Oryza sativa L. extracts inhibit nitric oxide production and inducible nitric oxide synthase expression in murine macrophage cells and lungs of antigen-challenged allergic mice

Cheol Yong Lee1, Min Jung Lee2, Ju Mi Kim2, Seul Min Choi3, Kyung Koo Kang2, Ok Jin Kim1, Byung Mu Lee3

*Corresponding author:
Seul Min Choi
1 Center for Animal Resources Development, Wonkwang University, 344-2, Sinyong-dong, Jeollabuk-do, Iksan, Republic of Korea
2 Research center, Dong-A ST, Yong-in, Gyeonggi-do, 446-905, Republic of Korea
3 Division of Toxicology, College of Pharmacy, Sungkyunkwan University, Chunchun-dong 300, Changan-ku, Suwon, Gyeonggi-do, 440-746, Republic of Korea

Abstract
Hyper-production of nitric oxide (NO) has been observed in the airways of patients with asthma and may contribute to airway inflammatory responses. Previous studies showed that DA-9201, an ethanolic extract of Oryza sativa L., significantly attenuated airway hyperresponsiveness, airway inflammation, and remodeling in murine models of asthma. The purpose of this study was to examine the effect of DA-9201 on the expression of inducible NO synthase (iNOS) and NO production in RAW 264.7 murine macrophage cells and ovalbumin (OVA)-sensitized allergic mice. The levels of NO and iNOS expression in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells were determined. Allergic inflammation was induced in mice by immunization with OVA and these mice were administered DA-9201 daily at 30, 100 or 300 mg/kg for 2 weeks. Mice were challenged with allergen by inhalation during the last 3 days of DA-9201 treatment and lungs were harvested 24 h after the last challenge. NO levels in blood plasma and iNOS expression in lung tissue were determined by ELISA and western blotting, respectively. DA-9201 inhibited the production of NO and iNOS gene and protein expression in a dose-dependent manner in LPS-stimulated cells. In the lungs of allergen-challenged mice, DA-9201 caused a significant decrease in iNOS protein expression. Furthermore, we found that DA-9201 significantly decreased NO levels in blood plasma. These results suggest that DA-9201 inhibits NO production by limiting the expression of iNOS, which may be one of the underlying mechanisms of its anti-asthmatic effect.

Keywords: Asthma, Oryza sativa, nitric oxide, iNOS expression, DA-9201

Introduction
Nitric oxide (NO) regulates many physiological processes [1] and is involved in diverse biological processes such as vasodilation, bronchodilation, and regulation of inflammatory-immune processes. In particular, NO plays a critical role in the regulation of airway function in both normal conditions and asthmatics [2]. NO is synthesized from L-arginine by the enzyme NO synthase (NOS), which exists in three distinct isoforms: constitutive neural NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) [3]. iNOS produces large amounts of NO, which have been closely correlated with the pathophysiology of a variety of diseases and inflammation, whereas constitutive NOS (cNOS) produces a small amount of NO, which is an important regulator of physiological homeostasis. iNOS can be induced in a variety of cell types by inflammatory cytokines, including interleukin-1beta (IL-1β), tumor necrosis-alpha (TNF-α), and interferon-gamma (IFN-γ) as well as microbial products such as lipopolysaccharide (LPS) [4]. Normally, cells do not express iNOS unless triggered by appropriate stimuli. In asthma, however, increased levels of IL-1β, TNF-α, and IFN-γ have been detected in the respiratory tract [5-6]. Increased iNOS production has been found in the airway epithelium of patients with asthma [7] and the high levels of NO produced by this NOS isoform is thought to reflect the high exhaled NO levels found in such patients [8-11]. This suggests that NO produced by iNOS may be marker of airway inflammation.

Black rice (Oryza sativa L.) belongs to the family Gramineae (syn. Poaceae). It is a dark purple-colored seed that is widely grown in South Asia, China, and Eastern Asia. It is traditionally known as enriched rice and has various medicinal properties such as anti-allergic, hematopoietic, anti-cancer, and anti-atherosclerotic effects [12-17].

DA-9201, an ethanol extract of Oryza sativa L., is currently being developed as an anti-asthma agent. Previously, we demonstrated the anti-asthmatic effect of DA-9201 in a murine acute asthma model, which was likely mediated by down-regulation of NF-κB expression [18-19]. In addition, using a chronic asthma model, we also showed that DA-9201 suppressed the progression of airway remodeling indicators such as goblet cell hyperplasia, smooth muscle hypertrophy and sub epithelial collagen deposition [20]. In this study, we investigated the effect of DA-9201 on the suppression of NO production and iNOS expression in the mouse.
The macrophage cell line RAW 264.7 as well as in lungs of antigen-challenged allergic mice.

