

NRF2 activation by 2-methoxycinnamaldehyde attenuates inflammatory responses in macrophages via enhancing autophagy flux

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A well-controlled inflammatory response is crucial for the recovery from injury and maintenance of tissue homeostasis. The anti-inflammatory response of 2-methoxycinnamaldehyde (2-MCA), a natural compound derived from cinnamon, has been studied; however, the underlying mechanism on macrophage has not been fully elucidated. In this study, LPS-stimulated production of TNF- α and NO was reduced by 2-MCA in macrophages. 2-MCA significantly activated the NRF2 pathway, and expression levels of autophagy-associated proteins in macrophages, including LC3 and P62, were enhanced via NRF2 activation regardless of LPS treatment, suggesting the occurrence of 2-MCA-mediated autophagy. Moreover, evaluation of autophagy flux using luciferase-conjugated LC3 revealed that incremental LC3 and P62 levels are coupled to enhanced autophagy flux. Finally, reduced expression levels of TNF- α and NOS2 by 2-MCA were reversed by autophagy inhibitors, such as bafilomycin A1 and NH4Cl, in LPS-stimulated macrophages. In conclusion, 2-MCA enhances autophagy flux in macrophages via NRF2 activation and consequently reduces LPS-induced inflammation. [BMB Reports 2022; 55(8): 407-412]

INTRODUCTION

Inflammation is an essential response of the body against infection, tissue injury, and cellular stress (1). Among the innate

immune cells, macrophages are rapidly recruited and mediate inflammatory responses in infectious diseases (2). Macrophages accomplish the initial microbial defense via tumor necrosis factor- α (TNF- α) and nitric oxide (NO) production (3, 4). A well-controlled inflammatory response, including adequate TNF- α and NO secretion, helps manage pathogenic microbial infection. However, excessive production of these molecules may lead to septic shock. Therefore, the control of inflammation is an important therapeutic target in infectious diseases.

The genus *Cinnamomum* include evergreen trees of about 250 species distributed worldwide. Notably, 2-methoxycinnamaldehyde (2-MCA) is a compound commonly identified in cinnamon (5); its anti-inflammatory, antioxidant, anti-osteoclastogenesis, anti-angiogenesis, and anti-aggregation activities have been studied in various types of cells, including macrophages, endothelial cells, tumor cells, and platelets (6-10). Particularly, it potentiates anti-inflammatory and antioxidant effects in macrophages. However, there is still no clear investigation regarding its mechanism of action.

Nuclear factor erythroid 2-related factor 2 (NRF2) regulates antioxidant gene expression to protect against oxidative damage induced by injury and inflammation (11). For its antioxidant response, NRF2 binds to antioxidant response elements (AREs) in gene promoter regions to produce various enzymes, such as heme oxygenase-1 (HO-1), NADH quinone oxidoreductase 1 (NQO1), and superoxide dismutase (SOD1) (12). Normally, NRF2 in the cytoplasm binds with Kelch-like ECH-associated protein 1 (KEAP1), which performs the ubiquitination and proteolysis of NRF2 (13). On the other hand, autophagosomal cargo protein P62/SEQUESTOSOME1 (P62/SQSTM1) acts on KEAP1 at the same binding site as NRF2-KEAP1 to competitively inhibit their interaction, consequently protecting NRF2 from its degradation (14). Upon stabilization, NRF2 increases the expression level of P62, which interacts with KEAP1 more frequently and further accelerates the activation of free NRF2 (15). Moreover, accumulated P62 and LC3 bind to each other to trigger the autophagosomal formation on its membrane (16).

In this study, we demonstrated that the 2-MCA-induced anti-

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inflammatory effect in macrophages is mediated via NRF2 activation. Furthermore, NRF2-mediated autophagy enhancement could modulate excessive inflammation by diminishing TNF- α and NO production.

