



## ORIGINAL ARTICLE

# Metformin or $\alpha$ -Lipoic Acid Attenuate Inflammatory Response and NLRP3 Inflammasome in BV-2 Microglial Cells

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## BV-2 미세아교세포에서 메트포르민 또는 알파-리포산의 염증반응과 NLRP3 인플라마솜 약화에 관한 연구

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## ARTICLE INFO

Received August 18, 2020  
Revised 1<sup>st</sup> August 25, 2020  
Revised 2<sup>nd</sup> August 31, 2020  
Accepted August 31, 2020

## Key words

 $\alpha$ -lipoic acid  
Amyloid beta  
Metformin  
NLRP3 inflammasome  
S100A9

## ABSTRACT

Alzheimer's disease (AD) is a chronic and progressive neurodegenerative disease that can be described by the occurrence of dementia due to a decline in cognitive function. The disease is characterized by the formation of extracellular and intracellular amyloid plaques. Amyloid beta ( $A\beta$ ) is a hallmark of AD, and microglia can be activated in the presence of  $A\beta$ . Activated microglia secrete pro-inflammatory cytokines. Furthermore, S100A9 is an important innate immunity pro-inflammatory contributor in inflammation and a potential contributor to AD. This study examined the effects of metformin and  $\alpha$ -LA on the inflammatory response and NLRP3 inflammasome activation in  $A\beta$ - and S100A9-induced BV-2 microglial cells. Metformin and  $\alpha$ -LA attenuated inflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6). In addition, metformin and  $\alpha$ -LA inhibited the phosphorylation of JNK, ERK, and p38. They activated the nuclear factor kappa B (NF- $\kappa$ B) pathway and the NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome. Moreover, metformin and  $\alpha$ -LA reduced the marker levels of the M1 phenotype, ICAM1, whereas the M2 phenotype, ARG1, was increased. These findings suggest that metformin and  $\alpha$ -LA are therapeutic agents against the  $A\beta$ - and S100A9-induced neuroinflammatory responses.

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## INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease, in which the onset and progression of dementia is determined by synapse loss and neuronal death. It is

pathologically characterized by abnormal protein accumulation, including extracellular and intracellular aggregation of amyloid beta ( $A\beta$ ) protein and hyperphosphorylated tau protein [1]. Inflammation plays a fundamental role in AD progression since microglia are able to be activated in the presence of  $A\beta$  [2]. When microglia are active in AD, they play a dual role. On the one hand, chronically activated microglia contribute to the release of pro-inflammatory cytokines, which initiates a pro-inflammatory cascade and subsequently

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induces neuronal death. These microglia are referred to as the M1 type of microglia. On the other hand, activated microglia can lead to decreased A $\beta$  accumulation through phagocytosis or clearance; these microglia are called the M2 type of microglia [3].

In addition to A $\beta$ , other danger-associated molecular patterns (DAMPs) including high mobility group box 1 (HMGB1) and the S100 family are known to be involved in neuroinflammation in neurodegenerative disorders such as AD [4, 5]. Among them, S100A9, a calcium-binding protein, has been suggested to act as an inflammation marker and to be an important innate immunity pro-inflammatory contributor [6]. Many studies have found that S100A9 is a potential contributor to AD development. In the AD brain, secretion of S100A9 promotes neurotoxicity and the formation of amyloid plaques due to its inherent amyloidogenicity [7]. Hence, in order to investigate therapeutic strategies to treat AD, molecules such as S100A9, in addition to A $\beta$ , should also be considered a target, and a modulator that attenuates activated microglia may be a suitable target.

Recent studies have suggested that autophagy and antioxidants play an important role in AD [8]. Metformin is considered an autophagy agent and  $\alpha$ -lipoic acid ( $\alpha$ -LA) is a strong antioxidant agent. Metformin, a biguanide-class anti-diabetic, acts on several major signaling pathways including AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR), and inflammatory signaling. Metformin activates the autophagic pathway by the activation of AMPK and subsequent suppression of mTOR. As a result, most A $\beta$ s are removed by autophagy [9].  $\alpha$ -LA has been suggested to have anti-dementia and anti-AD properties due to several actions, such as scavenging the reactive oxygen species (ROS), reducing inflammatory processes, and regenerating endogenous antioxidants [10]. To attenuate more effectively against A $\beta$ - and S100A9-induced microglia inflammation, we confirmed the effects of metformin and  $\alpha$ -LA.