Materials and methods

Test materials

The black rice (*Oryza sativa* L.) was purchased from the National Agricultural Cooperative Federation (Geochang, South Korea). The processed rice bran (1.348kg) was extracted with 70% ethanol (6.74 L) at room temperature with agitating. Next day, the extract was filtered through filter paper (Advantec, Japan), and the filtrate (5.13 L) was concentrated at 60°C using evaporative system. Finally, the dried extract powder was obtained in yields of 5.77% yield (77.80g). The amount of standard materials in the dried extract was determined by HPLC analysis with previously described conditions (Table 1) [20]. Chicken egg albumin (OVA, grade V), aluminum hydroxide gel (alum), dexamethasone, acetyl-β-methylcholine chloride (methacholine), protease inhibitor cocktail, bacterial LPS (*Salmonella typhimurium*) and the specific antibody for iNOS were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture media, fetal bovine serum (FBS) and media supplements for cell culture were obtained from Gibco BRL (Carlsbad, CA). The NO ELISA kit was purchased from OxisResearch (Foster City, CA).

<table>
<thead>
<tr>
<th>Table 1. The amounts of standard materials in DA-9201</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard materials</td>
</tr>
<tr>
<td>Cyanidine-3-O-β-glucopyranoside</td>
</tr>
<tr>
<td>Peonidine-3-O-β-glucopyranoside</td>
</tr>
<tr>
<td>Delphinidine-3-O-β-glucopyranoside</td>
</tr>
<tr>
<td>Pelargonidine-3-O-β-glucopyranoside</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
</tr>
<tr>
<td>Ferulic acid</td>
</tr>
</tbody>
</table>

Cell culture

RAW 264.7 cells (KCLB No. 40071) were purchased from the Korean Cell Line Bank (Seoul, Korea) and were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified atmosphere exposed to 5% CO2. For determination of nitrite concentration, iNOS protein and gene expression, cells were treated with various concentrations of DA-9201 (30, 100, 300 μg/ml) in the presence of LPS. DA-9201 was dissolved in dimethyl sulfoxide (DMSO).

Cell viability assay

The mitochondrial-dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan was used to measure cell respiration as an indicator of cell viability [21]. The cell suspension was plated in a 96-well microculture plate (1.8 × 10^5 cells per well) and incubated with DA-9201 (0.3, 3, 10, 30, 100, 300 μg/ml) with or without LPS (1 μg/ml) for 24 h. Following treatment, 0.5 mg/ml MTT was added to each well and incubated for 0.5-3 h at 37°C. The medium was then removed and DMSO added to dissolve the formazan. The absorbance of each sample was read at 560 nm in a SPECTRA max PLUS tunable microplate reader (Molecular Devices Corp., Sunnyvale, CA). The absorbance in cultures treated with LPS alone was regarded as 100% cell viability.

Nitrite determination

Nitrite accumulation was used as an indicator of NO production in the medium following previously described methods [22]. Briefly, cells were plated at 1 × 10^6 cells/ml in 24-well culture plates and cultured overnight. DA-9201 was added 1 h prior to exposure of the cells to 1 μg/ml bacterial LPS. Cells were then cultured for another 24 h and NO level in culture media supernatant was quantified using an NO ELISA kit.

Western blotting of iNOS in RAW 264.7 cells

To assess the effect of DA-9201 on iNOS protein expression, RAW 264.7 cells were cultured and treated as described above. For western blotting, cells were harvested in RIPA lysis buffer (Sigma-Aldrich) containing 1 mM EDTA and an EDTA-free protease inhibitor cocktail (Sigma-Aldrich) and incubated for 30 min at 4°C. Cell debris was removed by centrifugation at 13,000 g for 10 min and supernatants were used for experiments. Protein content of each sample was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Samples (30 μg per lane) were loaded onto 4-12% NuPAGE Novex Bis-Tris Gels (Invitrogen, Carlsbad, CA) and transferred electronically to nitrocellulose membranes (Invitrogen, Carlsbad, CA). The membranes were then blocked with 5% BSA in phosphate buffer solution/0.05% Tween-20 (PBST) at room temperature for 1 h. The membranes were incubated with mouse anti-iNOS antibody (Sigma-Aldrich) or mouse anti-beta-actin antibody (Sigma-Aldrich) in PBST for 2 h, followed by incubation with goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma-Aldrich) for 1 h. Protein was visualized with 5-bromo-4-chloro-3-indolyl-phosphatase and nitroblue tetrazolium in Tris buffer (BCIP/NBT) as the substrate (KPL, USA) and was quantified by densitometric analysis using a densitometer (Vilber Lourmat, France).

