ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science 2015 Vol. 25. No. 7. 801~809 DOI : http://dx.doi.org/10.5352/JLS.2015.25.7.801

Extract from *Prunus mume* Sieb. et Zucc. Fruit Prevents LPS-induced Homotypic Aggregation of Monocytic THP-1 Cells via Suppression of Nitric Oxide Production and NF-κB Activation

Hye-Rim Lee[1†], Youngsook Park[2†], Hyun Jeong Kim[1], Aram Lee[2], Jihea Choi[2], Jaeho Pyee[1], Heonyong Park[1] and Jongmin Kim[2*]

[1]*Department of Molecular Biology & Institute of Nanosensor and Biotechnology, Dankook University, 126, Jukjeon-dong, Suji-gu, Yongin-si, Gyeonggi-do 448-701, Korea*
[2]*Department of Life Systems, Sookmyung Women's University, 52 Hyochangwon-gil, Yongsan-gu, Seoul 140-742, Korea*

Received May 13, 2015 / Revised July 3, 2015 / Accepted July 8, 2015

Homotypic cell adhesion (homotypic aggregation) in activated monocytes plays a central role in physiological and pathological processes including inflammatory responses, differentiation and migration. The extract of the *Prunus mume* Sieb. et Zucc. fruit (Maesil) has potential benefits to human health; such as anti-viral, anti-microbial, and anti-cancer activities. Indeed, Maesil extract may modulate inflammatory responses via interference with homotypic aggregation in monocytes. In the present study, the molecular mechanisms underpinning the therapeutic efficacy of Maesil extract in inflammatory diseases were investigated. It was found that Maesil extract inhibited homotypic aggregation in lipopolysaccharide (LPS)-activated monocytes. This was mediated by reduction of nitric oxide (NO) production, partly via inhibition of inducible nitric oxide synthase (iNOS) expression in LPS-activated THP-1 cells. It was confirmed that NO inhibition is a key mechanism in Maesil induced blockade of monocyte aggregation through identification of reversal of this inhibitory effect by the NO-producing agent S-nitroso-N-acetyl penicillamine (SNAP). In addition, Maesil extract significantly attenuated LPS-induced IκB-α phosphorylation and NF-κB translocation into the nucleus. In conclusion, Maesil extract exerts anti-inflammatory effects via inhibition of homotypic aggregation of LPS-activated monocytes through mechanisms involving the suppression of NO production and NF-κB activity, suggesting Maesil extract as a potential therapeutic candidate for the prevention and treatment of chronic inflammatory diseases.

Key words : Homotypic aggregation, inflammationnitric oxide, maesil extract, NF-κB

Introduction

The fruit of *Prunus mume* Sieb. et Zucc. (Korean name, Maesil) belongs to the Rosaceae family and there are several varieties of Maesil in Korea, including Cheonmae and Cheongchuk. It has long been used as a traditional remedy and health food in Korea, China, Vietnam, and Japan [6, 21, 26, 34]. To date, a number of phytochemical, pharmacological, biological, and clinical studies on Maesil have been reported [5, 7, 13, 16, 17]. Many chemical constituents have been isolated from Maesil extract including volatile compounds [27], hydroxycinnamic acid derivatives [26], citric acid derivative (mumefural) [7], and triterpenoids [16]. Maesil extract has been reported to show anticancer [1, 13, 30], antiviral [38], anti-microbial [29], anti-oxidant [37], and cardiovascular protective [35] effects. Maesil extract has been used pharmaceutically in folk medicine for antitussive, expectoration, antiemetic, antidiarrheal, anthelmintic, and antipyretic actions [26, 37]. Despite the known beneficial effects of Maesil extract in chronic inflammatory diseases such as cancer and cardiovascular disease, there are very few reports concerning its antiinflammatory effects and the detailed mechanisms involved. The present study sought to determine a potential application for Maesil extract in phytoptherapy through identification of the anti-inflammatory responses elicited upon exposure of immune cells to multiple components of Maesil extract. Homotypic cell adhesion (homotypic aggregation) of activated monocytes is essential for physiological processes including immune response, differentiation, and monocyte migration. It is also intimately

