Master's Thesis

석사 학위논문

Caffeine reduces LPS-induced pro-inflammatory microglial response and autophagic suppression by inducing autophagy.

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Advisor : Professor Seong-Woon Yu

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by

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A thesis submitted to the faculty of DGIST in partial fulfillment of the requirements for the degree of Master of Science in the Department of Brain and Cognitive Sciences. The study was conducted in accordance with Code of Research Ethics[1)]

06. 30. 2016

Approved by

Professor Seong-Woon Yu (Advisor) _____(Signature)

Professor Ji-Woong Choi (Co-Advisor) _____(Signature)

[1)] Declaration of Ethical Conduct in Research: I, as a graduate student of DGIST, hereby declare that I have not committed any acts that may damage the credibility of my research. These include, but are not limited to: falsification, thesis written by someone else, distortion of research findings or plagiarism. I affirm that my thesis contains honest conclusions based on my own careful research under the guidance of my thesis advisor.

Caffeine reduces LPS-induced pro-inflammatory microglial response and autophagic suppression by inducing autophagy.

Leah Eunjung Kim

Accepted in partial fulfillment of the requirements for the degree of Master of Science

06. 30. 2016

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Abstract

Background Microglia are the resident immune cells in the brain that are sensitive to changes in their local environment. They respond to pathogen or danger signals by triggering brain immune response. Many drugs working on the nervous system can target microglia as well as neurons. Caffeine is the most widely consumed psychoactive drug across various age groups worldwide. However, little is known whether caffeine can affect microglia function. This study is aimed to examine whether caffeine regulates microglia functions and autophagy is involved in the action of caffeine on microglia, since autophagy plays a critical role in cellular immune response.

Results Caffeine increased autophagy flux and reduced lipopolysaccharide (LPS)-induced up-regulation of pro-inflammatory cytokines genes and their release. Suppression of autophagy using 3-methyladenine (3-MA) or by knockdown of Atg7 prevented the anti-inflammatory action of caffeine on microglia.

Conclusions Our findings suggest that caffeine can enhance microglial autophagy and reduce inflammation by down-regulating LPS-induced autophagy suppression. The anti-inflammatory effect of caffeine is autophagy-dependent.

Keywords: Autophagy, Microglia, Caffeine, Neuroinflammation, Lipopolysaccharide

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1. Introduction (Background and significance)

Microglia are mononuclear phagocytes the resident immune cells in the central nervous system (CNS) and they are estimated to comprise tenth of the total CNS cells. Their functions include cytokines/chemokines production, neurotropic factor release, phagocytosis and many others [1-3]. They are often compared to macrophages in the peripheral nervous system (PNS). Indeed, microglia and macrophages have several cell type markers in common and exert similar immune functions in response to changes in their environment. [1, 3, 4]. The cell surface receptors detect signals such as damage-associated molecular signal (DAMP) or pathogen-associated molecular pattern (PAMP). Despite existing dissenters to the term, the term 'activated microglia' is commonly used to describe microglial immune activation leading to their morphology changes to amoeboid shape. The activation does not always bring about benefiting outcomes although the activation happens primarily to dampen the infection or danger signals to protect other cells types in the CNS.

Autophagy is a cellular process for a self-engulfment or a bulk degradation of protein aggregates, long-lived proteins, damaged organelles, and invading microbes within a cell by forming autophagosomes and later autophagosomes through fusion with lysosomes to form autopysosomes [3, 5]. Increased autophagy rate is described as increased autophagic flux. Autophagic flux is generally increased in nutrient-deprived starvation condition to get nutrients as energy source from degraded materials. Therefore, the basal autophagy within a cell is considered to be beneficial for cell survival despite excessive autophagy leading to autophagic cell death (ACD), type II programmed cell death [6]. Autophagy is suggested to be closely linked to inflammatory immune response by degrading invading pathogens or toxic materials

such as mycobacteria and IL-1β [7, 8]. Autophagy disruption was related to neurodegeneration that neuronal autophagic dysfunction resulted in decreased neuronal survival [9]. However, there is a continuation of controversy for role of autophagy and the underlying mechanisms in immune cells although researches concerning both topics, immune cells and autophagy, are accumulating. Whether or not microglial autophagy is truly beneficial and involved in inflammation by the means of underlying mechanisms is to be revealed in accordance with very little research reported. Mice with microglia-specific Atg7 gene knock out showed impairment of Aβ fibrils clearance in the brain indicating that autophagy is crucial for intracellular clearance for fAβ [10]. Rapamycin-induced increase in autophagic flux in BV-2 murine microglial cells suppressed iNOS, IL-6 and cell death induced by bacterial endotoxin, lipopolysaccharide (LPS) [11]. These two reports suggest a beneficial role of autophagy in microglia. However, cocaine treatment in BV-2 and primary rat microglia showed high autophagic activity caused by to ER stress and that lead to enhancement of pro-inflammatory responses releasing more CCL2, IL-6 and TNFα [12].

Caffeine, one of the most commonly consumed psychoactive drugs, is well-known brain stimulator that causes wakefulness, alertness and increases heart rate. It is commonly found in food including chocolate, tea, soft drinks not to mention coffee, the most common source of caffeine [13, 14]. Effects and functions of caffeine has long been studied in various experimental settings due to the its psychoactive property and unfavorable effect it has during early development of the brain [15, 16]. However, accumulating studies support the beneficial role of caffeine. In neonatal rat model, acute high dose of caffeine before high oxygen exposure protected neuronal cell death in the immature brain and two weeks of chronic caffeine intake in rats before Alzheimer's disease (AD) induction significantly increased brain derived neurotrophic factor signaling and its receptor neural receptor protein-tyrosine kinase-beta

(TrkB) in hippocampus [17-19]. Caffeine reduced Aβ levels in plasma and brain and reversed cognitive impairment in mouse model of AD therefore the authors suggested that caffeine has therapeutic potential against AD [20-23]. Caffeine was also shown to have anti-inflammatory functions in microglia that caffeine markedly suppressed LPS-induced iNOS, COX-2 and TNF-α gene expression in BV-2 cells [24]. Also, chronic IP injection of caffeine in rats substantially reduced number of LPS-induced activated microglia in hippocampus [25]. However, the underlying mechanism of how caffeine exert neuroprotective effect in microglia are largely unknown. Caffeine lowered risk for nonalcoholic fatty liver disease (NAFLD) and reduced hepatosteatosis by increasing autophagy that enhanced lipid uptake in lysosomes [26, 27]. Treatment of caffeine lowered human prion protein-mediated human neuronal cell death and protected against prion-mediated neurotoxicity by caffeine-induced autophagy signals [28]. We therefore speculated that caffeine might have its neuroprotective function in microglia by inducing autophagy.

2. Materials and methods

Antibodies and reagents

Following antibodies are used: β-actin (sc-47778) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). mTOR (S2448); p62 (5114S) and ATG7 (8558S) were purchased from Cell Signaling Technology (Danvers, MA, USA); LC3B antibody was purchased from Novus Biologicals (Littleton, CO, USA); iNOS (ab15323), Iba-1 (ab5076), TNFα (ab9739). The followings are reagents used: LPS from *Escherichia coli* 0111:B4 was purchased from Calbiochem (La Jolla, CA, USA) and diluted in PBS; BafA1, caffeine and 3-methyladenine (3-MA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rapamycin (BML-A275-0005) was purchased from L*ife Sciences* Advanced Technologies, *Inc*. (*Saint Petersburg*, *FL*, *USA)*. Enzyme-linked immunosorbent assay (ELISA) kits for the mouse TNFα and IL-6 were purchased from R&D systems (Minneapolis, MN, USA).

Cell culture

BV-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) *(Corning, NY, USA) supplemented with* 10% heat-inactivated fetal bovine serum (FBS) (Hyclone; Logan, UT, USA) and 1% penicillin-streptomycin (Hyclone).