Analysis of messenger RNA levels for iNOS

Total cellular RNA from RAW 264.7 cells cultured and treated with DA-9201 as described above, was extracted with TRI reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommended procedure. Total RNA (1 g) was reverse-transcribed using oligo-(dT)18 primers and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA). After synthesizing cDNA at 42°C for 60
min, the RT mixture was incubated at 94°C for 5 min to inactivate the reverse transcriptase. PCR was performed using PerfectShot™ Ex Taq RT-PCR system (TaKaRa). PCR primers for mouse iNOS and β-actin were purchased from Genotech (Korea). The PCR primers were as follows: 5'-GTGTTCACGAGGATGTTG-3' (sense) and 5'-CTCTGTGCACACTGATTGTC-3' (antisense) for mouse iNOS, and 5'-TGTTAGGTGGGATGGTCGAG-3' (sense) and 5'-TTGTAGGTGACGCAGTTCC-3' (antisense) for mouse β-actin. The PCR mixture was incubated sequentially in a DNA thermal cycler (Perkin-Elmer) at 94°C for 2 min, followed by 35 reaction cycles (94°C for 30 s, 54°C for 30 s, 72°C for 1 min) and finally extended at 72°C for 7 min. The PCR product was analyzed using 1% agarose gel electrophoresis and ethidium bromide staining. The intensity of the band was quantitatively measured using a densitometer (Vilber Lourmat, France).

Animals

Female BALB/c mice (5 weeks old) were purchased from Charles River Japan and kept under standard conditions (temperature 23 ± 2°C, humidity range 40-70%, 12 h light/dark cycle with light between 7:00-19:00) at the animal facility at Dong-A Pharmaceutical Co Ltd. Food and UV-sterilized tap water were provided ad libitum. Studies were performed in accordance with the Institutional Standard Operation Procedure for Animal Care and Experiments (SOP-ANC) of Dong-A ST and following the “Guide for the Care and Use of Laboratory Animals” published by the National Institute of Health (Bethesda, Maryland).

Sensitization, study design, and allergen challenge

Mice (8/group) were immunized with 50 μg OVA mixed with 1 mg alum as an adjuvant in a total injection volume of 0.1 ml per mouse. Sensitized mice were administered booster doses 2 weeks after the primary immunization. Normal control mice were injected peritoneally with phosphate buffer saline (PBS, pH 7.0). Two weeks after the last sensitization, immunized mice were orally administered DA-9201 (30, 100, 300 mg/kg) for 14 consecutive days. Sham-treated mice were orally administered 0.5% hydroxypropyl methylcellulose (HPMC). During the last three days, mice were challenged with aerosolized 1% OVA for 30 min/day. OVA challenge was performed by placing mice in a Plexiglas box (29.22.18 cm) and aerosolizing OVA with an ultrasonic nebulizer (NE-U12; Omron, Japan) driven by compressed air for 30 min. Normal control mice were challenged with aerosolized PBS.

Measurement of NO level

Twenty-four hours after the last OVA challenge, mice were euthanized and plasma was prepared for measurement of NO level. NO was determined by an ELISA kit following the manufacturer’s instructions.

Western blot analysis of iNOS in lungs

To investigate the expression of iNOS, lung tissue isolated from control or DA-9201 treated mice was minced and incubated in cell lysis buffer (RIPA buffer®, Sigma-Aldrich) for 30 min at 4°C. Protein content was determined using the method of Bradford with BSA as the standard. Equal amounts of protein were loaded onto 4-12% NuPAGE Novex Bis-tris Gels (Invitrogen, Carlsbad, CA), followed by Western blotting under the conditions described above.

Statistical analysis

All the data were expressed as mean ± S.E.M. The comparison between the group means was conducted using a Dunnett multiple range test with a significance level of p = 0.05. All statistical analyses were performed using SigmaStat® (Jandel Corporation, San Rafael, CA).

