Intracellular NAD^+ depletion promotes NLRP3 inflammasome activation

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Directed by Professor Je-Wook Yu

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2 년 동안의 학위 과정을 함께 해준 실험실 식구들께도 감사의 마음 전합니다. 항상 넓은 마음으로 저의 부족한 점을 채워 주시고, 확신이 부족했던 제가 학위 과정을 잘 마칠 수 있도록 도와주신 심도완 박사님께 감사드립니다. 제 마음을 다잡는 데 많은 도움을 주신 정민오빠, 항상 밝은 모습으로 저의 어려운 순간에 많은 도움을 주신 인화언니, 학위 초반 저에게 큰 힘이 되어 주신 은주언니, 배울 점이 정말 많은 박학다식 승환오빠, 실험실에 적응할 수 있도록 도와주신 보름언니, 친절하게 실험실 생활을 알려주신 은지언니, 즐거운 학위 생활을 함께 해준 성현오빠, 그리고 대학원 생활 동안 서로 힘이 되어 주었던 후배이자 친구인 준철오빠, 보금언니, 병준, 세영, 창준, 경서에게도 심심한 감사 전합니다.

마지막으로 언제나 변함없이 저를 믿고 응원해주시는, 저의 버팀목과 같은 아버지, 어머니께 감사의 인사를 드립니다. 아낌없는 사랑에 보답할 수 있도록 항상 노력하겠습니다. 그리고 부족한 누나를 항상 잘 따라주는 동생 지화, 언제나 누나가 곁에 있다는 걸 잊지 말고, 중요한 길에 서 있는 너의 앞날을 응원할게.

제가 가질 수 있었던 배움의 기회에 대한 책임을 지고, 더 나은 사람이 되도록 노력할 것을 약속드리며 다시 한번 깊이 감사의 말씀을 드립니다.

2021 년 여름

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ABSTRACT

Intracellular NAD^+ depletion promotes
NLRP3 inflammasome activation

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Nicotinamide adenine dinucleotide (NAD^+) is an essential cofactor to various redox reactions as an electron carrier in all living cells. NAD^+ plays important roles in fundamental biological processes including glycolysis, fatty acid β-oxidation, and ATP synthesis. In addition, NAD^+ is consumed by several NAD^+-consuming enzymes in non-redox reactions. A large number of studies demonstrated that the level of NAD^+ is declined as one ages and the reduction of NAD^+ level is associated with aged-related diseases which were known to be caused by excessive inflammation. Recently, studies of the association between disruption of NAD^+ homeostasis and inflammation have consistently emerged, however, the detailed mechanism underlying the effect of NAD^+ depletion on immune response remains poorly understood. Here, I examined the potential role of intracellular NAD^+ depletion in the innate immune response. My data demonstrated that reduced NAD^+ level promotes

NLRP3 inflammasome activation, indicated by the activation of caspase-1 in macrophages. My observation showed that intracellular NAD^+ depletion does not activate TLR, RLR pathway, and it is not related to NLRC4, AIM2 inflammasome activation. However, reduced NAD^+ level could promote the NLRP3 inflammasome activation as an activator of priming step. In addition, cleaved GSMDM, the effector molecule of programmed lytic cell death, was observed in NAD^+ depleted macrophages. Interestingly, I have also observed that the mitochondria move toward perinuclear region and generates the clustering, and the NLRP3 is translocated into mitochondria upon NAD^+ depletion in macrophages. Along with previous studies that mitochondria movement and NLP3 translocation is important factor in inflammasome activation, NAD^+ depletion could be a potential contributor in inflammasome activation.

Taken together, this finding clearly proposes that depletion of intracellular NAD^+ promotes NLRP3 inflammasome activation in macrophages. Moreover, therapeutic strategy to restore intracellular NAD^+ level might provide opportunities for patients suffering from age-related diseases.

Key words: aging, NAD^+, NLRP3 inflammasome, macrophage, pyroptosis

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I. INTRODUCTION

Aging is a complex and diverse process characterized by accumulation of various forms of damage at the molecular, cellular, and tissue levels, resulting in dysfunction of the body[1]. Aging is not a disease in itself, however it can greatly increase the risk of promoting or developing various disease, such as diabetes, cancer, cardiovascular diseases, neurodegenerative diseases, and others[2-5]. Accumulating evidences suggest that change of various characteristics, such as epigenetic alteration, genomic instability, and reprogrammed metabolism, are known to be dependent on aging[6]. In particular, intracellular nicotinamide adenine dinucleotide (NAD^+) levels decrease, and many enzymes, related to NAD^+ synthesis and degradation, are changed as one ages[7]. Decreased levels of intracellular NAD^+ in various tissues, including heart,

brain, liver, skeletal muscle, kidney, lungs, skin, and extracellular fluids, in aged individuals have been reported[8,9]. Furthermore, the NAD^+ reduction in diverse tissue has been associated with development and progression of aged-related diseases, such as diabetes, atherosclerosis, and cognitive decline[10]. Moreover, a serious of clinical trials testing the efficacy of NAD^+-boosting strategies in human diseases are being conducted[11,12].