†**Authors contributed equally.**
***Corresponding author**
Tel : +82-2-710-9553, Fax : +82-2-2077-7322
E-mail : jkim@sookmyung.ac.kr

involved in the pathogenesis of chronic inflammatory diseases such as atherosclerosis [25]. Under chronic inflammation, proinflammatory stimuli, such as lipopolysaccharide (LPS), activate monocytes and induce the expression of cell adhesion molecules (CAMs) and the production of nitric oxide (NO) [9, 18, 36]. As a result, activated monocytes move to target tissues and induce heterotypic/homotypic cell adhesion [11], indicating that CAMs and NO are key regulators of the inflammatory processes that control cell-cell adhesion [18, 32, 36]. During this process, increased expression of lymphocyte function-associated antigen-1 (LFA-1) is correlated with the induction of homotypic aggregation of monocytes [22, 24]. In addition, integrin β2, and intercellular adhesion molecule-1 (ICAM-1) have also been shown to be involved in homo- and heterotypic cell adhesion [31]. During inflammatory processes, the expression of inducible nitric oxide synthase (iNOS) is significantly induced by various proinflammatory stimuli such as LPS, interleukin-1β, and tumor necrosis factor, and large amounts of NO are produced [3]. Excessive NO is associated with cytotoxicity and pro-inflammatory activity [10]. Nuclear factor (NF)-κB is considered as one of the most important factors in the immune response. Activation of NF-κB in response to pro-inflammatory stimuli is initiated by the rapid phosphorylation of IκB-α, through activation of IκB-α kinase. Phosphorylation of IκB-α results in its proteolytic degradation by ubiquitination [15], thus liberating the NF-κB complex and enabling translocation to the nucleus with subsequent activation of pro-inflammatory genes such as iNOS [15]. In addition, mitogen-activated protein kinase (MAPK) signaling is considered to play important roles in cell-cell adhesion [31, 33] and has been implicated in the regulation of iNOS expression [4, 5, 20]. In this study, the underlying mechanisms by which Maesil extract dampens the inflammatory response were investigated. It was found that Maesil extract inhibits the production of NO and NF-κB activation in LPS-stimulated monocytes, resulting in reduction of homotypic aggregation. These data support the proposal that Maesil extract has potential as a functional food material, prompting the need to expand research efforts in this area.

Materials and Methods

Preparation of *Prunus mume* fruit (Maesil) extract

Maesil extract was obtained from Institute of Native Genetic Resources Inc. (Yongin, Korea). Briefly, Maesil fruits of two different cultivars, Cheonmae and Cheongchuk (Aojiku) were obtained from local farmers in Suncheon and fruits without seeds were extracted through a series of processes, including freeze drying and grinding into powder followed by mixing and shaking in distilled water at 100°C for 2 hr or in 20%, 40% or 100% (v/v) ethanol for 20 hr. The extracts were filtered through a Whatman filter paper (GE Healthcare UK Limited, Buckinghamshire, U.K.), concentrated with rotary evaporator, and finally freeze dried, weighed, and stored in a refrigerator at -20°C until use.

Cell culture

THP-1 cells (obtained from Korean Cell Line Bank-KCLB No 40202) isolated from human monocytic leukemia were cultured in RPMI-1640 medium (Wel GENE Inc.) containing 10% fetal bovine serum (Wel GENE Inc.) and antibiotics (100 U/ml penicillin/streptomycin). The culture was maintained at 37℃ in a humidified 5% CO_2 atmosphere. For stimulation experiments, cells were incubated with LPS in the presence or absence of Maesil extract for the indicated time periods.

Cell viability assay

Cytotoxicity was measured using the WST-1 assay kit (Daeil LabService, Seoul, Korea) according to the manufacturer's instructions. THP-1 cells (1×10^5/ml) were treated with various concentrations of Maesil extract for 24 hr, followed by 1 hr incubation with WST-1 at 37°C and 5% CO_2. The absorbance was measured at 450 nm using an ELISA plate reader (Bio-Rad, Model 550). The cell viability was calculated as relative absorbance compared to control.