Results

Cell viability and NO production

The MTT assay was used to examine the possible cytotoxic effects of DA-9201. In the presence of LPS, DA-9201 at concentrations of up to 300 μg/ml did not cause any inhibition in growth of RAW 264.7 macrophages for 24 h (data not shown). On the basis of these results, concentrations of DA-9201 used in subsequent assays were 30, 100 and 300 μg/ml. LPS-induced NO production, normalized to 100% response, was 282.4 ± 7.39 μM and significantly higher than observed in control cells treated with vehicle (Figure 1). DA-9201 treatment significantly inhibited LPS-induced NO production in a dose-dependent manner (P < 0.05). Maximal effect observed at 300 μg/ml DA-9201 was a 51.8% reduction in NO production. The cell viability assay revealed that the decline in LPS-induced NO synthesis following DA-9201 treatment, was not caused by cell damage.

![Figure 1. Suppression of LPS-induced NO production by DA-9201](image-url)
iNOS protein expression

The immunoblot assay did not detect iNOS protein in untreated control RAW 264.7 macrophages while LPS in contrast, induced high level (Figure 2). In agreement with the results obtained for NO production, DA-9201 significantly suppressed (P < 0.05) iNOS protein expression in a dose-dependent manner. The iNOS protein level of cells treated with 300 μg/ml DA-9201 was 43.5% of the normalized value (100%) of cells treated with LPS alone.

Figure 2. Effect of DA-9201 on iNOS protein expression in LPS-stimulated RAW 264.7 cells
iNOS protein expression was determined by Western blot and bands were quantified by scanning densitometry. Figure shown is an autoradiograph of the gel image (A) and the relative unit between iNOS and β-actin expression (B). Values are expressed as the mean ± S.E.M. and shown as percentage of control. LPS represents stimulated cells in the absence of DA-9201. *P < 0.05 vs. LPS control. Lane 1: Control; lane 2: LPS; lane 3: LPS + DA-9201 30 μg/ml; lane 4: LPS + DA-9201 100 μg/ml; lane 5: LPS + DA-9201 300 μg/ml.

Analysis of messenger RNA levels for iNOS

mRNA levels were measured by RT-PCR to assess the effect of DA-9201 on iNOS gene expression. RAW 264.7 cells expressed high levels of iNOS mRNA when stimulated with LPS and DA-9201 significantly inhibited (P < 0.05) this LPS-induced iNOS mRNA expression in a dose-dependent manner (Figure 3). The iNOS gene expression levels of cells treated with increasing concentrations of DA-9201 were 71% (30 μg/ml), 62% (100 μg/ml) and 52% (300 μg/ml) of the normalized value of cells treated with LPS alone. RT-PCR analysis revealed that DA-9201 inhibited iNOS gene expression and NO production in a similar manner.

Figure 3. Inhibition of iNOS gene expression by DA-9201 in LPS-stimulated RAW 264.7 cell
Total RNA was isolated and iNOS mRNA was determined by RT-PCR while bands were quantified by scanning densitometry. β-actin mRNA was used as control. Figure shown is an autoradiograph of the gel image (A) and the relative unit between iNOS and β-actin expression (B). Values are expressed as the mean ± S.E.M. and shown as a percentage of control. LPS represents stimulated cells in the absence of DA-9201. *P < 0.05 vs. LPS control. Lanes 1-2: Control; lanes 3-4: LPS; lanes 5-6: LPS + DA-9201 30 μg/ml; lanes 7-8: LPS + DA-9201 100 μg/ml; lanes 9-10: LPS + DA-9201 300 μg/ml.

Plasma NO level in asthmatic lungs

NO level was significantly increased (P < 0.05) in the OVA-sensitized and -challenged mice compared to normal control mice. In contrast, mice treated with DA-9201 showed significant, dose-dependent attenuation in NO generation (Figure 4).
**Figure 4.** Effect of DA-9201 on plasma NO level
Values are expressed as mean ± S.E.M. and shown as percentage of control. *P < 0.05 vs. OVA control. HPMC; negative control, OVA; positive control.

**Western blot analysis of iNOS in lungs**

As shown in Figure 5, iNOS protein expression was very low in lungs of control mice not sensitized by OVA, while significant elevation (*P < 0.05) in iNOS protein expression was observed in OVA-sensitized and -challenged mice. Treatment with DA-9201 at the highest concentration tested, significantly reduced (*P < 0.05) iNOS protein expression by 65%.