Inflammasomes are associated with a number of auto-inflammatory and autoimmune diseases that can

cause inflammatory diseases. Among inflammasomes, the NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome has been most widely characterized because it is involved in several human diseases [11]. The NLRP3 inflammasome oligomerizes in response to DAMPs or pathogen-associated molecular patterns (PAMPs) to form apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC). This can convert pro-caspase-1 into its cleaved caspase-1 form, which plays a role in the maturation of pro-IL-1 $\beta$  and pro-IL-18 into mature forms [12].

In this study, we aimed to investigate the mechanisms underlying inflammatory responses and NLRP3 inflammasome activation by metformin and  $\alpha$ -LA against A $\beta$ - and S100A9-induced microglial activation.

## MATERIALS AND METHODS

### 1. Materials

Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) were provided from Corning (USA). Penicillin (100 U/mL)/streptomycin (100  $\mu$ g/mL) and heat-inactivated fetal bovine serum (FBS) were purchased from Gibco (Life Technologies Inc., Gaithersburg, USA). A $\beta$ <sub>25-35</sub>, metformin, and  $\alpha$ -lipoic acid were obtained from Sigma-Aldrich (St. Louis, USA). The S100A9 protein was synthesized and purified as previously described [13]. TNF- $\alpha$  was measured by an enzyme-linked immunosorbent assay (ELISA) using the mouse TNF- $\alpha$  DuoSet ELISA kit (R&D systems, Minneapolis, USA), according to the manufacturer's instructions. The following antibodies were used for Western blot assays: anti-pERK1/2, anti-ERK1/2, anti-p-p38, anti-p38, anti-NF- $\kappa$ B, and anti-NLRP3 from Cell Signaling Technology (USA); and anti-p-JNK, anti-JNK, anti-Lamin-B1, and anti- $\beta$ -actin from Santa Cruz Biotechnology (Dallas, USA).

### 2. Cell culture

BV-2 microglial cells were obtained from the Department of Biochemistry and Molecular Biology, University

of Ulsan College of Medicine (Seoul, South Korea) and cultured in DMEM supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), and 10% FBS in a humidified incubator at 37°C in 5% CO<sub>2</sub>.

### 3. Enzyme-linked immunosorbent assay (ELISA)

BV-2 microglial cells were seeded in a 6-well plate ( $5 \times 10^5$  cells/well) and incubated in metformin (1 mM) or in  $\alpha$ -LA (500 µM) with A $\beta$  (20 µM) and S100A9 (10 µg/mL). After incubation for 24 h, the cultured cell supernatant was collected and the level of TNF- $\alpha$  was measured with the mouse TNF- $\alpha$  DuoSet ELISA kit, according to the manufacturer's instructions at 450 nm absorbance value using an ELISA microplate reader (Molecular Device, Sunnyvale, USA).

### 4. Western blot analysis

Following treatment with metformin (1 mM) or  $\alpha$ -LA (500 µM) with A $\beta$  (20 µM) and S100A9 (10 µg/mL) for 24 h, BV-2 microglial cells were lysed using a RIPA buffer containing phosphatase and a protease inhibitor. Protein concentrations were determined using the Lowry protein assay. Thereafter, equal amounts of proteins were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto a nitrocellulose membrane. The membranes were then incubated overnight at 4°C with the primary antibody. The membranes were washed and then incubated with either the anti-rabbit IgG HRP or the anti-mouse IgG HRP secondary antibodies for 1 h at room temperature. The protein bands on the membrane were developed with a chemiluminescent ECL reagent using an enhanced chemiluminescence detection system (Vilber Lourmat, Marne-la-Vallée, France).

### 5. Real-time quantitative PCR (qPCR) analysis

RNA was extracted from BV-2 microglial cells using Trizol according to the manufacturer's instructions. The RNA was then quantified using NanoDrop<sup>TM</sup> One, and cDNA was synthesized using DiaStar<sup>TM</sup> 2X RT Pre-Mix (Biofact). Real-time PCR was conducted in a

