in the regulation of a variety of genes that are involved in inflammatory responses. Since chamomile is a nontoxic and pharmacologically active compound that has demonstrable inhibitory effects on iNOS gene expression, and since NO plays an important role in mediating inflammatory responses, our study supports the utilization of chamomile as a potentially effective therapeutic anti-inflammatory agent.

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