All procedures for the care and use of laboratory animals were approved by the Institutional Animal Care and Use Committee (IACUC) at Daegu Gyeongbuk Institute of Science and Technology (DGIST). Pregnant C57BL/6 mice were purchased from Koatech (Namyangju-si, Gyeonggi-Do, Republic of Korea) and the brains of 1-day old neonatal mice were prepared for primary microglia cultures. In brief, neonatal brains are dissected in a serum-free media, and dissociated with gentle pipetting. Suspended cell pallet was seeded with medium consists of DMEM supplemented with 10% FBS (Hyclone) and 1% penicillin-streptomycin (Hyclone). Five days after plating, whole medium was changed with fresh medium containing 10% FBS and 1% penicillin-streptomycin. After, half of the medium for each plate was changed every other day with fresh medium. Primary microglia were isolated from mixed glial culture by tapping the plate. The cultures and isolations were continued up to three weeks until enough microglia were isolated for designed experiments.

Plasmids and magnetofection

pMXs-puro GFP-DFCP1 and pEGFP-LC3 for mouse was purchased from Addgene (Cambridge, MA, USA). pMXs-puro GFP-DFCP1 and mRFP-GFP-LC3 constructs were incubated with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Nano-sized magnetic particles CombiMAG (Chemicell, Berlin, Germany) were then introduced to the mixture and incubated for additional 30 min in 37℃incubator for combining of the DNA-Lipofectamin 2000 mixture. DNA-Lipofectamine 2000-magnetic particles mixture was then delivered into BV-2 cells. Transfection was performed in DMEM without FBS and antibiotics for 3 h. Then, cells were replaced with normal culture medium for 24 h and re-seeded onto glass coverslips for immunocytochemistry experiments.

Lentiviral production

Lentivirus vector pLKO.1 scramble shRNA (plasmid 1864), enveloper vector pMD2.G (plasmid 12259), and packaging vector psPAX2 (plasmid 12260) were obtained from Addgene. pLKO.1 sh-Atg7 vector (Sigma-Aldrich TRCN0000092164) was purchased from Sigma-Aldrich. Lentiviruses were produced according to the manufacturer's instruction and ultra-centrifuged for 5 h at 2,5000 × g and re-suspended in BV-2 cells culture medium. The BV-2 cells were infected with the virus for 12 h and the fresh virus-free medium replaced virus-containing infection medium. After 48 h incubation, 0.5μg/ml puromycin was added for selection procedure and the puromycin-containing medium was changed 6 h later. After selection with 1μg/ml puromycin in the next cell passaging, the surviving cells were used for the knockdown experiments.

Western blotting

BV-2 and primary microglia were harvested and lysed in radioimmunoprecipitation assay buffer (RIPA buffer, Sigma-Aldrich) with 1× protease cocktail inhibitors (Thermo Scientific, Waltham, MA, USA) and 1× phosphatase cocktail inhibitors (Thermo Scientific), 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride for 15 min on ice. After centrifugation (16,100 × g, 10 min), each sample's lysate was collected. BCA protein assay reagent (Thermo Scientific) were used to measure protein concentration in the lysate. Prepared samples were loaded into the gel and electro-transferred to polyvinylidene difluoride (PVDF) membrane with a

semi-dry electrophoretic transfer cell (Bio-Rad, Richmond, CA). Membranes were blocked with 5% nonfat dry milk powder dissolved in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature. The membranes were incubated with primary antibodies for overnight in 4℃ on a shaking incubator. The membranes were washed with TBST for 3 times, 10 min each. Peroxidase-conjugated secondary antibodies diluted in blocking solution was applied for 1 h at room temperature. After washing, proteins of interest were detected using either chemiluminescence detection kit (Thermo Scientific) or WesternBright ECL (Advansta ICL, Menlo Park, CA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

To assess various cytokines expression after stimulation with caffeine or/end LPS, RNA was isolated for qRT-PCR using the ImProm-II Reverse Transcriptase kit (Promega, Madison, WI, USA) and cDNA was synthesized using oligo dT. PCR primers were commercially synthesized (Cosmo genetech, Seoul, Republic of Korea). qRT-PCR was performed on the RT product using Taq Polymerase (Invitrogen) and primers specific for mouse Arg1, BDNF, CCL2, IL-10, IL-6, TNFα and β-actin cDNAs. TOPreal™ qPCR 2× PreMIX (SYBR Green with low ROX) (Enzynomics, Daejeon, Republic of Korea) was used. 50-cycle amplification was applied for all primers using the CFX96 Real-Time System (Bio-Rad). β-actin was used as the reference gene for normalization. Primers used for Arg1, BDNF, CCL2, IL-10, IL-6, TNFα and β-actin were listed in the table below:

Gene	5'- Sense primer sequence -3'	5'- Antisense primer sequence -3'
Arg1	CGCCTTTCTCAAAAGGACAG	CCAGCTCTTCATTGGCTTTC
BDNF	GGGTCACAGCGGCAGATAAA	GCCTTTGGATACCGGGACTT
CCL2	AACTCTCACTGAAGCCAGCTCT	CGTTAACTGCATCTGGCTGA
IL-10	AGTGAACTGCGCTGTCAATG	TTCAGGGTCAAGGCAAACTT
IL-6	GACAACTTTGGCATTGTGG	ATGCAGGGATGATGTTCTG
TNFα	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
β-actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT

ELISA

BV-2 cells were seeded onto 24-well plate and cultured supernatant from each well was collected at the end of scheduled experiments for the measurement of TNFα and IL-6 concentration by ELISA (Enzyme-linked immunosorbent assay) following manufacturer's instructions (R&D systems).

Statistical analysis

At least three independent experiments were performed for each condition of experiments and data were presented as mean ± standard deviation (SD) values. Statistical analysis was performed by either unpaired Student's t-test or one-way analysis of variance (ANOVA) and the statistical significance was obtained using Graphpad Prism (GraphPad Software, San Diego, CA, USA)

3. Results

LPS suppressed autophagic flux in BV-2 and primary microglia

LPS is the major component of gram-negative bacteria and is endotoxin that LPS is used as the same word for endotoxins nowadays [29, 30]. LPS is recognized as pathogen associated molecular pattern (PAMP) by pattern recognition receptors (PPRs) in immune cells, among them are toll like receptors [31]. LPS is the ligand of toll-like receptor 4 (TLR4) and is very widely used to activate immune cells such as microglia [29, 30]. Before the commencement of investigation of caffeine's effect on autophagy, we first tested the effect of LPS on autophagy because there was not a single study reporting the level of microglial autophagy following LPS treatment despite almost firmly settled LPS's autophagy-inducing effect in macrophage in several reports [32-34]. To measure autophagic flux in LPS treated BV-2 and primary murine microglia, LPS was treated for 6 h as in the schematic of experimental procedure (Fig. 1a). Bafilomycin A1 (BafA1) is autophagy inhibitor that prevents the fusion between autophagosomes and lysosomes therefore blocks the late step of autophagy [35]. During autophagy, microtubule-associated protein 1A/1B-light chain 3 (LC3-I) is conjugated to phosphatidylethanolamine and is then converted to LC3-phosphatidylethanolamine conjugate (LC3-II). LC3-II is recruited to autophagosomal membranes and intra-phagosomal materials, including LC3-II, are degraded following autophagosomes fuse with lysosomes to form autolysosomes [36-38]. Thus, LC3-II is an important autophagosomal marker. Detection of lysosomal turnover of LC3-II gives good indication of autophagic flux that the accumulation LC3-II level following BafA1 treatment indicates an increase in autophagic flux and no accumulation vice versa. [35]. Beyond our expectation and opposite of autophagy-inducing effect of LPS in macrophage, LPS treatment significantly decreased