CFX96<sup>TM</sup> real-time system employing the SsoAdvanced<sup>TM</sup> Universal SYBR Green Supermix for cDNA quantification. The following primers were used: IL-6 (Forward: 5'-GTCCTTCCTACCCCAATTTCCTCA-3', Reverse: 5'-TAAC-GCACTAGGTTTGGCCGA-3'), IL-1 $\beta$  (Forward: 5'-ATGC-CACCTTTTGACAGTGATG-3', Reverse: 5'-TGTCCT-GCTGCGAGATTTGA-3'), ICAM-1 (Forward: 5'-AGC-ACCTCCCCACCTACTTT-3', Reverse: 5'-AGCTTGAC-GACCCTTCTAA-3'), ARG-1 (Forward: 5'-ATGGGCAA-CCTGTGTCCTTT-3', Reverse: 5'-TCTACGTCTCGC-AAGCCAAT-3') and GAPDH (Forward: 5'-TCACCA-CCATGGAGAAGGC-3', Reverse: 5'-GCTAAGCAGTTG-GTGGTGCA).

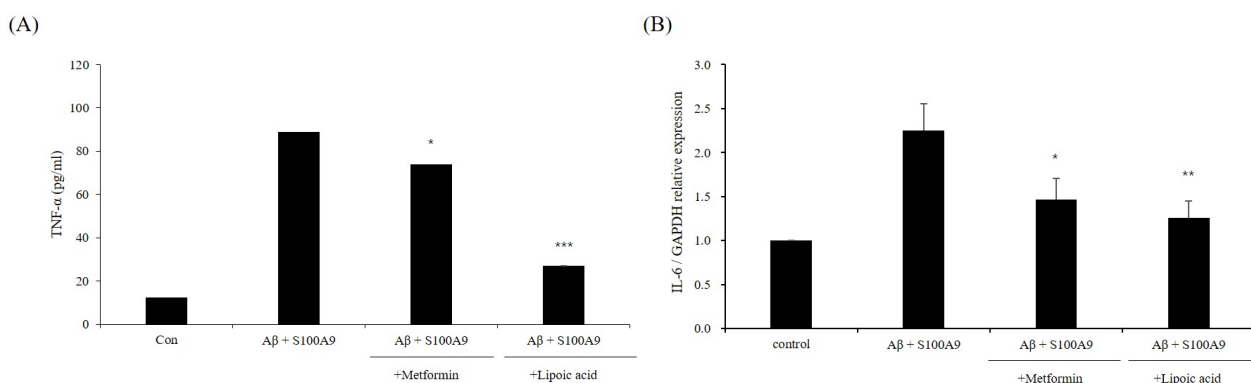
### 6. Statistical analyses

All data are presented as mean  $\pm$  standard error and are representative of three independent experiments. The SPSS statistical software package (Version 18.0, Chicago, USA) was used for the analysis of variance (ANOVA), as appropriate. Additionally, individual differences among each group were compared through one-way ANOVA, followed by Scheffe and Dunnett T3 methods. Results with  $P < 0.05$  were considered statistically significant.

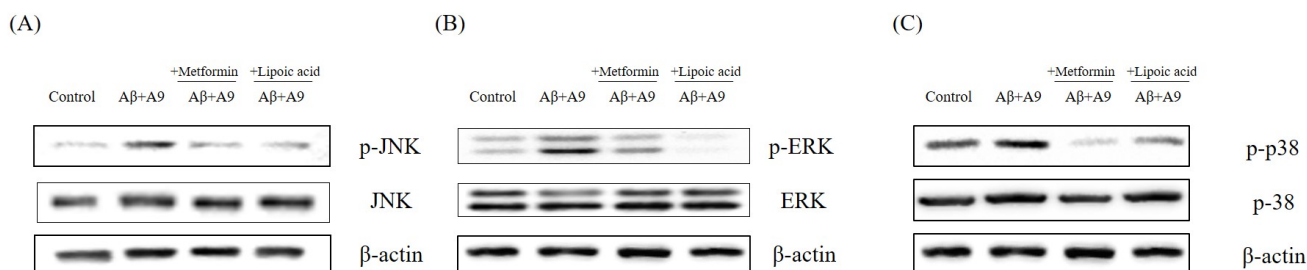
## RESULTS

### 1. Metformin and $\alpha$ -LA inhibit the secretion of pro-inflammatory cytokines in A $\beta$ - and S100A9-induced BV-2 microglial cells