RESULTS

2-MCA reduces TNF- α and NO secretion in macrophages

We searched several natural compounds in PubMed by using the following keywords: natural compound, inflammation, phytochemical, and TNF- α . Based on the results, six compounds were selected: β -caryophyllene, hydroxycitric acid, ruscogenin A, maslinic acid, β -myrcene, and 2-MCA (Supplementary Fig. 1). To confirm the anti-inflammatory effect of each compound in macrophages, we first examined TNF- α production together with the cell viability in lipopolysaccharide (LPS)-stimulated RAW264.7 cells and bone-marrow macrophages (BMMs). In RAW264.7 cells, two compounds, maslinic acid and 2-MCA, significantly reduced TNF- α production (Supplementary Fig. 2). However, only 2-MCA successfully reduced TNF- α production without significant cell death in LPS-exposed BMMs (Supplementary Fig. 3). Then, we further examined the concentration-dependent cytotoxicity of 2-MCA in both cells. In all tested conditions, 2-MCA did not affect cell viability (Fig. 1A). Next, concentration-dependent TNF- α and NO secretion levels were measured to ensure the anti-inflammatory effect of 2-MCA.

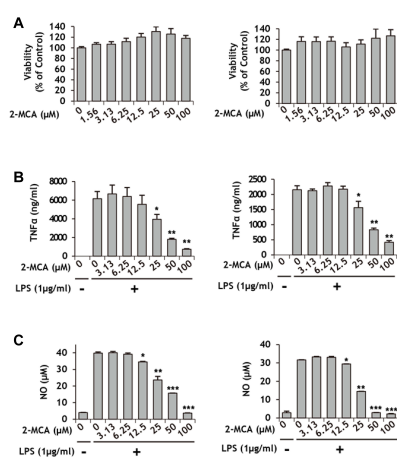


Fig. 1. Anti-inflammatory effects of 2-MCA in LPS-stimulated macrophages. (A) RAW264.7 cells (left) and BMMs (right) were incubated with 2-MCA as indicated for 24 h. The absorbance produced by MTS was measured at 490 nm. The relative cell viability is shown as the mean \pm SD ($n = 3$, with triplicates in each experiment). (B) RAW264.7 cells (left) and BMMs (right) were pre-incubated with 2-MCA as indicated for 4 h, followed by incubation with or without LPS (1 μ g/ml) for 24 h. The secreted TNF- α in the culture media was analyzed by ELISA; * $P < 0.05$, ** $P < 0.01$. (C) RAW264.7 (left) and BMMs (right) were incubated with 2-MCA as indicated for 4 h, followed by incubation with or without LPS (1 μ g/ml) for 24 h. The released NO was evaluated by Griess Reagent-based analysis; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Consistently, 2-MCA inhibited TNF- α and NO secretion in a concentration-dependent manner in LPS-stimulated macrophages, especially from concentrations of 12.5 to 100 μ M (Fig. 1B, C). As a result, we selected the 50- μ M concentration to investigate the anti-inflammatory mechanism of 2-MCA.

2-MCA maintains LPS-induced MAPK, NF- κ B, and AP-1 pathways

Next, we evaluated whether 2-MCA primarily affects the well-known LPS-related signaling pathway, including the MAPK, NF- κ B, and AP1 pathways. In the MAPK signaling pathway, phosphorylation levels of p38, ERK, and JNK were increased by LPS in both RAW264.7 cells and BMMs; all of them were unchanged by 4-h pretreatment of 2-MCA (Fig. 2A, B). Similarly, LPS-initiated degradation of I κ B and induction of c-Jun and c-Fos in NF- κ B and AP-1 signaling, respectively, were also unaffected by 4-h pretreatment of 2-MCA in macrophages (Fig. 2C, D). These data suggest that LPS-induced conventional pathways were not the primary targets in anti-inflammation by 2-MCA.