Homotypic aggregation assay

THP-1 cells were plated in 6-well plates. The cells were treated 1 μg/ml LPS (Sigma - Aldrich) along with each extract at the indicated concentrations for 24 hr. The homotypic aggregation of THP-1 cells was observed by microscope (Zeiss Autoplan 2) at 10X magnification or quantified by counting the number of adhesive cells.

Western blotting

After cell activation for the indicated time periods, THP-1 cells were washed twice in ice-cold PBS and then lysed by a 30 minute rocking incubation in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride). Nuclear and cytoplasmic extracts were prepared using Nuclear Complex Co-IP Kit (Active Motif) according to man-

ufacturer's instructions. Cell debris was removed by centrifugation and total protein concentration of the soluble cell lysate was measured using the BCA assay (Intron). Equal amounts of protein (10~25 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene difluoride membrane (Millipore, USA) at 300 mA for 1 hr. The membrane was blocked with 5% bovine serum albumin (BSA) and probed with antibodies specific to LFA-1, Integrin β2, ICAM-1 (Abcam), Tubulin, p-IκB-α, NF-κB p65, GAPDH (Cell Signaling Technology), Lamin A/C (Santa Cruz Biotech). After washing, the membranes were incubated with HRPconjugated secondary antibodies (Cell Signaling Technology) and immunoreactivity was detected using enhanced chemiluminescence (Amersham, USA).

Measurement of NO production

Intracellular NO content was monitored via the fluorescence intensity of DAF-2DA (Calbiochem). Cells were pre-incubated with DMSO or Maesil extract and were washed with HEPES buffer (5 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM Glucose, pH7.4) and treated with LPS. Following this, cells were incubated at 37°C in 5% CO_2 with DAF-2DA for 30 min, harvested, and lysed by sonication. The fluorescence of collected supernatants was measured using a spectrofluorophotometer (RF 5301PC Shimadzu) at excitation and emission of 495 and 515 nm (slit 10 nm), respectively. NO content was calculated via fluorescence intensity [19].

Results and Discussion

Maesil extract inhibits homotypic aggregation of LPS-activated THP-1 cells

Cell-cell adhesion plays central roles in the inflammatory response and migration of monocytes. A homotypic aggregation assay is a useful method to identify the efficacy and molecular mechanisms of an extract or the active constituents of a medicinal plant against inflammation. Accordingly, the effects of Maesil extract on homotypic aggregation of LPS-activated THP-1 cells were investigated, with focus on the following: 1) different cultivars of Maesil; 2) different extraction methods (hot water or ethanol); and 3) different concentrations of Maesil extract. Two different Maesil varieties, namely Cheonmae and Cheongchuk (Aojiku), were compared. Hot water extract from Cheonmae (CME) substantially inhibited homotypic aggregation of LPS-activated THP1 cells; Hot water extract from Cheongchuk (CCE) also exhibited inhibitory activity, but to a much lesser extent than Cheonmae hot water extract (Fig. 1A). These data suggest that Cheonmae hot water extract (referred to as Maesil extract from here onwards), and to some extent Cheongchuk hot water extract, may confer potent anti-inflammatory activity against pathological processes in activated monocytes. Maesil extract has traditionally been consumed as a sauce, a syrup, and as juice, with extraction methods employing hot water. However, it is also consumed as a liquor, which is extracted using alcohol. Thus, in the present study, Cheonmae Maesil was also extracted using water, 20%, 40%, or 100% ethanol and these preparations were investigated for their effect on homotypic aggregation of LPS activated THP-1 cells. As shown Fig. 1B, all of these extracts showed a similar inhibitory effect on homotypic aggregation, indicating that the anti-inflammatory properties of Maesil are evident regardless of the solvent used for extraction. Finally, the effect of various concentrations of Maesil (Cheonmae hot water) extract on homotypic aggregation of LPS-activated THP-1 cells was determined. Incubation of THP-1 cells with Maesil extract resulted in dose-dependent inhibition of LPS-induced homotypic aggregation. Indeed, aggregation was inhibited up to 50% when concentrations of 1,040 μg/ml Maesil extract were used (Fig. 1C), suggesting that a concentration of 10 μg/ml Maesil (Cheonmae hot water) extract is sufficient to inhibit LPS-activated homotypic aggregation of THP-1 cells.