To confirm the effect of metformin or  $\alpha$ -LA on A $\beta$ - and S100A9-induced activation of microglia, BV-2 microglial cells were treated with metformin (1 mM) or  $\alpha$ -LA (500 µM) with A $\beta$  (20 µM) and S100A9 (10 µg/mL) for 24 h [14, 15]. It is known that activated BV-2 microglial cells release pro-inflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), and interleukin-1beta (IL-1 $\beta$ ). As shown in Figure 1, A $\beta$  and S100A9 induced an upregulation of TNF- $\alpha$  secretion and IL-6 mRNA level from BV-2 microglial cells but the upregulation was reduced by metformin



**Figure 1.** Metformin and  $\alpha$ -LA prohibit the expression of pro-inflammatory cytokine in A $\beta$ - and S100A9- treated BV-2 microglial cells. BV-2 microglial cells were treated with metformin (1 mM) or  $\alpha$ -LA (500  $\mu$ M) with A $\beta$  (20  $\mu$ M) and S100A9 (10  $\mu$ g/mL) for 24 h. After stimulation, the supernatants and cells were harvested and analyzed by ELISA or RT-qPCR. (A) Cytokine production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was evaluated using ELISA, and (B) interleukin-6 (IL-6) was evaluated using RT-qPCR. Data from three independent experiments are presented as means  $\pm$  S.D. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 are related with A $\beta$  and S100A9 treated cells.



**Figure 2.** Effects of metformin and  $\alpha$ -LA on MAPKs signaling in A $\beta$ - and S100A9-induced BV-2 microglial cells. BV-2 microglial cells were incubated for 24 h with metformin (1 mM) or  $\alpha$ -LA (500  $\mu$ M) with A $\beta$  (20  $\mu$ M) and S100A9 (10  $\mu$ g/mL). Cells were harvested and lysed to whole lysates with RIPA buffer. The protein expression level of (A) p-JNK, JNK, (B) p-ERK1/2, ERK1/2, (C) p-p38 and p-38 was detected by Western blotting. Data from three independent experiments are presented as means  $\pm$  S.D. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 are related with A $\beta$ - and S100A9-induced cells.

and  $\alpha$ -LA. These results demonstrate that metformin and  $\alpha$ -LA reduced the TNF- $\alpha$  and IL-6 expression in A $\beta$ - and S100A9-stimulated BV-2 microglial cells.

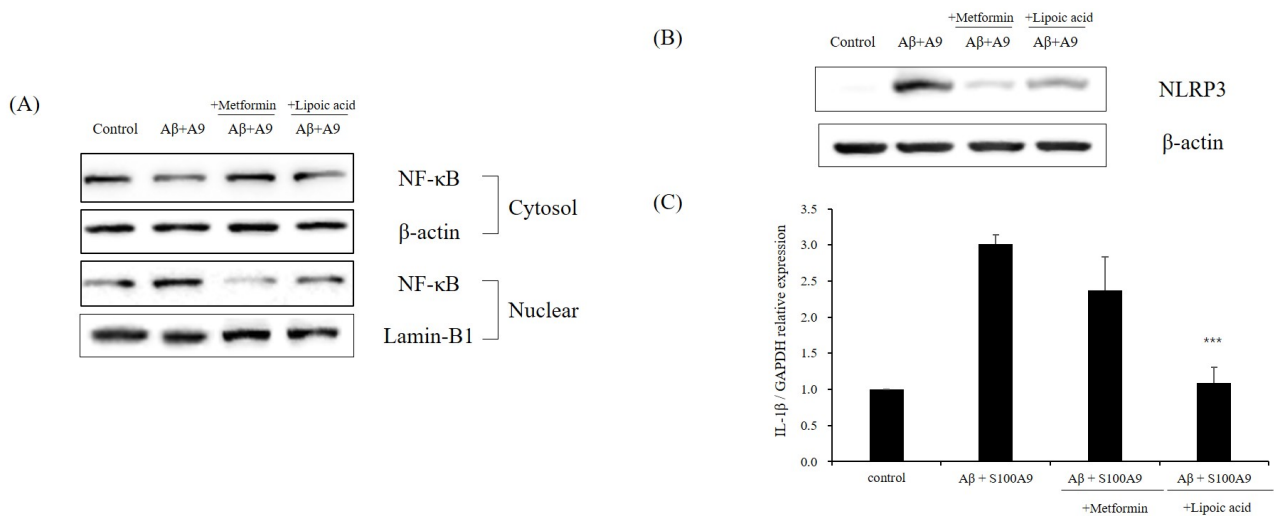
## 2. Metformin and $\alpha$ -LA regulate MAPKs signaling in A $\beta$ - and S100A9-induced BV-2 microglial cells