2-MCA activates NRF2/HO-1 signaling axis in LPS-stimulated macrophages

A previous study revealed that the NRF2/HO-1 pathway was activated in vascular endothelial cells by 2-MCA (17). Thus, we investigated whether 2-MCA would also activate the NRF2/HO-1

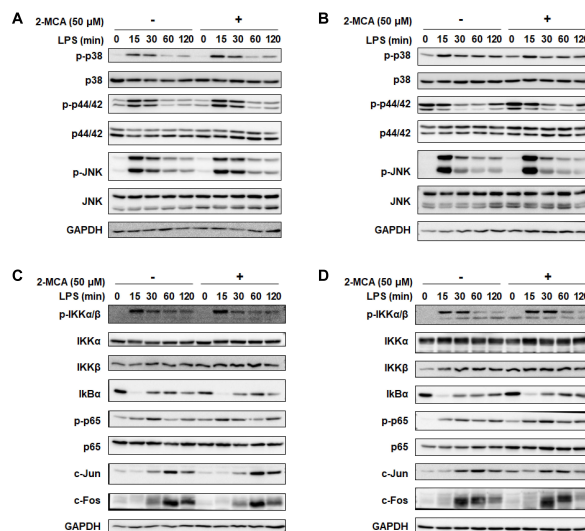


Fig. 2. Effect of 2-MCA on activation of MAPK, NF- κ B, and AP-1 pathways in RAW264.7 and BMMs. (A, B) RAW264.7 cells (A) and BMMs (B) were pre-treated with or without 2-MCA (50 μ M) for 4 h. After LPS (1 μ g/ml) treatment within the indicated time, the phosphorylation levels p38, p44/42, and JNK were analyzed using immunoblot assay. (C, D) RAW264.7 cells (C) and BMMs (D) were pre-treated with or without 2-MCA (50 μ M) for 4 h. After LPS (1 μ g/ml) treatment within the indicated time, the phosphorylation levels of IKK α , IKK β , and p65, degradation of I κ B α , and expression levels of c-Jun and c-Fos were analyzed using immunoblot assay.

pathway in macrophages. We found that 2-MCA activated the NRF2/HO-1 axis, regardless of LPS stimulation (Supplementary Fig. 4 and 5A). Interestingly, activated NRF2 by 2-MCA further increased ATF3 expression level in LPS-stimulated conditions (Fig. 3A, B). In addition, 2-MCA further increased the expression level of HO-1, which was slightly increased by LPS stimulation. On the other hand, LPS-induced NOS2 expression level was significantly decreased by 2-MCA (Fig. 3C, D).

Recently, autophagy-associated genes, such as *P62*, *LC3A/B*, and *ATG5*, have been identified as targets of NRF2 (18, 19).

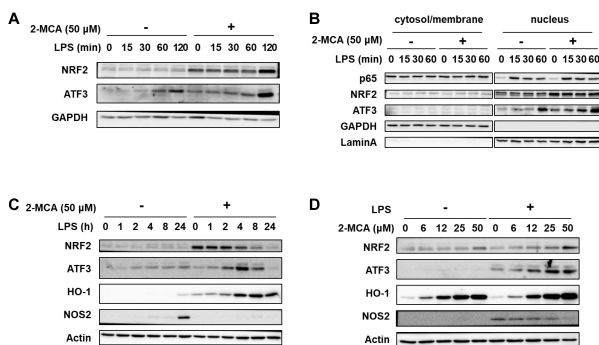


Fig. 3. Effect of 2-MCA on the expression of NRF2 and ATF3 in RAW264.7. (A) RAW264.7 cells were pre-treated with or without 2-MCA (50 μM) for 4 h. After LPS (1 μg/ml) treatment within the indicated time, the kinetic expression levels of NRF2 and ATF3 were analyzed using immunoblot assay. (B) RAW264.7 cells were pre-treated with or without 2-MCA (50 μM) for 4 h. After LPS (1 μg/ml) treatment within the indicated time, cells were fractionated into cytosol/membrane and nucleus fractions. The nucleus translocations of p65, NRF2, and ATF3 were assessed. (C) RAW264.7 cells, whether pre-treated with or without 2-MCA (50 μM) for 4 h, were stimulated with LPS (1 μg/ml) as indicated. The expression levels of NRF2, ATF3, HO-1, and NOS2 were evaluated using immunoblot assay. (D) RAW264.7 cells, whether pre-incubated with or without 2-MCA as indicated for 4 h, were stimulated with LPS (1 μg/ml) for 4 h. The expression levels of NRF2, ATF3, HO-1, and NOS2 were assessed using immunoblot assay.