In vitro cytotoxic effect of Maesil extract against THP-1 cells

Next, it was investigated whether or not Maesil extract is capable of eliciting a cytotoxic effect on THP-1 cells. To assess this, THP-1 cells were treated with Maesil extract (0.1200 μg/ml) for 24 hr and then an alteration of LPS-induced cytotoxicity was analyzed. It was found that Maesil extract at concentrations of 0.110 μg/ml appeared non-cytotoxic, but Maesil extract at concentrations of 100-200 μg/ml was, if minimal, cytotoxic in THP-1 cells (Fig. 2). Therefore, to exclude an experimental noise by Maesil extract-induced cytotoxicity, further experiments were conducted with Maesil extract at less than noncytotoxic concentrations (≤ 10 μg/ml). In addition, it is worthy of note that a range of concentrations (≤10 μg/ml) of Maesil extract employed in this study are likely to be physiologically and clinically rele-

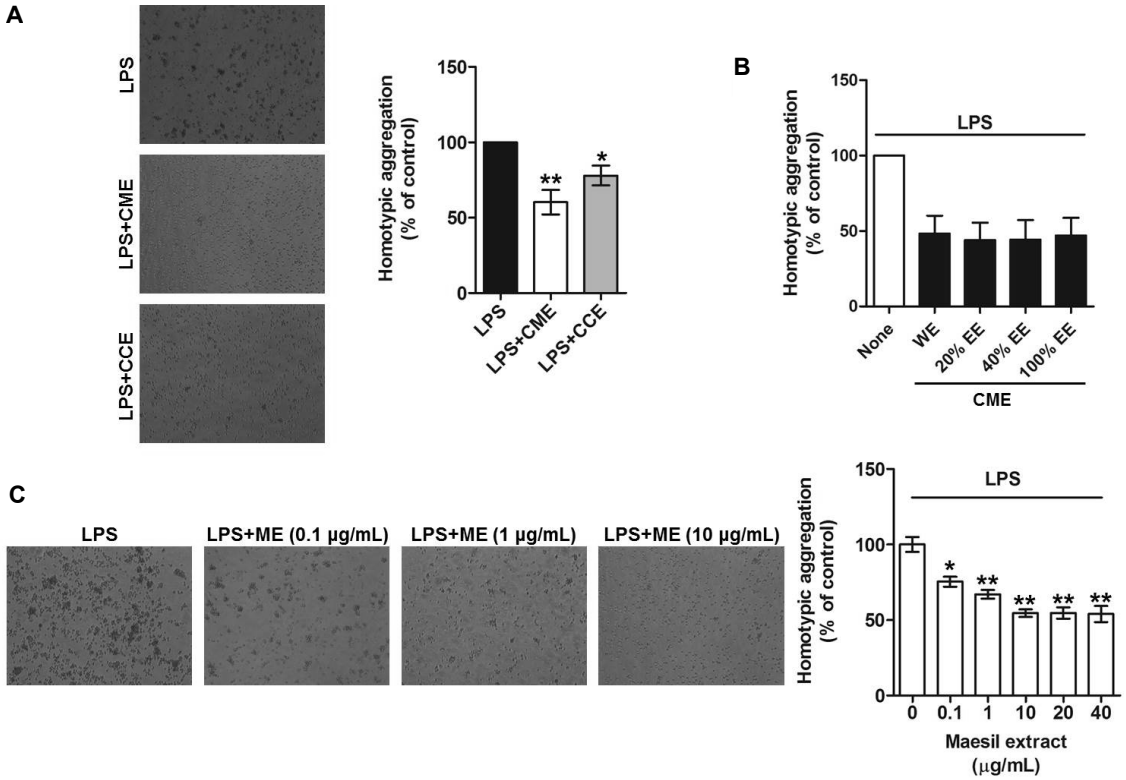

Fig. 1. Inhibition of LPS-activated THP-1 cell homotypic aggregation by Maesil extract. THP-1 cells were incubated with 1 μg/ml LPS along with Cheonmae hot water extract (CME) or Cheongchuk hot water extract (CCE) (A) or Cheonmae ethanol extracts (EE) (0%-WE, 20%, 40% and 100%) (B), various concentrations of Maesil (Cheonmae hot water) extract (ME, 0.1-40 μg/ml) (C) for 24 hr. Homotypic aggregation of LPS activated THP-1 cells was observed under the microscope and quantified by counting the adhesive cells. Images are representative of at least 3 different observations. Bar graphs show mean ± S.E. (n=3). *p<0.05. **p<0.01.