LC3-II in both BV-2 and primary microglia meaning that there is either a marked decrease in autophagic flux or an autophagy impairment. BafA1 (20 nM for 2 h) treatment confirmed that the decrease was the result of a severe reduction in autophagic flux since there was less accumulation of LC3-II in LPS+BafA1 group than BafA1 only group in both BV-2 and primary microglia (Fig. 1b). We found a paper indicating microRNA-Let7A as a positive regulator for autophagy induction in BV-2 murine microglia cell line. Although the paper did not pay any attention to LPS effect on autophagy in microglia, their LPS treatment data showed a significant decrease of Beclin1 and ATG3, both known as positive regulators for autophagy [39]. We next investigated the autophagy suppression at later time point. There was continuation of significant autophagy impairment at 24 h in primary microglia (Fig. 1c). However, the impairment was not shown in BV-2 cells at 24 h possibly due to the toxic environment and extensive occurrence of cell death at 24 h of LPS treatment (data not shown). In our knowledge, it is the first report indicating that LPS suppressed autophagy in microglia. To our surprise, there was only one paper indicating LPS induced autophagy in microglia although the result is contrast to ours [11]. Our result, however is clearly in contrast to previous studies reporting LPS-induced autophagy in other types of immune cells [11, 33, 40]. An in vivo study demonstrating an impairment of autophagy in the tissue homogenate of hippocampus and cortex using LPS-injected mice was the only paper that are in line with our result [41].

Monomeric RFP-GFP tandem fluorescent LC3 (mRFPGFP-LC3) transfection is a convenient method for monitoring autophagic flux other than just imaging autophagosomes by GFP-LC3. Both GFP and RFP signal in autophagosomes before maturation, but due to the low stability in acid-sensitive nature of green florescent protein leading to fluorescence quenching, only RFP signals in acidic autolysosomes [42]. The construct was transfected into BV-2 cells

and LC3 puncta were counted. There were less LC3 puncta but higher yellow puncta ratio in LPS treated cells indicating a decreased flux and impairment of autophagosome maturation (Fig. 1d-e). Quantification of LC3 puncta revealed that the percentage of yellow puncta was significantly higher but the total number of puncta was significantly less than control reflecting a decrease in autophagy (Fig. 1e). We also observed very intriguing effect of LPS treatment in microglia that p62, an autophagy adaptor protein, is highly accumulated time and dose-dependent manner (Fig. 1f, 2e). Decrease in LC3-II was also observable from 3 to 8 h (Fig. 1f). To clarify the suppression of autophagy by LPS is TLR4 mediated, Pam3CSK4, a ligand of TLR1/2, was treated in primary microglial cell. Pam3CSK4 treatment also showed autophagy-suppressing manner similar to LPS treatment (Fig. 1g). Therefore, we speculate that there is a direct link between specific TLR ligands and autophagy. Another evidence is that HEK293 cells exhibiting LPS responsive elements showed almost no LC3-II even in the basal level (Fig. 1h). These data indicate a very interesting fact that mentioned nowhere that LPS greatly suppressed autophagy in microglia.

Caffeine induced autophagy in BV-2 cells and primary microglia

Caffeine is the most consumed psychoactive drug worldwide and it is still controversial if caffeine is beneficial to the brain function. Recently autophagy is revealed to play a very important role in inflammation and immune response that some autophagy-relevant genes are indeed associated with immune diseases [43-45]. Caffeine's effect on microglial autophagy has not been reported elsewhere and underlying mechanisms of caffeine neuroprotective properties are largely unexplored. Therefore, we investigated caffeine-autophagy axis in BV-2 and primary microglia. 1 mM of caffeine induced increased autophagy in both BV-2 and primary

microglia in time-dependent manner (Fig. 2a). 6 h of caffeine treatment showed the highest accumulation of LC3-II and therefore was selected for the treatment time for later experiments. To investigate the increase in LC3-II is due to increase of autophagic flux and not the impairment of late step of autophagy, BafA1 was treated. BafA1 treatment to caffeine-treated BV-2 and primary microglia showed a greater increase of LC3-II than BafA1 only treatment, indicating increased autophagic flux (Fig. 2b). RFP-LC3 puncta were significantly increased in caffeine treated BV-2 cells where rapamycin was used as positive control (Fig. 2c). Rapamycin is an autophagy inducer that inhibits mammalian target of rapamycin (mTOR) thereby mimicking the starvation condition [46, 47]. A significant increase of red puncta by caffeine treatment is correlated with a higher autophagic flux. Indeed, quantification analysis of puncta shows a significantly higher number of total puncta but significantly less percentage of yellow puncta indicating an increase in autophagic flux (Fig. 2d).

Autophagy inhibition blocked Caffeine-induced autophagy

Our data indicate that LPS suppresses autophagy and caffeine increases autophagic flux in microglia. Autophagy is critically controlled by phosphatidylinositol 3-kinases (PtdIns3Ks). While Class I PtdIns3K inhibits autophagy by activating Akt-TOR kinases signaling cascade, Class III PtdIns3K (Vps34) plays a central role in autophagosome formation by generating the phospholipid PtdIns(3)P [48, 49]. 3-Methyladenine (3-MA) is an inhibitor of PtdIns3K therefore is widely used as an autophagy inhibitor [50]. 3-MA (1 mM) was pretreated one hour before caffeine treatment in BV-2 and primary microglia (Fig. 3a). 3-MA treatment decreased LC3-II level and accumulated p62. Caffeine treatment following 3-MA treatment did not show any increase of LC3-II, indicating that caffeine-induced autophagy in microglia is PtdIns3K-

mediated (Fig. 3b). To further confirm autophagy-inducing function of caffeine is truly autophagy-related gene-dependent, Atg7, one of the core autophagy regulator proteins, was knockdown by using lentivirus expressing Atg7 targeting shRNA in BV-2 cells. Atg7 knockdown abrogated the increase of LC3-II by caffeine and failed to exhibit caffeine-induced autophagy (Fig. 3c). We were curious about caffeine's effect on apoptosis other than autophagy and also we speculated caffeine's anti-apoptotic function based on other studies [51]. Staurosporine, an apoptosis inducer, was treated to BV-2 cells and cell death was measured at 12 h. Interestingly, caffeine markedly reduced apoptosis in control BV-2 cells whereas the effect was gone in Atg7 knockdown BV-2 cells (Fig. 3d). To our knowledge, it is the first report demonstrating caffeine's anti-apoptotic effect in microglia and it was autophagy-dependent.

Caffeine rescued LPS-induced autophagic suppression and ameliorated LPS-induced inflammation

It is understood that autophagy is beneficial to the cell survival and plays important role in immune response [9, 52]. Autophagy promotes cell survival by removing damaged organelles or protein aggregates and recycle them as nutrients to the cells. Based on our data that LPS suppressed autophagy in microglia, caffeine's autophagy inducing function was test in LPS-treated condition. As our expectation, caffeine rescued LPS-induced autophagic suppression (Fig. 4a). The enhancement of autophagy was in parallel with decreases in p62 level (Fig. 4a-b). To find the detailed mechanisms how LPS suppressed autophagy phosphorylation of Akt, mTOR, ULK1 was investigated. Phosphorylation of Akt and its downstream mTOR and ULK1 was markedly increased (Fig. 4c). Between two mTOR complexes, mTORC2 is the protein complex responsible for autophagy induction and its phosphorylation at serine2448 by AKT

results in autophagy inhibition [46]. LPS treatment largely increased mTOR phosphorylation whereas caffeine decreased mTOR phosphorylation (Fig. 4d). The phosphorylation gives a good indication of the source of autophagy suppression which is revealed to be upstream signals (Fig. 4c-d). Double FYVE-domain-containing protein 1 (DFCP1) marks omegasome and is used as autophagy initiation marker due to its involvement in the early step of autophagosome formation by interacting with PtdIns(3)P [48, 53]. DFCP1 level was analyzed by imaging. DFCP puncta were increased in caffeine treated BV-2 cells whereas LPS treatment showed almost no punctate formation (Fig. 4e). These data indicate that LPS suppressed autophagy by altering the early step of autophagy initiation which can be restored by caffeine.