Thus, we further investigated whether such genes could be increased by 2-MCA. We found that the mRNA expression levels of *Sqstm1* and *Map1lc3a*, which encode P62 and LC3A, respectively, were sufficiently increased by 2-MCA; results were also similar with ARE activity (Supplementary Fig. 5). Since P62 and LC3, important proteins for autophagy, are known to be transcribed by both NRF2 and TFEB, we examined whether 2-MCA could activate TFEB-dependent transcription in macrophages. Thus, mRNA expression levels of TFEB-target genes, *Tfeb* and *Lamp2*, were evaluated; no obvious enhancement by 2-MCA was observed (Supplementary Fig. 6A, B). Moreover, TFEB activity analysis using 5xCLEAR (Coordinated Lysosomal Expression and Regulation) reporter, which contained five repeats of TFEB-responsive elements (20), showed that rapamycin successfully increased TFEB activity; in contrast, 2-MCA did not demonstrate similar results (Supplementary Fig. 6C).

Therefore, these data suggest that 2-MCA increases NRF2 activity followed by increasing the expression levels of canonical genes, such as ATF3 and HO-1, and autophagy-associated genes, such as P62 and LC3A.

Elevated autophagy flux by 2-MCA reduces pro-inflammation in LPS-stimulated macrophages

Based on the enhanced expression of autophagy-associated genes, including P62 and LC3A, we evaluated whether their incremental expression could accompany the changes in autophagy flux. Thus, we adopted the luciferase-conjugated LC3 reporter (21). We found that the luciferase-LC3 reporter successfully chased autophagy flux by reporting inverse correlated luminescence signals with changes in autophagy flux; results revealed reduced signals by Earle's balanced salt solution (EBSS) and enhanced signals by bafilomycin A1 (BafA1), which accelerate and diminish during autophagy flux, respectively (Supplementary Fig. 7). Thus, we evaluated 2-MCA-dependent autophagy flux in LPS-stimulated RAW264.7 cells. Interestingly, LPS diminished autophagy flux, whereas 2-MCA completely enhanced autophagy flux to levels of the absence of LPS (Fig. 4A).

We investigated whether this increase in autophagy flux was

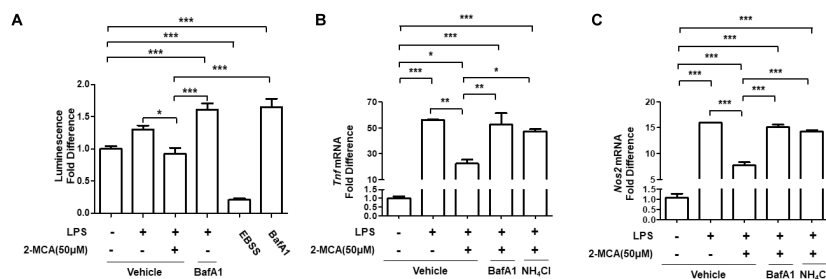


Fig. 4. Effects of 2-MCA on Inducing Autophagy Flux in RAW264.7 (A) RAW264.7 cells containing luciferase-LC3 were pre-treated with or without 2-MCA (50 μM) for 4 h. After LPS (1 μg/ml) or BafA1 (40 nM) treatment for 8 h, autophagy flux was measured using luciferase assay. (B, C) RAW264.7 cells were pre-treated with or without 2-MCA (50 μM) for 4 h. After LPS (1 μg/ml), BafA1 (40 nM), or NH₄Cl (10 mM) treatment for 8 h, the expression levels of *Tnf* and *Nos2* were analyzed using qRT-PCR assay. The results from three independent experiments are presented as means ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001.

