**Solanum nigrum** induces macrophage activation through TLR4-mediated activation of JNK and macrophage autophagy through TLR4-mediated activation of p38 and JNK

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**ABSTRACT**

In this study, we investigated the effect of *Solanum nigrum* aerial parts (SNAP) on macrophage activation and macrophage autophagy in RAW264.7 cells. SNAP increased the production of immunostimulatory factors and phagocytosis in RAW264.7 cells. TLR4 inhibition blocked SNAP-mediated production of immunostimulatory factors. In addition, the JNK inhibition reduced the SNAP-mediated production of immunostimulatory factors, and the SNAP-mediated JNK activation was blocked by the TLR4 inhibition. SNAP activated macrophage autophagy. TLR4 inhibition blocked SNAP-mediated macrophage autophagy and inhibition of p38 and JNK attenuated SNAP-mediated macrophage autophagy. These findings indicate that SNAP may induce TLR4/JNK-mediated macrophage activation and TLR4/p38 and JNK-mediated macrophage autophagy.

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**1. Introduction**

Improving the immune system and immune response of the human body through immunostimulants is important for protecting the human body from the occurrence of diseases and restoring health (Batatinha et al., 2019). Macrophages are known to participate in the immune response of the human body such as innate and adaptive immune responses directly or indirectly (Medzhitov & Janeway, 2000; Zhang et al., 2019). Activated macrophages remove foreign pathogens through phagocytosis (Liu et al., 2016). In addition, various immunostimulatory factors such as nitric oxide (NO), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) secreted by activated macrophages contribute to the activation of T-cells and B-cells involved in adaptive immune responses.
Therefore, the development of natural immunostimulants capable of activating macrophages has been required.

One of the Solanum plants in the Solanaceae family, it has been reported that Solanum nigrum exhibits various pharmacological activities such as hepatoprotective activity, anticancer activity, antulcer activity, antidiarrheal activity, anti-inflammatory activity, larvicidal activity, anticonvulsant activity, anti-HCV activity, analgesic activity, and antioxidant activity (Mani et al., 2022). Recently, it was reported that polysaccharides isolated from Solanum nigrum increased the production of immunomodulatory factors such as NO, TNF-α, and IL-6 in murine macrophages, RAW264.7 cells (Pu et al., 2020; Razali et al., 2014). However, research on how Solanum nigrum activates macrophages is insufficient. Thus, in this study, we report that Solanum nigrum activates macrophages through TLR4/JNK signalling. And additionally, we report that Solanum nigrum induces macrophage autophagy through TLR4/JNK signalling.

2. Materials and methods

2.1. SNAP preparation

Aerial parts of Solanum nigrum were purchased from a local market located in Dongdae-mun-gu, Seoul. After grinding the purchased aerial parts of Solanum nigrum, 10 g of the powder was immersed in 200 ml of distilled water and left at 80 °C for 6 h. After extraction for 6 h, the extracts were centrifuged at 15,000 rpm at 4 °C for 10 min, and then the clear supernatant was freeze-dried. The freeze-dried extracts from aerial parts of Solanum nigrum (SNAP) were dissolved in distilled water for treatment of the cells and stored at −80 °C.

2.2. Cell line and cell culture

RAW264.7 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). RAW264.7 cells were cultured in a CO₂ incubator (5% CO₂, 37 °C, Humidified atmosphere) with DMEM/F-12 medium containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

2.3. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

To evaluate the cytotoxicity of SNAP, an MTT assay was performed. Briefly, RAW264.7 cells (5×10⁵ cells/ml) cultured for 24 h in a 96-well plate were treated with SNAP in the presence or absence of LPS, and then further cultured in an incubator (5% CO₂, 37 °C, Humidified atmosphere) for 24 h. After 24 h, 50 μl of MTT solution (1 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) was added to each well and then incubated in an incubator (5% CO₂, 37 °C, Humidified atmosphere) for 4 h. After 4 h, the culture medium was removed, and then 100 μl of dimethyl sulfoxide was added to each well to dissolve the purple formazan. The absorbance of dissolved purple formazan was measured at 570 nm using a UV/Visible spectrophotometer (Human Cop., Xma-3000PG, Seoul, Korea).
2.4. Griess assay

To evaluate the effect of SNAP on NO production, a Griess assay was performed. Briefly, RAW264.7 cells (5×10^5 cells/ml) cultured for 24 h in a 96-well plate were treated with SNAP in the presence or absence of LPS, and then further cultured in an incubator (5% CO₂, 37 °C, Humidified atmosphere) for 24 h. After 24 h, the cell culture medium and Griess solution (Sigma-Aldrich) were mixed in a 1:1 ratio and left at room temperature for 15 min. After 15 min, the absorbance of the mixture was measured at 540 nm using a UV/Visible spectrophotometer (Human Cop., Xma-3000PC).