Ablation of autophagy blocked caffeine's anti-inflammatory effect

Microglia are immune cells in the brain and they play a critical role in inflammation. Caffeine has been reported to have anti-inflammatory effect in neuroinflammation [24, 25]. We investigated caffeine's immunoregulatory function in microglia. Caffeine pretreatment reduced LPS-induced iNOS and TNFα in miroglia (Fig. 5a-b). BDNF is essential for growth, survival and neuronal cell defferentiation and is involved in learning and memory. A recent study reported a protective effect of caffeine against AD that caffeine increased BDNF [19]. Arg1+ microglia uptaked more Aβ in rat brain of AD [54]. Caffeine treatment in BV-2 cells induced brain derived neurotrophic factor (BNDF) and Arginase 1 (Arg1) which are regarded as anti-inflammatory (Fig. 5c). Caffeine treatment also significantly reduced pro-inflammatory cytokines, TNFα, IL-6 and CCL2 (Fig. 5c) and reduced release of TNFα and IL-6 (Fig.5d). These data strongly indicate caffeine's anti-inflammatory effect in microglia.

To investigate the anti-inflammatory effect of caffeine is autophagy-dependent,

pharmacological and genetic inhibition of autophagy were performed. Both 3-MA treatment and Atg7 knockdown ablated increase of BDNF and Arg1 by caffeine, also, reduction of pro-inflammatory cytokines, TNFα, IL-6 and CCL2, was abrogated (Fig. 6A-B). In conlcusions, caffeine protects against LPS-induced inflammation in microglia by enhancing autophagy.

4. Discussion

In this study, we report few key findings in microglia. LPS suppresses autophagy and caffeine induces autophagy in microglia. LPS-induced neuro-inflammation is largely down-regulated by activation of autophagy by caffeine and knockdown of Atg7 abrogated the anti-inflammatory effect of caffeine. Therefore, we concluded that caffeine's anti-inflammatory function is autophagy-dependent.

Caffeine can freely pass through the blood brain barrier (BBB) and function in the brain. One of the action of caffeine is to replace a structurally similar molecule, adenosine, and acts as an adenosine receptor antagonist [13]. However, the effect and detailed mechanisms for its revealed anti-inflammatory effect in microglia remain still unexplored [24]. Caffeine reduced LPS-induced generation and release of pro-inflammatory molecules such as iNOS, TNFα, IL-6 and CCL2 in microglia. These data suggest that caffeine's neuroprotective effect in microglia. Furthermore, caffeine treatment in microglia was enough to up-regulated neurotrophic factor such as BDNF and anti-inflammatory molecule, Arg1, even at the basal condition. All these anti-inflammatory effect of caffeine was abrogated by knockdown of a core autophagy protein, Atg7, indicating that anti-inflammatory effect is mediated by autophagy. Further investigations are needed to explain how caffeine induces autophagy in microglia. It is also possible that caffeine-induced autophagy enhances degradation of detrimental cytokines within the microglia.

A remarkable suppression of autophagy by LPS in microglia has not been reported and this significant and interesting finding would lead to further observation on how LPS impairs autophagy. We revealed that LPS blocked early autophagy signal shown by little DFCP1 puncta formation and increased phosphorylation of mTOR. We strongly believe that effect of LPS, a

TLR4 ligand, on autophagy reduction is related with TLR signaling cascade rather than inflammation-related signaling. It is due to the observation that Pam3CSK4 showed the same autophagy-reducing effect as LPS while Pam3CSK4 is not as potent as LPS to induce inflammation.

In conclusion, we proposed that caffeine reduces inflammation by activating microglial autophagy. Caffeine can therefore be classified as a neuroprotective drug at least when considering microglia.

5. Figure legends

Figure 1. LPS suppressed autophagy in BV-2 cells and primary microglia.

(A) Schematic diagram of experimental procedure **(B)** LPS treatment significantly suppressed autophagy. **(C)** Continuation of autophagy suppression by LPS. **(D)** BV-2 cells transfected with mRFP-GFP-LC3. GFP is degraded in acidic pH therefore RFP puncta indicate autolysosome. Scale bar, 10 μm. **(E)** Quantitative red and yellow puncta numbers. **(F)** Time-dependent p62 protein accumulation following LPS treatment. Decrease in autophagic flux was observed at 3 – 8 h. **(G)** Pam3CSK4, TLR1/2 ligand, suppressed autophagy in primary microglia at 6 h of treatment. **(H)** Dose-dependent treatment of LPS in HEK293 cells. Expression of TLR4 in HEK293 cells decreased LC3-II.

Figure 2. Caffeine induced autophagy in BV-2 cells and primary microglia.

(A-B) Caffeine increased autophagic flux. **(C)** More red puncta after caffeine treatment indicated an increase in autophagic flux. Scale bar, 10 μm. **(D)** Quantitative red and yellow puncta numbers. **(E)** Autophagy suppression by LPS at 12 h treatment in BV-2 cells and caffeine-induced increase in LC3-II.

Figure 3. 3-MA, PtdIns3K inhibitor, and knockdown of Atg7 blocked caffeine-induced autophagy and anti-apoptotic effect.

(A) Schematic diagram of experimental procedure. **(B)** Caffeine's autophagy-inducing effect was abrogated by 3-MA treatment. **(C)** Caffeine's autophagy-inducing effect was abrogated by Atg7 gene knockdown**. (D)** Staurosporine (STS) was treated to induce apoptosis in BV-2 cells. Caffeine significantly decreased apoptotic cell death but Atg7 knockdown abolished the anti-apoptotic effect of caffeine.

Figure 4. Caffeine rescued LPS-induced autophagic suppression.

(A) Caffeine increased autophagy in LPS-treated microglial cells and **(B)** reduced accumulation p62 puncta. Scale bar, 10 μm. **(C)** LPS treatment induced phosphorylation of Akt, mTOR, ULK1. **(D)** Autophagy suppression by LPS is the result of upstream mTOR activation. **(E)** Increase of DFCP1 puncta by caffeine is indicative of higher autophagic flux. Scale bar, 10 μm.