MAPKs are involved in inflammatory responses, and activated MAPKs can release pro-inflammatory cytokines. To determine the effects of metformin or  $\alpha$ -LA on MAPKs, we measured the protein levels of p-JNK, p-ERK, and p-p38. A $\beta$ - and S100A9-induced the phosphorylation of JNK, ERK, and p38. By contrast, metformin or  $\alpha$ -LA treatment inhibited the phosphorylation (Figure 2). These results demonstrated that metformin and  $\alpha$ -LA inhibit MAPKs and reduce the

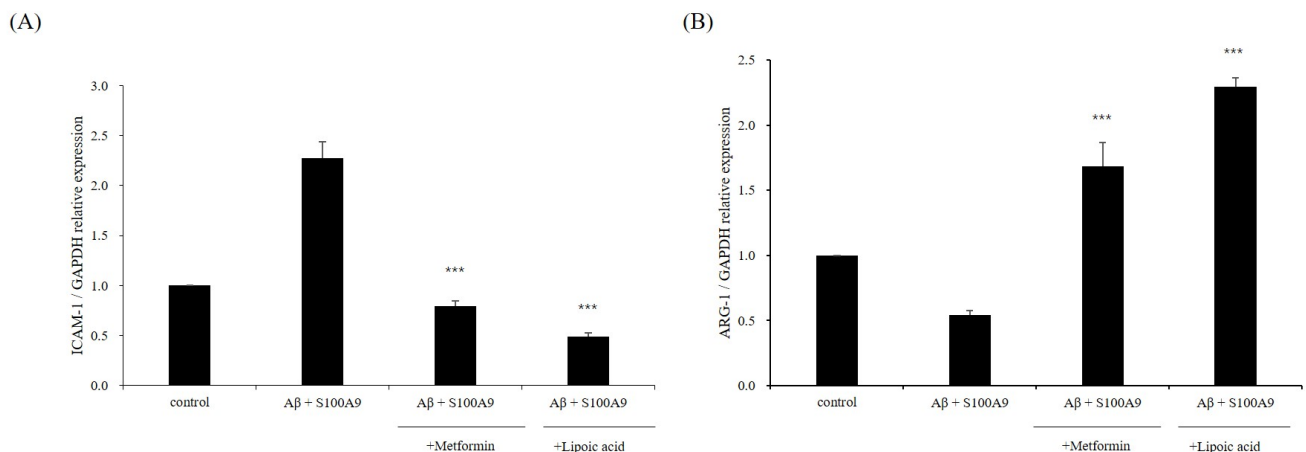
inflammatory response in BV-2 microglial cells co-treated with A $\beta$  and S100A9.

## 3. Metformin and $\alpha$ -LA attenuate NF- $\kappa$ B activation in A $\beta$ - and S100A9-induced BV-2 microglial cells

We investigated the repressive effects of metformin and  $\alpha$ -LA in the activation of the NF- $\kappa$ B pathway in A $\beta$ - and S100A9-induced BV-2 microglial cells. As shown in Figure 3A, A $\beta$ - and S100A9-induced provoked the translocation of nuclear factor kappa B (NF- $\kappa$ B) into the nucleus, whereas metformin or  $\alpha$ -LA treatment blocked this process. Western blot analysis demonstrated that the expression of NLRP3 was increased in response to A $\beta$ - and S100A9-stimulation but that metformin and  $\alpha$ -LA markedly downregulated A $\beta$ - and S100A9-induced



**Figure 3.** Effects of metformin and  $\alpha$ -LA on NLRP3 protein, IL-1 $\beta$  transcription and NF- $\kappa$ B translocation in A $\beta$ - and S100A9-induced BV-2 microglial cells. BV-2 microglia cells were stimulated with metformin (1 mM) or  $\alpha$ -LA (500  $\mu$ M) with A $\beta$  (20  $\mu$ M) and S100A9 (10  $\mu$ g/mL) for 24 h. (A) The translocation of NF- $\kappa$ B was analyzed through Western blotting. Cells were lysed to cytosol extracts and nucleus extracts.  $\beta$ -actin and Lamin-B1 were used as an internal controls. (B) The expression of NLRP3 was detected by Western blot and (C) the cytokine IL-1 $\beta$  was assessed through RT-qPCR. Data from three independent experiments are presented as means  $\pm$  S.D. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 are related with A $\beta$ - and S100A9-induced cells.