correlated with the anti-inflammatory effect of 2-MCA. We evaluated the mRNA expression levels of *Tnf* and *Nos2* in RAW264.7 cells, which affect TNF- α and NO secretion. As a result, LPS highly elevated *Tnf* and *Nos2* mRNA expression levels, whereas these were significantly diminished by 2-MCA. In addition, BafA1, an autophagy inhibitor that impedes V-ATPase, deteriorated the 2-MCA-induced anti-inflammatory effects to levels of the LPS-only stimulation (Fig. 4B). Moreover, another well-established autophagy inhibitor, NH₄Cl, which prevents lysosomal acidification, also reversed the anti-inflammatory functions of 2-MCA in RAW264.7 cells (Fig. 4C). Therefore, these data suggest that 2-MCA potentiates anti-inflammation via NRF2-mediated enhancement in autophagy flux. Thus, incremental autophagy flux could be strategically used to deal with excessive inflammation.

DISCUSSION

The function of inflammation is to eliminate the initial etiologic cause and repair damaged tissue. This inflammatory response is initiated and amplified by secretory factors, such as TNF- α and NO, released by various immune cells, including macrophages. Therefore, regulating these factors is important to limit the excessive inflammatory response (22, 23). Infection is the entry of pathogenic microorganisms into the body, whether through wounded skin, airways, or other entry points. LPS, an endotoxin which constitutes the outer membrane of bacteria, mediates infectious diseases by triggering an innate immune response within the body (24). In macrophages, LPS activates MAPK, NF- κ B, and AP1-related pathways to secrete NO and pro-inflammatory cytokines, such as TNF- α , IL-6, and IL-1 (25-27). Secreted NO promotes the recruitment of circulating immune cells by dilating blood vessels (28). Inflammatory responses by TNF- α and IL-1 mediate recruitment, activation, and adhesion of circulating phagocytes to remove pathogenic microorganisms (29, 30). However, the uncontrolled hyperinflammatory reaction by cytokine storms and high NO secretions causes high fever, chills, and low blood pressure, which could lead to septic shock (31, 32). Therefore, the appropriate control of inflammation is crucial for treating infectious diseases.

In this regard, we measured the levels of TNF- α and NO, representative factors mediating cytokine storm and low blood pressure, to evaluate the inflammatory response in macrophages. Among the investigated natural compounds expected to regulate hyperinflammation, only 2-MCA significantly reduced TNF- α and NO levels without obvious cytotoxicity. Thus, we deeply investigated how 2-MCA reduces TNF- α and NO levels in macrophages. Our results illustrated that 2-MCA significantly reduced TNF- α and NO levels in macrophages primarily via NRF2 pathway with unaltered conventional LPS-mediated signaling, including MAPK, NF- κ B, and AP-1 pathways. Although 2-MCA did not alter the conventional LPS-mediated signaling in a short duration of 4-h pretreatment, the substantial changes due to the secondary effect of NRF2 activation in a longer du-

ration cannot be ruled out. Moreover, autophagy-related genes, such as P62 and LC3A/B, contain AREs in their promoter regions, which induce transcription upon NRF2 stabilization (18, 19). Stabilized NRF2 increases the expression level of P62, which interacts with KEAP1 more frequently and thus further accelerates the activation of free NRF2 (15). Therefore, we confirmed the activation of AREs in response to NRF2 as well as the increased expression levels of P62 and LC3A by 2-MCA. However, 2-MCA did not affect other autophagy-associated genes containing TFEB promoter regions, such as *TFEB* or *LAMP2*.