**Figure 5.** Effect of DA-9201 on iNOS protein expression in the lung of allergen challenged allergic mice
iNOS protein expression was determined by Western blotting and bands were quantified by scanning densitometry. Figure shown is an autoradiograph of the gel image (A) and the relative unit between iNOS and β-actin expression (B). Values are shown as a percentage of control of the relative ratio between iNOS and β-actin expression and expressed as the mean ± S.E.M. *P < 0.05 vs. OVA control. *P < 0.05 vs. LPS control. Lane 1: Control; lane 2: LPS; lane 3: LPS + DA-9201 30 μg/ml; lane 4: LPS + DA-9201 100 μg/ml; lane 5: LPS + DA-9201 300 μg/ml.

**Discussion**

NO has been implicated in many physiological and pathophysiological processes and it plays a crucial role in airway functioning in both health and disease [2]. In healthy conditions, low levels of NO derived from cNOS control airway smooth muscle tone [23, 24]. However, high levels of NO derived from the iNOS isoenzyme are a major contributor to the inflammatory process seen in asthma [25]. Indeed, it is well known that iNOS expression in airway epithelium is elevated in experimentally induced asthmatic animal models and asthmatic patients compared with normal controls [7, 26]. In previous studies, DA-9201 showed anti-asthmatic effects including reduction of bronchial hyperresponsiveness, suppression of the airway eosinophilia, and attenuation of progression of airway remodeling as well as other features of pulmonary inflammation in murine models of asthma, which are related to downregulation of NF-κB expression. In this study, we provide additional data showing that DA-9201 inhibits NO production and iNOS expression in a murine macrophage cell line and in lungs of allergen-challenged allergic mice.

In the murine macrophage cell line, expression of iNOS and activation of NO production was induced by LPS, corroborating previous findings. Notably, DA-9201 clearly inhibited NO production in macrophages treated with LPS. Similar to effects observed with NO level, DA-9201 dose-dependently and significantly suppressed iNOS protein expression. Furthermore, RT-PCR analysis revealed that DA-9201 significantly decreased iNOS gene expression. Treatment with DA-9201 did not reduce cell viability, indicating that the inhibitory effect of DA-9201 on NO production and iNOS expression was not mediated by cell death. In the lungs of allergen-challenged allergic mice, DA-9201 also reduced NO production and iNOS expression, as shown by ELISA and Western blot, respectively. These results suggest that DA-9201 exerts its effect through the inhibition of iNOS mRNA transcription.

The intracellular cascade that leads to NO production is triggered by LPS in macrophages and involves a number of critical steps, including activation of NF-kB and subsequent iNOS mRNA expression [1]. The promoter of the iNOS gene contains two transcriptional regions, an enhancer, and a basal promoter [27]. There are many binding sites for transcriptional factors including kB sites and NF-kB essential for LPS-mediated NO production [28]. In addition, NF-kB is the most important transcription factor in the regulation of iNOS gene expression [29]. It has been demonstrated that some flavonoids decrease iNOS induction and inhibit NO production. Polyphenols also decrease iNOS levels by reducing the expression of iNOS mRNA, possibly through prevention of binding.
of NF-kB to the iNOS promoter, thereby inhibiting iNOS transcription [30, 31]. A similar finding has been reported for dexamethasone, which decreased the activity of the iNOS promoter and reduced the formation of cytokine-induced NF-kB complex [32]. Our previous study demonstrated that DA-9201 inhibited the DNA binding activity of NF-kB, as assessed in a nuclear extract with an EMSA assay [18]. Collectively, these reports and our results, strongly suggest that the reduced expression of iNOS and decreased NO production in LPS-stimulated cells and lung tissue following treatment with DA-9201 could be due to the inhibition of transcription of the iNOS gene, mediated by inhibition of NF-kB.

**Conclusion**

In this study, we have demonstrated that DA-9201 has anti-asthmatic activity through inhibition of NO production accompanied with suppression of iNOS in protein and gene level.

**Authors’ contributions**

Conception and design of the study: Seul Min Choi, Kyung Koo Kang
Performing experiments: Cheol Yong Lee, Min Jung Lee, Ju Mi Kim
Data analysis: Ok Jin Kim
Contribution of reagents/materials/analysis tools: Byung Mu Lee
Writing the manuscript: Min Jung Lee

All authors have approved the final manuscript.

**Funding support**

This study received no external financial support.

**Conflict of Interest**

The authors declare no potential conflicts of interest exist.

**References**


[25]. Zuo L, Koozechian MS, Chen LL. Characterization of reactive nitrogen species in allergic asthma. Ann Allergy Asthma Immunol 2014; 112: 18–22