2.5. Neutral red assay

To evaluate the effect of SNAP on phagocytosis, a neutral red assay was performed. Briefly, RAW264.7 cells (5×10^5 cells/ml) cultured for 24 h in a 96-well plate were treated with SNAP, and then further cultured in an incubator (5% CO₂, 37 °C, Humidified atmosphere) for 24 h. After 24 h, the cells were cultured with 0.01% of the neutral red solution in an incubator (5% CO₂, 37 °C, Humidified atmosphere) for 2 h. After 2 h, the cells were washed with 1 X PBS three times and then treated with lysis buffer (50% ethanol acid: 1% acetic acid = 1:1) at room temperature for 2 h to elute the neutral red adsorbed on the cells. The absorbance of the eluted neutral red was measured at 540 nm using a UV/Visible spectrophotometer (Human Cop., Xma-3000PC).

2.6. SDS-PAGE and western blot analysis

Protein changes in SNAP-treated RAW264.7 cells were analyzed by Western blot analysis using specific antibodies such as p-JNK (#9251), LC3 (#2775), p62/SQSTM1 (#39749) and β-actin (#5125) purchased from Cell Signalling (Beverly, MA, USA). The total protein extracted from RAW264.7 cells was quantified using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA USA). The protein (30 μg) was separated on 12% SDS-PAGE and subsequently transferred to a nitrocellulose membrane (Thermo Fisher Scientific). After blocking at room temperature for 1 h, the membranes were incubated with the primary antibodies (1:1,000) at 4 °C overnight. After that, the membranes were incubated with anti-rabbit IgG, HRP-linked antibody as the secondary antibody (#7074, Cell Signalling) at room temperature for 1 h. After treating ECL Western blotting substrate on the membrane, the protein band was visualised using LI-COR C-DiGit Blot Scanner. Quantitative analysis of visualised protein bands was performed using the software UN-SCAN-IT gel version 5.1 (Silk Scientific Inc. Orem, UT, USA).

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

mRNA changes in SNAP-treated RAW264.7 cells were analyzed by RT-PCR. Total mRNA from RAW264.7 cells was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Extracted mRNA was quantified using a GeneQuant™ 1300 (Biochrom, Cambridge, England). Total mRNA (1 μg) was reverse transcribed into cDNA using a Verso cDNA Kit (Thermo Scientific). The PCR amplification of the cDNA was
performed using a PCR Master Mix Kit (Promega, Madison, WI, USA). The sequences of primers used in the PCR amplification of the cDNA were as follows: iNOS: forward 5’-tttgtgcatgacctgacctgaa-3’ and reverse 5’-gacctttgcattgcaagc-3’, COX-2: forward 5’-gaactggctgtctgtggc-3’ and reverse 5’-caccatactgcagctcggaa-3’, IL-1β: forward 5’-gcagccatcattcact-3’ and reverse 5’-ccaaagcagtcattt-3’, IL-6: forward 5’-gaggtaccactcccaagacc-3’ and reverse 5’-aatgtgcatctggtctttataca-3’, TNF-α: forward 5’-tggaactggcagaagggca-3’ and reverse 5’-tcctctccacctgggtt-3’, TLR2: forward 5’-gcctgacctacc-3’ and reverse 5’-cagcagcagaatagc-3’, TLR4: forward 5’-tgctcctccacttggtggtt-3’ and reverse 5’-gcctctccacctacc-3’, GAPDH: forward 5’-gcctgacctggcc-3’ and reverse 5’-aactccagcgaatcagc-3’. The PCR bands were visualised using agarose gel electrophoresis. The density of the PCR bands was calculated using the software UN-SCAN-IT gel version 5.1 (Silk Scientific Inc. Orem).

2.8. Statistical analysis

All the data are shown as mean ± SD (standard deviation). Statistical significance was determined by Student’s t-test. Differences with *P < 0.05 and **P < 0.05 were considered statistically significant.

3. Results

3.1. Effect of SNAP on the production of immunostimulatory factors in RAW264.7 cells

To evaluate whether SNAP affects the production of immunostimulatory factors such as NO, iNOS, COX-2, IL-1β, IL-6, and TNF-α in RAW264.7 cells, we performed Griess assay and RT-PCR analysis. As shown in Figure 1A, SNAP increased NO production in RAW264.7 cells in a dose-dependent manner. In addition, the mRNA expression of iNOS, COX-2, IL-1β, IL-6, and TNF-α was increased in SNAP-treated RAW264.7 cells (Figure 1B). Moreover, activation of phagocytosis was observed in SNAP-treated RAW264.7 cells (Figure 1C). Excessive immunostimulatory factors secreted by macrophages cause cytotoxicity, so it was verified whether SNAP was cytotoxic to RAW264.7 cells. However, as shown in Figure 1D, no cytotoxicity of SNAP to RAW264.7 cells was observed.