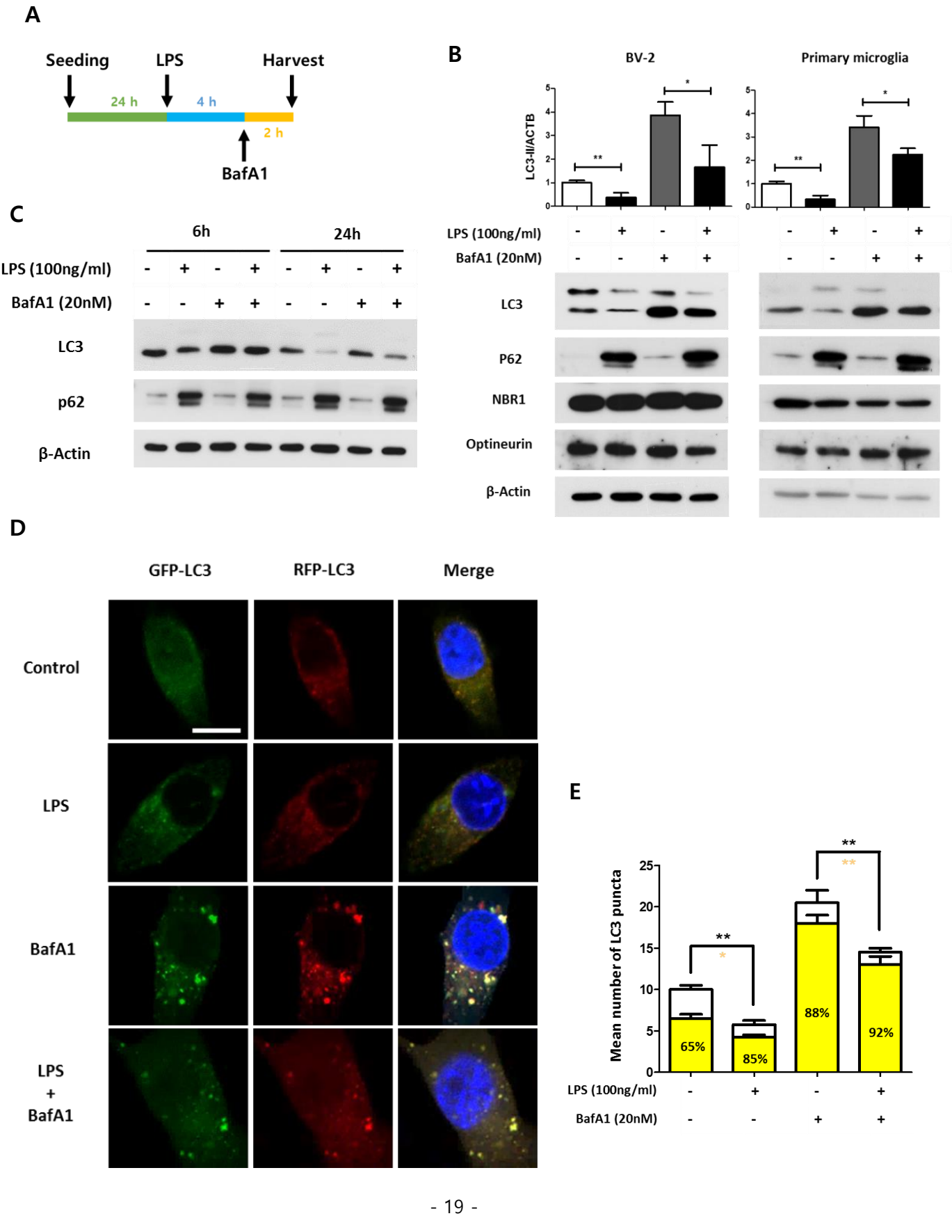
Figure 5. Caffeine ameliorated LPS-induced inflammation.

(A) Caffeine reduced LPS-induced nitric oxide (NO) production in BV-2 cells. **(B)** Caffeine-treated primary microglia showed reduced iNOS level compared to LPS treatment. Scale bar, 20 μm. **(C)** Caffeine treatment increased anti-inflammatory factors, BDNF and ARG1, and reduced pro-inflammatory cytokines, TNFα, IL-6 and CCL2. **(D)** Caffeine reduced TNFα and IL-6 release measured by ELISA assay.

Figure 6. Ablation of autophagy blocked the caffeine's anti-inflammatory effect.

(A) 3-MA treatment in BV-2 cells blocked increases in the expression of anti-inflammatory genes and reversed decreases in the expression of pro-inflammatory genes by caffeine. **(B)** Atg7 knockdown abolished the anti-apoptotic effect of caffeine in BV-2 cells.

6. Figures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LPS
Harvest
24 h
4 h
2 h
BafA1
B
BV-2
Primary microglia
LC3-II/ACTB
LPS (100ng/ml)
BafA1 (20nM)
LC3
P62
NBR1
Optineurin
β-Actin
C
6h
24h
LPS (100ng/ml)
BafA1 (20nM)
LC3
p62
β-Actin
D
GFP-LC3
RFP-LC3
Merge
Control
LPS
BafA1
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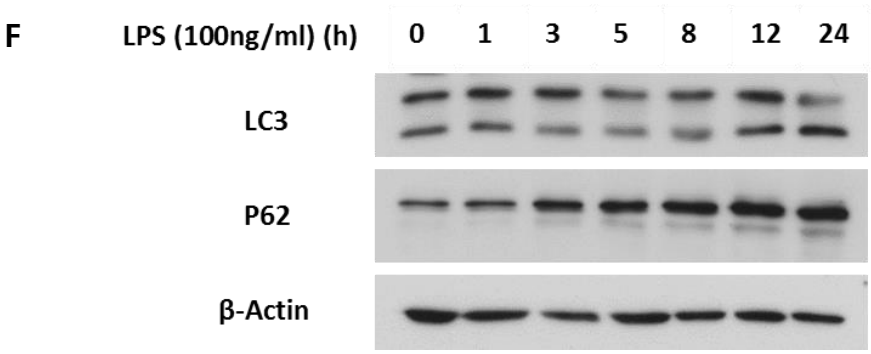
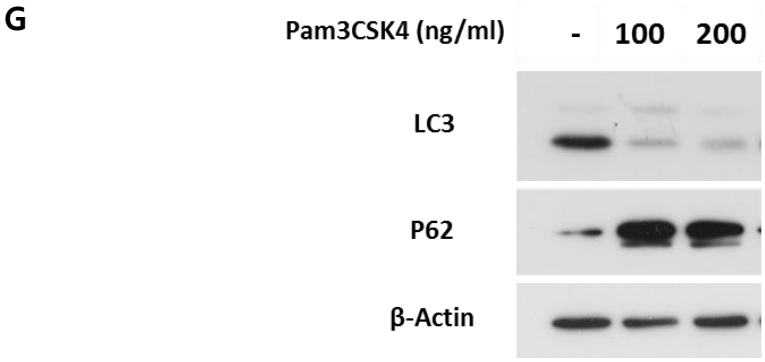
85%
88%
92%
LPS (100ng/ml)
BafA1 (20nM)





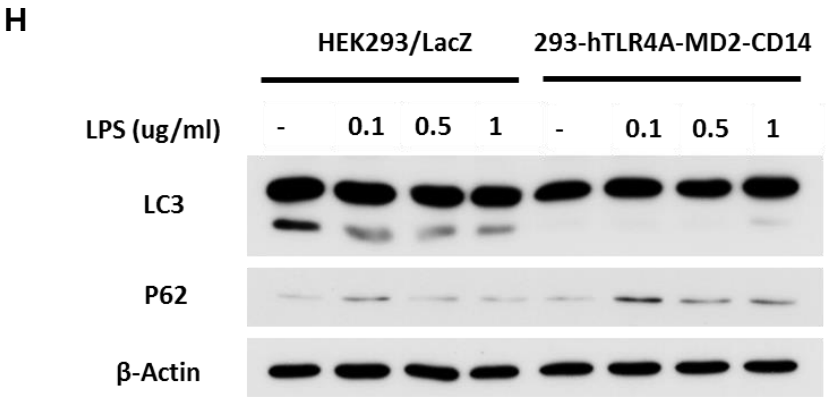


Figure 1. LPS suppressed autophagy in BV-2 cells and primary microglia.