vant in THP-1 cells. A recent report has shown that MK615, an extract of the *Prunus mume,* is able to relieve severe symptoms of patients with cancer when 6.5 g of MK615 was administered twice per day [12]. Based on this and other data, the effective concentrations (≤10 μg/ml) of Maesil extract used in this study are comparable to doses administrated in clinical and in vivo studies [12, 23]. It is also noteworthy that an advantage of using whole extracts seems likely to appear unexpected efficacy caused by unknown compounds in the whole extracts. Many studies have shown that such synergistic effects from unknown compounds in whole extracts occasionally render the crude extract more efficacious when comparing to a single compound isolated at the equivalent dose [8, 18].

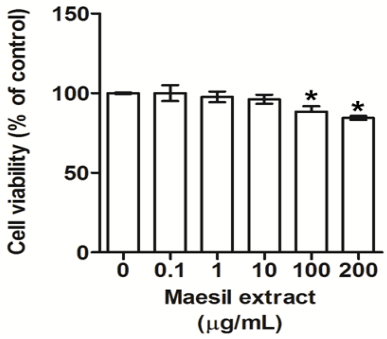

Fig. 2. Effect of Maesil extract on THP-1 cell viability. THP-1 cells were treated with various doses of Maesil extract for 24 hr and cell cytotoxicity was measured by the WST-1 assay. Data were plotted as bar graphs (mean ± S.E., n=3). *p<0.05.

Maesil extract markedly inhibits the production of nitric oxide via suppression of iNOS expression in LPS-activated THP-1 cells, but not integrin beta 2 family members and ICAM-1

Given that homotypic aggregation of activated monocytes is regulated by NO production and CAM expression [18, 32, 36], it is plausible that Maesil extract regulates these mediators, thereby crucially modifying homotypic aggregation of monocytes. Previous studies have shown that the integrin

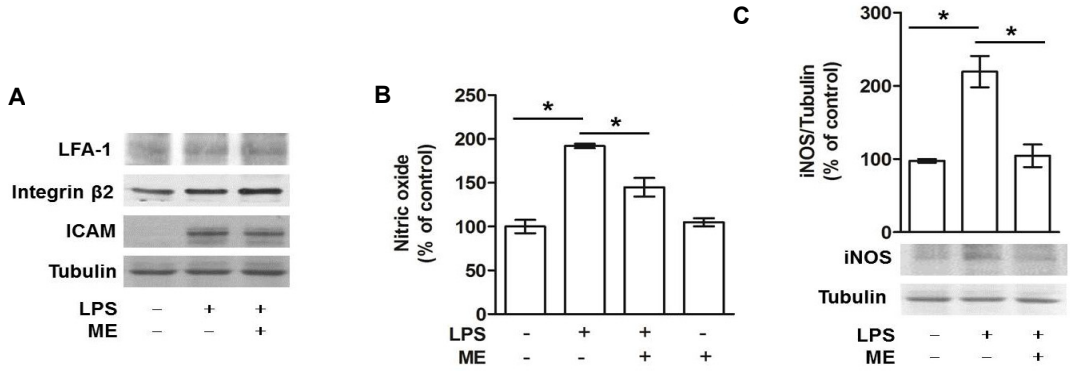

Fig. 3. Effect of Maesil extract on protein expressions of inducible nitric oxide synthase (iNOS) and cell adhesion molecules (CAMs) and production of nitric oxide in LPS activated THP-1 cells. THP-1 cells were incubated for 24 hr with 1 μg/ml LPS alone or in the presence of 10 μg/ml Maesil extract (ME). Protein levels of LFA-1, Integrin β2, ICAM-1 (A), iNOS (C) and tubulin were determined. Cells were treated with LPS alone or in the presence of 10 μg/ml ME extract and NO was measured (B). Data are plotted as bar graphs (mean ± S.E., n=3). *p<0.01.