3.2. Effect of TLR2 and TLR4 on SNAP-mediated production of immunostimulatory factors in RAW264.7 cells

To investigate the effect of Toll-like receptor (TLR) 2 and 4 on the production of immunostimulatory factors by SNAP in RAW264.7 cells, the cells were pretreated with C29 (TLR2 inhibitor, Sigma-Aldrich) or TAK-242 (TLR4 inhibitor, Sigma-Aldrich) for 2 h and then co-treated with SNAP for 24 h. As shown in Figure 2(A and B), SNAP increased the production of NO, iNOS, COX-2, IL-1β, IL-6, and TNF-α regardless of whether TLR2 was inhibited by C29. However, the TLR4 inhibition by TAK-242 attenuated the production of NO, iNOS, IL-1β, and IL-6, but not COX-2 and TNF-α.
3.3. Effect of MAPK signalling on SNAP-mediated production of immunostimulatory factors in raw264.7 cells

To investigate the effect of mitogen-activated protein kinase (MAPK) signalling on the NO production by SNAP in RAW264.7 cells, the cells were pretreated with PD98059 (ERK1/2 inhibitor, Sigma-Aldrich), SB203580 (p38 inhibitor, Sigma-Aldrich) or SP600125 (JNK inhibitor, Sigma-Aldrich) for 2 h and then co-treated with SNAP for 24 h. As shown in Figure 3A, SNAP increased the NO production regardless of whether ERK1/2 and p38 were inhibited by PD98059 and SB203580. However, SNAP-

Figure 1. Effect of SNAP on macrophage activation in RAW264.7 cells. RAW264.7 cells were treated with SNAP for 24 h. NO level (A), mRNA level (B), phagocytic activity (C) and cell viability (D) were measured by Griess assay, RT-PCR, neutral red assay, and MTT assay, respectively.

Figure 2. Effect of TLR2 and TLR4 on SNAP-mediated production of immunostimulatory factors in RAW264.7 cells. RAW264.7 cells were pretreated with C29 (TLR2 inhibitor, 100 μM) or TAK-242 (TLR4 inhibitor, 5 μM) for 2 h and co-treated with SNAP (100 μg/ml) for 24 h. NO level (A) and mRNA level (B) were measured by Griess assay and RT-PCR, respectively.
mediated NO production was blocked in RAW264.7 cells in which JNK was inhibited by SP600125. Thus, we investigated the effect of JNK on the SNAP-mediated expression of iNOS, COX-2, IL-1β, IL-6, and TNF-α. As a result (Figure 3B), the expression of iNOS, IL-1β, and IL-6 by SNAP was decreased but not COX-2 and TNF-α in presence of SP600125. And we investigated the effect of SNAP on JNK activation and the effect of TLR4 on SNAP-induced JNK activation. As shown in Figure 3(C and D), SNAP increased the phosphorylation level of JNK and TLR4 inhibition by TAK-242 blocked SNAP-induced phosphorylation of JNK in RAW264.7 cells.

3.4. Effect of SNAP on macrophage autophagy in raw264.7 cells

To evaluate the effect of SNAP on the autophagy of RAW264.7 cells, the levels of microtubule-associated protein 1A/1B-light chain 3 (LC3) and p62/Sequestosome 1 (SQSTM1) were investigated using Western blot analysis. As shown in Figure 4A, LC3-II increased from 15 min to 3 h after SNAP treatment and decreased thereafter. On the other hand, the level of p62/SQSTM1 increased from 3 h to 6 h after SNAP treatment and decreased thereafter. SNAP dose-dependently increased the level of LC3-II and p62/SQSTM1 (Figure 4B). SNAP increased the level of p62/SQSTM1 regardless of whether TLR2 was inhibited by C29. However, the increase in the level of p62/SQSTM1 by SNAP was attenuated in the TLR4 inhibition by TAK-242 (Figure 4C). In addition, SNAP increased the level of p62/SQSTM1 regardless of whether ERK1/2 was inhibited by PD98059. However, SNAP-mediated increase of p62/SQSTM1 level was blocked in...
RAW264.7 cells in which p38 and JNK were inhibited by SB203580 and SP600125, respectively.

4. Discussion

Since the improvement of the immune response plays a pivotal role in preventing or treating diseases, interest in the search and development of agents capable of inducing immunostimulation is increasing (Hong et al., 2017).