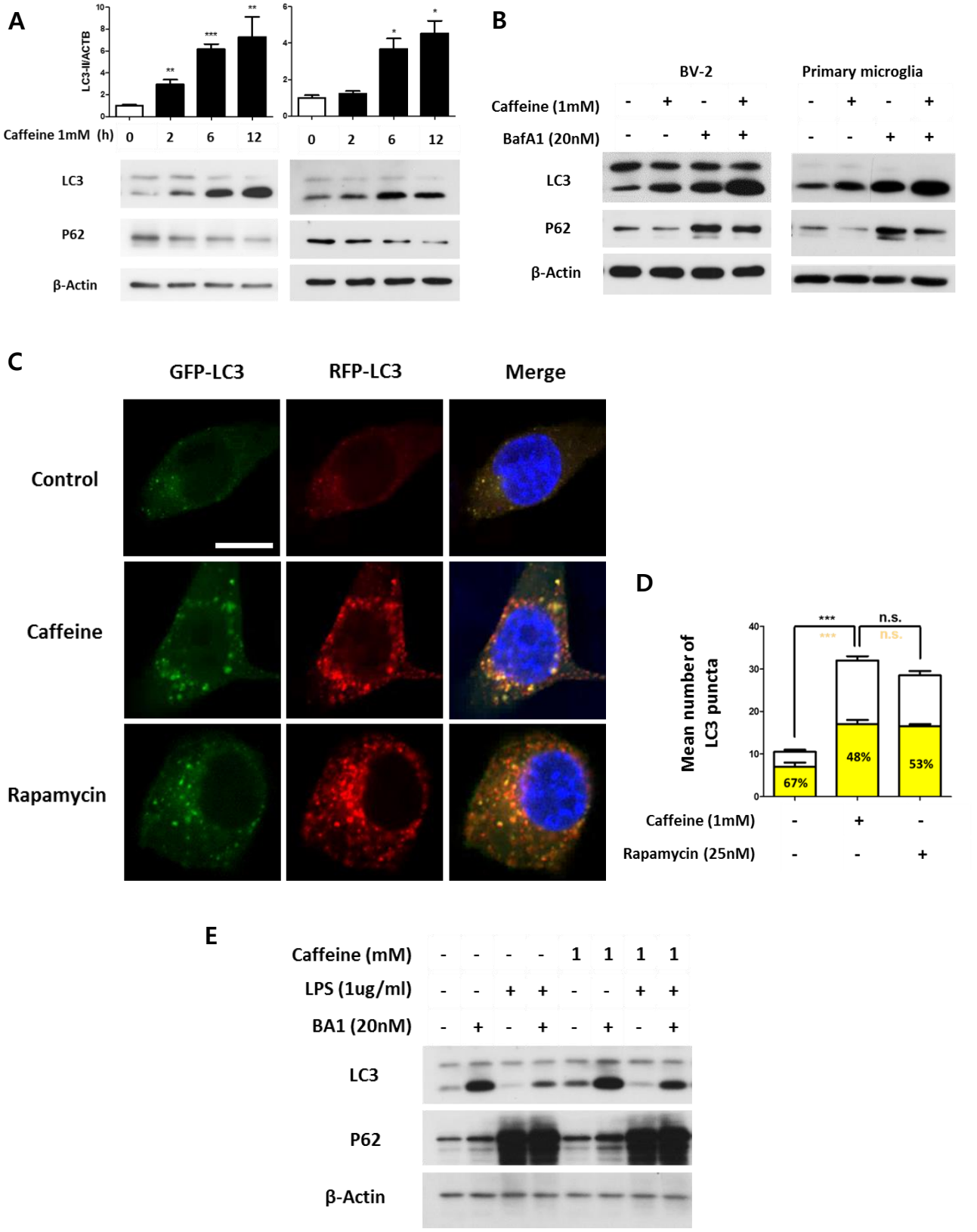


Figure 2. Caffeine induced autophagy in BV-2 cells and primary microglia.

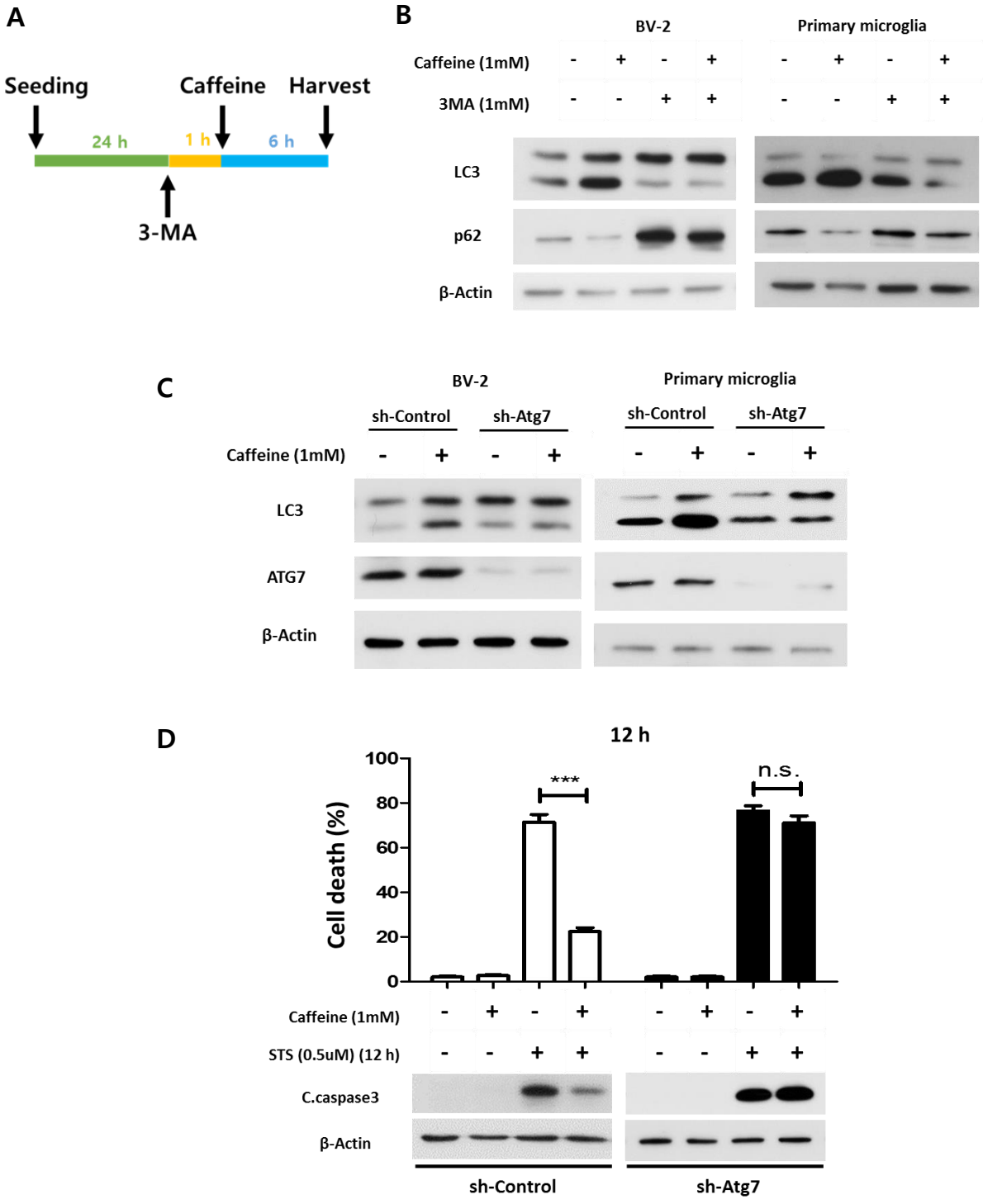


Figure 3. 3-MA and knockdown of Atg7 blocked caffeine-induced autophagy and anti-apoptotic effect.