beta 2 family members including LFA-1, and integrin β2, as well as ICAM-1 are key molecules in the regulation of monocyte adhesion [22, 31]. Therefore, the effects of Maesil extract on the expression of LFA-1, integrin β2, and ICAM-1 in LPS activated THP-1 cells were investigated. THP1 cells were treated with 1 μg/ml LPS, alone or in the presence of 10 μg/ml Maesil extract for 24 hr and subjected to western blot analysis. The protein levels of the three CAMs were increased by LPS treatment, whereas Maesil extract had no effect on the protein expression of any of the adhesion molecules examined (Fig. 3A). These findings suggest that Maesil extract at the low concentrations (10 μg/ml) regulates homotypic aggregation of activated monocytes through, if any, other CAMs than examined in this study. Accordingly, further experiments will be needed to identify cell adhesion molecules specifically involved in this event. To characterize underlying molecular mechanisms for the inhibition of homotypic aggregation of LPS-activated monocytes by exposure to Maesil extract, it was assessed whether Maesil extract regulates the production of NO in these cells. It is known that NO plays an important role in inflammatory responses via mediation of monocyte activation and cell adhesion [32, 36]. Many studies have shown that monocytes produce low levels of NO, but treatment with LPS induces high levels of NO in these cells [3, 10]. Consistent with previous studies, NO production was found to be remarkably elevated in THP-1 cells upon treatment with LPS. This LPSinduced NO production was shown to be substantially diminished in the presence of Maesil extract, whereas Maesil extract alone had no effect on the basal NO level (Fig. 3B). Given that enhanced production of NO in LPSactivated monocytes requires the induction of iNOS protein [3], the effect of Maesil extract on the expression level of iNOS was explored in these cells. Treatment of monocytes with LPS appeared to markedly enhance the expression of iNOS; an effect that was completely abrogated by concurrent treatment with Maesil extract (Fig. 3C). These data suggest that Maesil extract inhibits excessive NO production and dampens inflammatory pathways in LPS activated monocytes. However, appropriate NO production plays a crucial role in physiological functions [2, 28] and thus a fine balance in NO production is fundamental to maintaining homeostasis in monocytes. In addition, phenolic compounds and terpenoids, which are known chemical constituents of Maesil extract, seems likely to have beneficial effects on chronic inflammatory diseases associated with excessive NO production [14]. It is therefore assumed that phenolic compounds and terpenoids may be key active constituents of Maesil extract that plays important roles in the regulation of NO production in inflammatory conditions. Detailed molecular mechanisms remain to be identified.

Maesil extract inhibits homotypic aggregation via inhibition of nitric oxide production in LPS-activated THP-1 cells

To confirm whether inhibition of NO production by Maesil extract is a crucial factor for homotypic aggregation, a reversal effect of an NO-donor called S-nitroso-N-acetyl penicillamine (SNAP) on homotypic aggregation in THP-1 cells was examined. As expected, basal homotypic aggregation of monocytes was increased upon treatment with SNAP; co-treatment of THP-1 cells with LPS and SNAP was