**Figure 4.** Metformin and  $\alpha$ -LA inhibit the M1 polarized BV-2 microglial cells. The effects of metformin and  $\alpha$ -LA on microglial polarization was analyzed through RT-qPCR. The BV-2 microglial cells were treated with metformin (1 mM) or  $\alpha$ -LA (500  $\mu$ M) with A $\beta$  (20  $\mu$ M) and S100A9 (10  $\mu$ g/mL) for 24 h. (A) The M1 phenotype ICAM-1 and (B) M2 phenotype ARG1 mRNA expression were detected by RT-qPCR. Data from three independent experiments are presented as means  $\pm$  S.D. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 are related with A $\beta$ - and S100A9-induced cells.

protein expression (Figure 3B). Furthermore, we confirmed the increase of IL-1 $\beta$  at the RNA level (Figure 3C). These results suggest that metformin and  $\alpha$ -LA reduced NLRP3 protein expression and IL-1 $\beta$  mRNA expression, it is thought that these will affect NLRP3 inflammasome formation and activation via NF- $\kappa$ B activation.

#### 4. Metformin and $\alpha$ -LA suppress M1 polarization and promotes M2 polarization in BV-2 microglial cells

Reactive microglia phenotype is distinguished by the expression of either inflammatory cytokines or cell surface markers. To evaluate M1/M2 polarization, we confirmed the expression of M1 and M2 cytokines and surface markers using RT-qPCR. ICAM-1 is expressed



in M1-positive BV-2 microglial cells, while ARG-1 is expressed in M2-positive BV-2 microglial cells. We observed that ICAM-1 expression increased in A $\beta$ - and S100A9-induced BV-2 microglial cells, but that treatment with metformin or  $\alpha$ -LA decreased their expression levels (Figure 4A). By contrast, the expression of ARG-1 is decreased in the presence of A $\beta$  and S100A9, and is increased in metformin- or  $\alpha$ -LA-induced BV-2 microglial cells (Figure 4B). These results indicate that metformin and  $\alpha$ -LA inhibited M1 polarization and activated M2 polarization.

## DISCUSSION

In microglia, the neuro-inflammatory response caused by A $\beta$  may induce neurodegeneration and the production of various pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-6, that cause neuronal cell death [16]. Moreover, microglia activation was more synergistic with other proinflammatory stimuli, such as lipopolysaccharide (LPS), interferon gamma (IFN- $\gamma$ ), and S100A9, together with A $\beta$  [17–19]. S100A9 is classified as a danger-associated molecular pattern, which is recognized by a variety of pathogen-recognition receptors (PRRs). S100A9 is known as an inflammation-mediated calcium binding protein that is a major contributor to inflammation [20]. Upregulation of S100A9 has been observed in amyloid plaques and adjacent activated microglial cells in the AD brain [21]. S100A9 homodimer as well as S100A8/S100A9 heterodimer is found to be upregulated surrounding amyloid plaques but S100A9 increases much more strongly in AD brain [5]. In a combined treatment of A $\beta$  and S100A9 in BV-2 microglial cells, the effect of a therapeutic target should be considered. Recent studies have suggested that metformin and  $\alpha$ -LA play a neuroprotective role in several neurologic diseases [22, 23]. Therefore, we examined the effects of metformin and  $\alpha$ -LA following A $\beta$ - and S100A9-mediated inflammation and NLRP3 inflammasome activation in BV-2 microglial cells.

Metformin is a drug that activates the autophagy

process. When autophagy occurs at abnormal levels, it can lead to various metabolic diseases and may play a role in degenerative neurological diseases such as Parkinson's disease and Alzheimer's disease. Accordingly, it has been reported that autophagic dysfunction contributes to AD [24].  $\alpha$ -LA is a natural antioxidant. Oxidative stress is an imbalance between oxidants and antioxidants and is associated with an increased ROS production. In case of ROS production and antioxidant imbalance, the overproduction of ROS combined with insufficient antioxidant defenses causes oxidative stress [25]. Oxidative stress is one of the earliest events in AD. Some risk factors for AD can promote oxidative damage [26]. Therefore, in this study, we confirmed that NF- $\kappa$ B translocation and MAPK signaling molecules, such as p-JNK, p-ERK, and p-p38, were inhibited by metformin and by  $\alpha$ -LA in A $\beta$  and S100A9 stimulated BV-2 microglial cells.