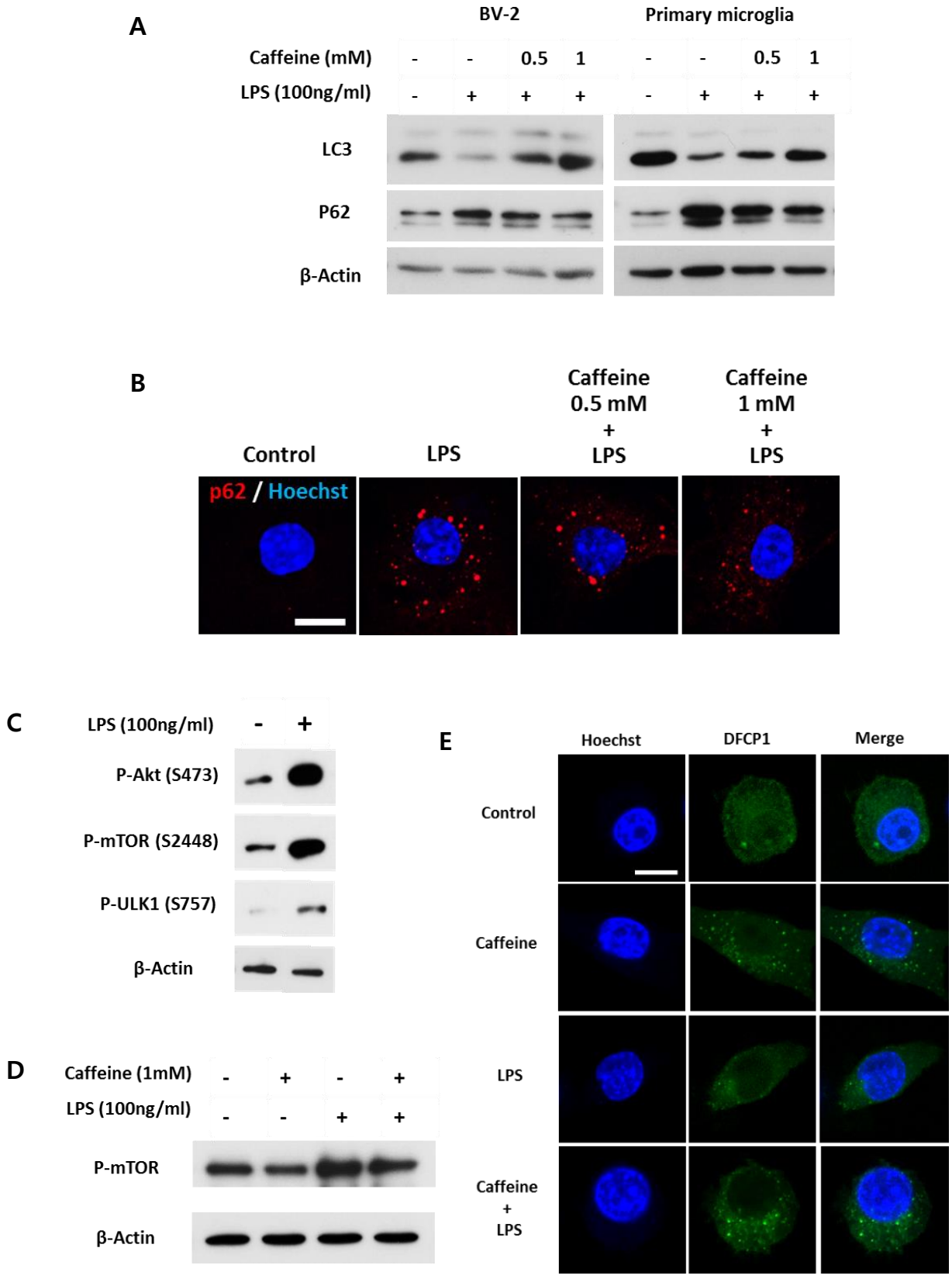


Figure 4. Caffeine rescued LPS-induced autophagic suppression.

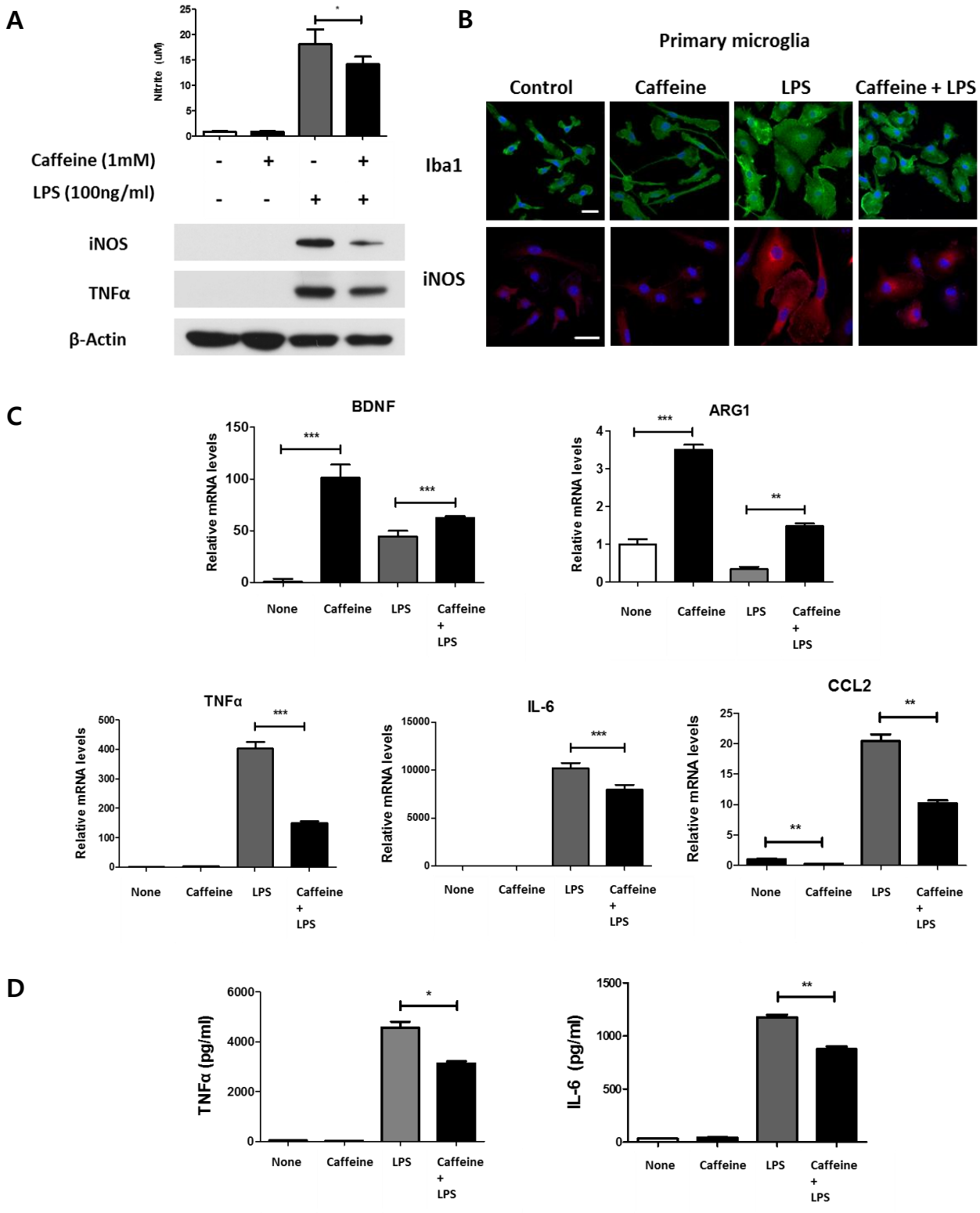


Figure 5. Caffeine ameliorated LPS-induced inflammation.