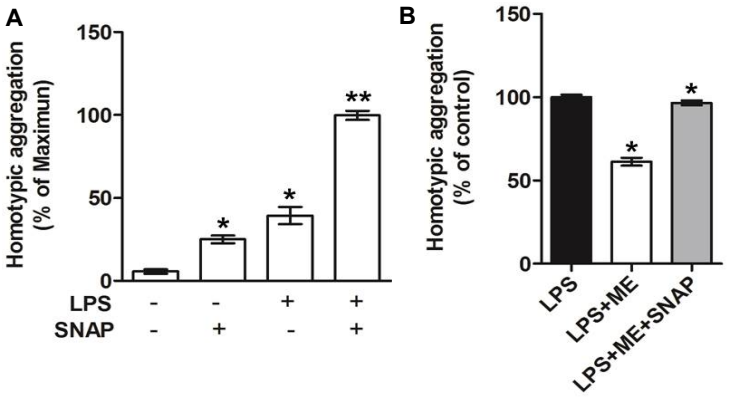

Fig. 4. Effect of Maesil extract and the NO-producing agent S-nitroso-N-acetyl penicillamine (SNAP) on homotypic aggregation in LPS activated THP- 1 cells. THP-1 cells were incubated for 24 hr with 1 μg/ml LPS alone or in the presence of 200 mM SNAP (A), 10 μg/ml Maesil extract, or both Maesil extract and 200 mM SNAP (B). Homotypic aggregation of LPS activated THP-1 cells was observed under the microscope and quantified by counting the adhesive cells. Images are representative of at least 3 different observations. Bar graphs show mean ± S.E. (n=3). *p<0.05. **p<0.01.

shown to considerably enhance homotypic aggregation (Fig. 4A). In addition, the inhibitory effect of Maesil extract on LPS induced homotypic aggregation of monocytes was abrogated by treatment of THP 1 cells with SNAP (Fig. 4B). These data suggest that Maesil extract-induced blockade of inflammatory responses such as an inhibition of homotypic aggregation occurs through the regulation of NO production.

Attenuation of LPS-induced IκB-α phosphorylation and NF-κB translocation into the nucleus by Maesil extract

The participation of NO signaling in homotypic cell adhesion is under tight regulation, involving several intercellular signaling pathways. To determine the signal transduction pathways underlying the inhibition of LPS-induced homotypic aggregation by Maesil extract, several key signaling molecules in the cell-cell adhesion pathway [18, 31] were studied. LPS-induced phosphorylation of p38 MAPK, ERK, and JNK was not altered by Maesil extract at a concentration of 10 ug/ml (Data not shown), whereas IκB-α phosphorylation was dramatically reduced by the co-treatment with Maesil extract for 60 min (Fig. 5A). Phosphorylation of IκB-α leads to its degradation and this event releases NF-κB from the NF-κB/IκB-α complex in the cytoplasm, thus allowing

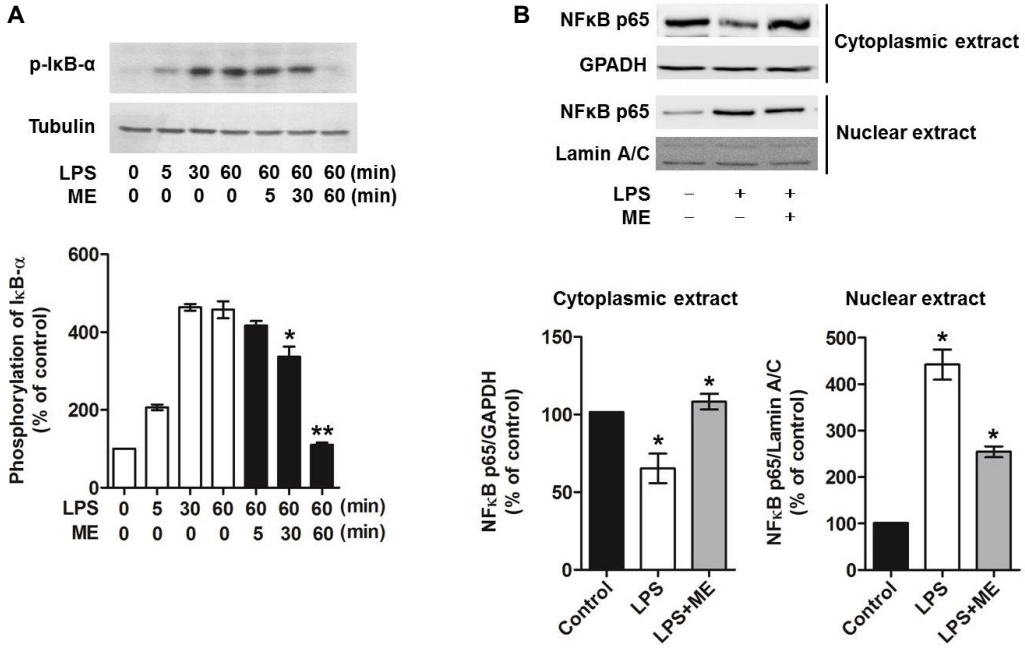

Fig. 5. The inhibitory effect Maesil extract on LPS-induced IκB-α phosphorylation in LPS activated THP-1 cells. Serum-depleted THP-1 cells were treated with 1 μg/ml LPS along with Maesil extract for various periods of time. Western blotting was performed with p-IκB-α (A) and NF-κB p65 antibodies in LPS activated THP-1 cytosolic and nuclear extracts (B). Each band was quantified by densitometry. Bar graphs show mean ± S.E. (n=3), *p<0.05. **p<0.001.