Macrophages, one of the immune cells, are involved in both the innate immune response, which is an antigen-specific immune response and the adaptive immune response, which is an antigen-specific immune response (Chen et al., 2012). Activated macrophages secrete various immunostimulatory factors such as NO, iNOS, COX-2, IL-1β, IL-6, and TNF-α (Medzhitov, 2007). NO kills virus-infected cells, cancer cells, and foreign pathogens (Yin et al., 2019). Thus, NO secreted by macrophages is used as a good indicator of the activation state of macrophages (Lee et al., 2012). It is known that iNOS plays an important role not only in NO production but also in normal immune function and host defense against foreign pathogens (Apetoh et al., 2017). IL-1β increases the survival of T cells, one of the adaptive immune cells, and induces activation of innate immune cells. In addition, IL-1β promotes the antigen-presenting activity of macrophages (Seillet et al., 2014). IL-6 promotes the differentiation of T
cells and B cells, and the phagocytosis of macrophages (Neurath, 2014; Wang et al., 2018). Since TNF-α contributes to the removal of foreign pathogens by activating the phagocytosis of macrophages, TNF-α production is known to be beneficial for host defense in the initial infection (Barbara et al., 1996). In this study, we observed that SNAP increased the production of NO, iNOS, COX-2, IL-1β, IL-6, and TNF-α, and activated the phagocytosis in RAW264.7 cells. These results indicate that SNAP may induce macrophage activation, which may contribute to enhancing innate and adaptive immune responses.

It has been reported that macrophage TLRs recognising pathogen-associated molecular patterns of foreign pathogens directly contribute to the activation of host defense mechanisms (Medzhitov, 2007). Activation of TLRs not only stimulates the innate immune response by secreting NO, but also stimulates the adaptive immune response by inducing the secretion of IL-1β, IL-6, and TNF-α in macrophages (Medzhitov, 2007). Among TLRs, TLR4 has been used as a major target to improve immune function in patients with immune disorders (Li et al., 2019). Some drugs targeting TLR4 are being used in clinical practice (Li et al., 2019). In this study, we observed that TLR2 inhibition did not affect the SNAP-mediated production of NO, iNOS, COX-2, IL-1β, IL-6, and TNF-α, but TLR4 inhibition blocked the production of NO, iNOS, IL-1β, and IL-6. These results indicate that SNAP-mediated production of NO, iNOS, IL-1β, and IL-6 may be dependent on TLR4 stimulation. However, we observed that the production of COX-2 and TNF-α by SNAP did not change upon TLR4 inhibition, which indicates that the production of COX-2 and TNF-α by SNAP may occur through a mechanism separated from TLR4 stimulation.

The MAPK signalling pathway, which plays an important role in the immune system, is known as one of the signalling pathways that are activated through the stimulation of TLR4 (Xu et al., 2020). Activation of the MAPK signalling pathway induces macrophage activation and promotes macrophages-mediated secretion of immunostimulatory factors such as NO, iNOS, COX-2, IL-1β, IL-6, and TNF-α (Li et al., 2017). In this study, inhibition of JNK blocked the production of NO, iNOS, IL-1β, and IL-6, but not COX-2 and TNF-α. In addition, SNAP phosphorylated JNK and TLR4 inhibition blocked SNAP-mediated phosphorylation of JNK. These results suggest that SNAP may induce macrophage activation through TLR4/JNK signalling pathway.

The adjuvant plays a role in enhancing the adaptive immune response, which is an antigen-specific immune response, by stimulating T cells and B cells (Chou et al., 2020). Many adjuvants currently developed and used in clinical applications activate pattern recognition pattern receptors that recognise foreign pathogens (Chou et al., 2020). Among various pattern recognition pattern receptors, TLR4 is an important target for the development of adjuvants included in vaccines (Toussi & Massari, 2014). Lipopolysaccharide (LPS) with potent immunostimulatory activity through TLR4 stimulation has been used in many vaccine clinical trials, but the toxicity of LPS has limited use (Toussi & Massari, 2014). In a recent study, it was reported that autophagy can increase T cell response by regulating the functions of antigen-presenting cells and T cells (Merkley et al., 2018). It has been reported that activation of macrophage autophagy through TLR4 stimulation enhances innate and adaptive immune responses by increasing macrophage antigen processing and presentation. (Chou et al., 2020). In this study, we observed that SNAP increased the levels of LC3-II and p62/SQSTM1, which are known as major indicators of autophagy in RAW264.7 cells. In addition, the inhibition of TLR4, p38, and JNK
attenuated the SNAP-mediated increase of p62/SQSTM1 level in RAW264.7 cells. These results indicate that SNAP may activate macrophage autophagy through TLR4-mediated activation of p38 and JNK.

5. Conclusion

In conclusion, SNAP increased macrophage-released immunostimulatory factors through TLR4-mediated activation of JNK and activated macrophage autophagy through TLR4-mediated activation of p38 and JNK in RAW264.7 cells. These findings indicate that SNAP has immunostimulatory activity and vaccine adjuvant activity. In addition, it is believed that SNAP can be developed as an agent related to immune-enhancement and vaccine adjuvant in the future. However, animal-based preclinical studies related to SNAP’s immunostimulatory activity and vaccine adjuvant activity are required since this study was cell-based.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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