In addition, neurodegenerative diseases have been associated with the NLRP3 inflammasome. The NLRP3 inflammasome is an important factor that influences and induces an innate immune response to A $\beta$ . A $\beta$  fibrils activate NLRP3 inflammasomes involved in the innate immune response in order to increase IL-1 $\beta$  release [27]. A two-signal model has been proposed for NLRP3 inflammasome activation. The first signal (priming) is provided by microbial components or endogenous cytokines that prime NLRP3 and pro-IL-1 $\beta$  expression through NF- $\kappa$ B activation. The second signal is triggered by extracellular ATP, pore-forming toxins, or particulate matter, which activate NLRP3 inflammasomes [28]. In the present study, we showed that metformin and  $\alpha$ -LA decrease the expression of NLRP3 and IL-1 $\beta$  in A $\beta$ - and S100A9-induced BV-2 microglial cells.

Activated microglia have two opposite states. The M1 (pro-inflammatory) state is characterized by production of inflammatory cytokines (IL-6, IL-12, IL-1 $\alpha$ , IL-1 $\beta$ , and TNF- $\alpha$ ), chemokines, and high levels of inducible NO (iNOS) for nitric oxide (NO). By contrast, the M2 (anti-inflammatory) state is induced by treatment with anti-inflammatory cytokines such as IL-4 and IL-13, which

induce an upregulation of arginase 1 (ARG1) [29, 30]. Our results show that metformin and  $\alpha$ -LA treatment following A $\beta$ - and S100A9-stimulated BV-2 microglial cells reduced the marker levels of the M1 phenotype, ICAM1, while the M2 phenotype marker, ARG1, was increased. In addition, the increased expression level of ARG1 shows that metformin and  $\alpha$ -LA inhibit NLRP3 inflammasome activation.

In summary, we demonstrated that metformin and  $\alpha$ -LA reduce pro-inflammatory cytokine release, NF- $\kappa$ B levels, MAPK signaling, and NLRP3 inflammasome activation in A $\beta$ - and S100A9-induced BV-2 microglial cells. Furthermore, these results confirmed that antioxidants such as  $\alpha$ -LA are more effective than the autophagy inducer metformin, specifically in a combined scenario involving A $\beta$  and S100A9 in BV-2 microglial cells. Moreover, metformin and  $\alpha$ -LA modulate microglia M1/M2 polarization. Therefore, our study results indicate that metformin and  $\alpha$ -LA may be candidate drugs for neurodegenerative diseases.

## 요약

알츠하이머 병은 인지 기능 저하로 인한 치매 발생으로 설명할 수 있는 만성 및 진행성 신경 퇴행성 질환이다. 알츠하이머 병의 특징은 세포 외 및 세포 내 아밀로이드 플라크의 형성이다. 아밀로이드 베타는 알츠하이머 병의 특징이며 미세아교세포는 아밀로이드 베타의 존재하에 활성화될 수 있다. 활성화된 미세아교세포는 전 염증성 사이토카인을 분비한다. 게다가, S100A9는 염증의 중요한 선천성 전 염증 기여자이며 알츠하이머 병에 잠재적인 기여자로 알려져 있다. 이 연구는 아밀로이드 베타 및 S100A9이 처리된 BV-2 세포에서 염증반응 및 NLRP3 인플라마솜 활성화에 대한 메트포르민 및 알파리포산의 효과를 조사했다. 메트포르민과 알파-리포산은 중앙 괴사 인자-알파 및 인터루킨-6와 같은 염증성 사이토카인을 억제시킨다. 또한 메트포르민과 알파-리포산은 JNK, ERK, p38의 인산화를 억제하고, NF- $\kappa$ B 경로 및 NLRP3 인플라마솜의 활성화를 억제했다. 또한 메트포르민과 알파-리포산은 M1 표현형인 ICAM1의 수준을 감소시킨 반면 M2 표현형인 ARG1은 증가시켰다. 이러한 발견은 메트포르민과 알파-리포산이 아밀로이드베타 및 S100A9에 의한 신경 염증 반응에 대한 치료제가 될 수 있음을 시사한다.

**Acknowledgements:** This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2018R1D1A3A0300069213).

**Conflict of interest:** None

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