translocation to the nucleus and subsequent promotion of pro-inflammatory gene expression, including iNOS. To be sure, the effects of Maesil extract on NF-κB translocation into the nucleus in LPS activated THP-1 cells were investigated. It was found that the nuclear fraction of NF-κB was increased by LPS. To the contrary, Maesil extract significantly attenuated the NF-κB translocation into the nucleus in LPS-stimulated monocytes (Fig. 5B). These data suggest that Maesil extract inhibits the NF-κB pathway through inhibition of LPS-induced phosphorylation of IκB-α and NF-κB translocation into the nucleus [15, 18]. However, further studies will be required to elucidate whether inhibition of the NF-κB pathway is directly involved in Maesil extract mediated inhibition of LPS-induced THP-1 cell homotypic aggregation via NO regulation. In conclusion, the present study provides an evidence for the following mechanisms: (1) Maesil extract inhibits homotypic aggregation of LPS-activated THP-1 cells; (2) Maesil extract reduces the production of NO in LPS activated-THP-1cells and this event is mediated by the suppression of iNOS expression; and (3) Maesil extract inhibits LPS induced phosphorylation of IκB-α and NF-κB translocation into the nucleus. Taken together, there is strong evidence to suggest that Maesil extract could be utilized as a phytotherapeutic agent for the prevention of inflammatory diseases.

Acknowledgments

I thank Dr. Danielle McLean for critical reading of the manuscript and thoughtful discussions. This Research was supported by the Sookmyung Women's University Research Grants (1-1503-0049).

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초록 : 매실 추출물의 산화질소 생성과 NF-κB 활성 조절을 통한 LPS유도성 THP-1 세포 동형성 응집의 억제 효과

이혜림[1†] · 박영숙[2†] · 김현정[1] · 이아람[2] · 최지혜[2] · 피재호[1] · 박헌용[1] · 김종민[2*]
([1]단국대학교 분자생물학과, [2]숙명여자대학교 생명시스템학과)

활성화된 단핵구의 동형성 세포 부착(동형성 응집)은 염증반응, 분화, 이동과 같은 생리학적, 병리학적 과정에서 중요한 역할을 한다. 매실 추출물은 항바이러스, 항균, 항암작용과 같은 효과를 보인다고 알려 져있다. 따라서, 매실 추출물은 단핵구의 동형성 응집 억제를 통해 염증반응을 조절할 가능성을 가진다. 본 연구에서는, 염증성 질환에서 매실 추출물의 치료효능을 뒷받침할 수 있는 분자적 기전을 조사하였다. 매실 추출물이 지질다당질(LPS)로 활성화된 단핵구의 동형성 응집을 억제함을 확인하였다. 이러한 효과는 LPS로 활성화된 THP-1 세포의 iNOS 단백질 발현 억제를 통해, 산화질소(NO) 생산의 감소로 조절되는 것을 발견하였다. 또한 NO 생성물질인 SNAP 처리 실험을 통해 단핵구 동형성 응집을 억제하는데 매실에 의한 NO 억제가 필수적인 기작임을 확인하였다. 게다가, 매실 추출물은 LPS로 유도된 IκB-α 의 인산화와 NF-κB의 핵내로의 이동을 현저하게 감소시키는 것을 확인하였다. 매실 추출물은 NO생성과 NF-κB 활성 억제를 통해 LPS로 활성화된 단핵구의 동형성 응집을 저해하고 이를 통해 항염증 효과를 유도할 수 있다는 결론으로부터 만성 염증성 질환의 치료와 예방에 매실 추출물의 효능을 제시하고자 한다.