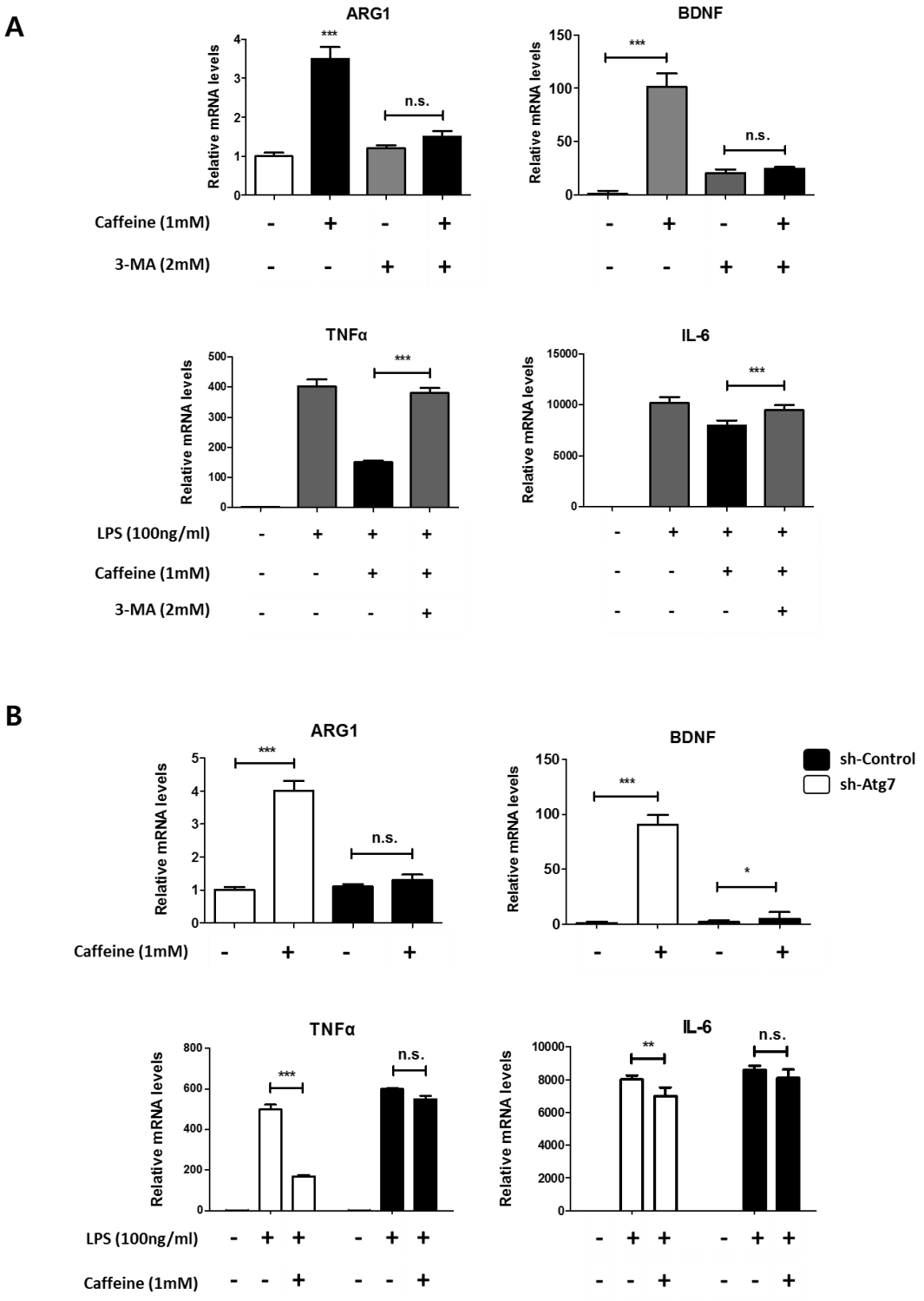


Figure 6. Ablation of autophagy blocked caffeine's anti-inflammatory effect.

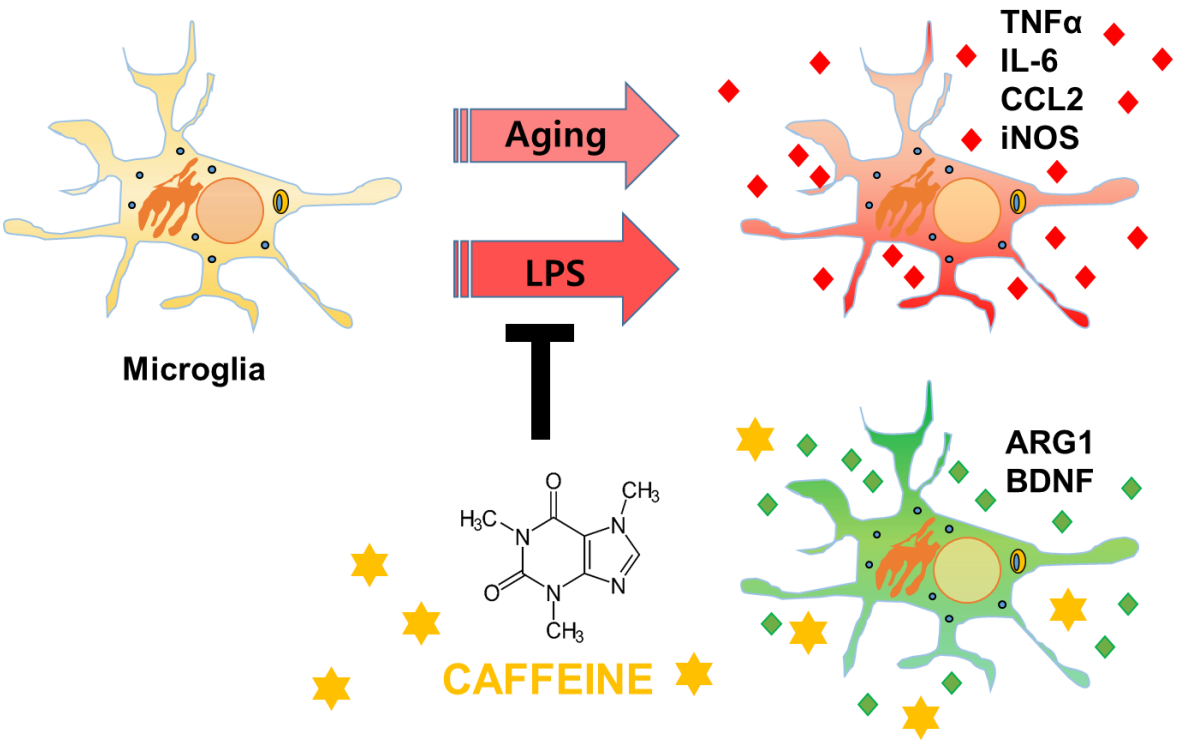


Figure 7. Schematic presentation illustrating anti-inflammatory effect of caffeine in microglia.

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요 약 문

우리 뇌에는 크게 신경세포와 비신경세포인 신경교세포(글리아세포)들이 있는데 신경교세포인 미세아교세포(마이크로글리아)는 중추신경계의 면역세포로서 말초신경계의 대식세포와 견주어진다. 미세아교세포는 주변환경의 변화와 염증에 민감하게 반응을 함으로서 신경독성의 지표로 잘 알려져있다. 활성화된 미세아교세포는 다양한 면역물질들을 분비하며 염증을 완화 또는 악화 시킬 수 있다.

카페인은 현대인들이 가장 자주 섭취하는 교감신경계 자극 물질로서 신진대사를 활발하게하고 적당한 양의 카페인은 염증을 감소시켜 뇌의 기억중추인 해마의 손상을 억제시킨다고 보고된 바 있다. 본 논문은 뇌자극제인 카페인이 미세아교세포에 미치는 영향을 다루었다. 카페인을 미세아교세포 배양 컬쳐에 넣어 주었을 경우 자가소화작용이 늘어남을 관찰하였다. 자가소화작용은 세포에 에너지를 보충하고, 손상된 세포를 수리하는 작업을 한다. 카페인의 자가소화작용을 늘리는 영향은 박테리아 내독소로 발생한 미세아교세포의 심각한 염증반응까지 완하시켜 주었다. 따라서, 미세아교세포에서의 카페인의 염증완화기전은 자가소화작용을 통한 것이라는 것을 본 논문이 밝힌는 바이다.

핵심어: 미세아교세포, 카페인, 뇌염증, 자가소화작용

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