In the current study, evaluation of autophagy flux using endogenous LC3 proteins was problematic owing to their increased expression levels (Supplementary Fig. 8). In this case, it is helpful to measure autophagy flux using exogenous LC3, which shows a constant expression level. Previous reports were based on Renilla luciferase-conjugated LC3 (33). On the other hand, we adopted the firefly luciferase, which is more applicable for luminescence *in vivo* (34). Despite the usage of a different luciferase, our system also reported cellular autophagy flux. Thus, we found that LPS reduces autophagy flux and that 2-MCA successfully ameliorated this process to levels of the absence of LPS. Accordingly, enhanced autophagy suppresses LPS-induced inflammation (35). In addition, autophagy acceleration ameliorated the damage caused by sepsis (36). Together with our current results, autophagy is suspected to reduce inflammation in infectious diseases.

In conclusion, NRF2-associated autophagy flux by 2-MCA successfully reduced macrophage-mediated inflammation. Therefore, NRF2 activation may treat acute infectious diseases by reducing oxidative stress and inducing autophagy flux.

MATERIALS AND METHODS

Cell culture

RAW264.7 cells (Korean Cell Line Bank, Seoul, Korea) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA) at 37°C containing 5% CO₂, with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco). BMMs were isolated from femurs of mice and cultured in DMEM/F12 medium (GenDEPOT, Barker, TX) at 37°C containing 5% CO₂, supplemented with 10% FBS, 20% L929-conditioned medium, 1 × GlutaMAX (Gibco), and 1% penicillin/streptomycin.

Measurement of TNF- α secretion

To evaluate the effect of 2-MCA, RAW264.7 cells and BMMs were cultured in 96-well plates at 1×10^4 and 2×10^4 cells/well for 12 h. Then, the plates were pre-treated with serially diluted 2-MCA. After a 4-h culture, media was added with or without LPS (1 μ g/ml). After a 24-h incubation period, the cultured supernatant was collected. The amount of secreted TNF- α was measured using Mouse TNF- α ELISA MAX kit (BioLegend, San Diego, CA) according to the manufacturer's instructions.

NO assay

To evaluate the effect of 2-MCA, RAW264.7 cells and BMMs were plated into 60-mm dishes at 5×10^5 and 1×10^6 cells/dish for 12 h and were then pre-treated with serially diluted 2-MCA. After a 4-h culture, media was added with or without LPS (final concentration, 1 $\mu\text{g/ml}$). After a 24-h incubation period, the cultured supernatant was collected. Cells were then removed via centrifugation at 500 g for 3 min. Then, 100 μl of the cultured supernatant was mixed with 100 μl of Griess reagent (1:1 mixture of 1% sulfanilamide in 30% acetate and 0.1% N-1-naphthyl ethylenediamine dihydrochloride in 60% acetate) at room temperature for 10 min. The absorbance of the incubated samples was measured at 540 nm using a microplate reader. A standard curve drawn with known concentrations of sodium nitrite was applied to calculate the concentration of nitrite, which is the stable end-product.

Luminescence-based autophagy flux analysis

RAW264.7 cells stably expressing luciferase-conjugated LC3 (Supplementary Fig. 9) were seeded into a white, 96-well plate at 2×10^4 cells/well for 12 h and were then pre-treated with serially diluted 2-MCA. After a 4-h culture, media was added with or without LPS (1 $\mu\text{g/ml}$) and BafA1 (40 nM). After an 8-h incubation period, the medium was changed to phenol red-free DMEM, which contained *D*-luciferin (100 $\mu\text{g/ml}$). Upon further incubation at 37°C for 15 min, luminescence was measured using a microplate reader (Tecan Spark, Tecan, Switzerland).

Statistical analysis

The results are expressed as mean \pm standard deviation (SD). Statistical analysis was performed using an unpaired Student's *t*-test or a one-way analysis of variance with Tukey's post-hoc test using GraphPad Prism software (GraphPad Software, La Jolla, CA). The minimum significance level was set at $P = 0.05$; at least three independent replications were performed for each experiment.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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