Nicotinamide adenine dinucleotide (NAD^+) plays vital roles as an electron acceptor in redox reactions, including glycolysis, fatty acid β-oxidation, and ATP synthesis[8,13,14]. NADH, reduced form, serves as an essential hydride donor for ATP synthesis in energy metabolism[15]. In addition, NAD^+ acts a critical roles as a co-substrate for various enzymes including CD38, Poly (ADP-ribose) polymerases (PARPs), sirtuins (SIRTs), and others[16]. Accordingly, it is well-established that the decreased level of NAD^+ can, both directly and indirectly, influence major cellular processes including DNA repair, cellular senescence, and immune cell function[7]. In particular, studies demonstrating the correlation between intracellular NAD^+ level and inflammation have been published[17-19]. Some studies suggested that the boosting of intracellular NAD^+ level improves mitochondrial respiration and attenuates pro-inflammatory activity of PBMCs during heart failure (HF)[11]. However, another study suggested that increased NAD^+ level promotes IL-1β transcription and secretion in LPS-primed monocytes[20]. Thus, recent evidences have suggested that it could act both as a good or a bad contributor in innate immune system.

The innate immune system is the first protective defense line against invading pathogens, and this system is mainly induced by myeloid cells, including macrophages and dendritic cells (DCs)[21,22]. These cells have pattern recognition receptors (PRRs) expressed on the cell surface or in the cytoplasm, and recognize the

pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs)[23,24].There are several types of PRR, including the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Retinoic acid inducing gene (RIG)-I-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)[25]. Tissue injury or pathogen infection can activate Inflammasome, cytosolic multiprotein complex, consisting of a particular set of sensor proteins[26,27]. It is well-established that there are five such sensor proteins, such as NOD-like receptors (NLRs), absent in melanoma 2 (AIM2), and pyrin[28,29]. Activation of these sensor molecules leads to the cleavage of caspase-1, which subsequently processes inactive forms of pro IL-1β, pro IL-18 and, gasdermin D (GSDMD) into active forms[30-32]. In particular, the cleaved N-terminal GSDMD fragment oligomerizes in the plasma membrane to form membrane pores, through which the secretion of mature IL-1β or IL-18 is induced[33]. GSDMD pore-mediated release of these cytokines is known to be responsible for the initiation of inflammation[34]. Furthermore, GSDMD pore triggers plasma membrane rupture and pyroptosis, the inflammatory caspase-dependent lytic cell death[30,34].

Previous studies reported that NLRP3 recognizes DAMPs, such as ATP, amyloid β and uric acid, triggering NLRP3 inflammasome activation[35-37]. However, if the inappropriate NLRP3 inflammasome activation by deposited DAMPs cause excessive inflammation, it will result in various inflammatory metabolic diseases including type 2 diabetes, atherosclerosis, and neurodegenerative diseases[38-41]. The current mechanism suggests that NLRP3 inflammasome is activated by potassium efflux, mitochondrial reactive oxygen species (ROS), and rupture of lysosome in response to various stimuli[42-44]. Accumulating studies have shown that NLRP3 is assembled and activated by alternating the intracellular environment that occur in response to various stimuli[45,46]. Although a large number of studies have been

conducted on NLRP3 inflammasomes, many unknown challenging areas still remain poorly obscure.

Previous studies suggest that depleted intracellular NAD^+ level leads to stimulate inflammasome activation, promoting secretion of IL-1β which is pro-inflammatory cytokine in macrophages[47,48]. Moreover, NAD^+ precursor including NAM, NMN, and NR could reduce pro-inflammatory effect[49]. However, the exact role of NAD^+ in inflammasome activation in myeloid cell is remains still unclear. Therefore, I investigate potential role of depleted NAD^+ level on NLRP3 inflammasome assembly or activation in macrophage.

II. MATERIAL & MATHODS

1. Mice

C57BL/6 and NLRP3 -/- mice were from The Jackson Laboratory. All mouse strains were maintained at Yonsei University College of Medicine under specific pathogen-free conditions. All experimental procedures were administered by the Institutional Ethical Committee, Yonsei University College of Medicine. The procedures for animal experiments were performed in accordance with the Guide for care and Use of the Institutional Ethical Committee.

2. Cell cultures

Mouse primary bone marrow-derived macrophages (BMDMs) were isolated from femurs of C57BL/6, NLRP3 $^{-/-}$ mice. Bone marrow progenitor cells were maintained in 8% L929-conditioned DMEM (Corning, NY, USA) supplemented with 10% fetal bovine serum (Gibco), and 100 U/mL penicillin and streptomycin (Gibco, Gran Island, NY, USA). Culture medium was replaced with fresh 5% L929-conditioned DMEM containing 10% FBS and antibiotics 3 days after seeding. Then, enriched cells were detached by 0.25% trypsin-EDTA and plated appropriate plates with 5% L929-conditioned DMEM. Immortalized NLRP3-GFP expressing BMDMs were maintained in L929-condituined DMEM.

3. Reagents and Antibodies

FK866, LPS, Nig, ATP, poly (dA:dT) and poly(I:C) were purchased from Sigma-Aldrich (St. Louis, MO). Flagellin purified from P. aeruginosa was obtained from Invivogen (San Diego, CA, USA). Monoclonal anti-mouse caspase-1 and anti-mouse NLRP3 antibody were purchased from Adipogen (San Diego, CA, USA). Rabbit monoclonal anti-ASC antibody and Anti-mouse ERK1/2 antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Polyclonal Anti-mouse IL-1β antibody was obtained from R&D Systems (Minneapolis, MN, USA). Anti-mouse GSDMD antibody was purchased from Abcam. Monoclonal anti-mouse β-actin antibody was purchased from Santa Cruz (Santa Cruze, CA, USA). Anti-VDAC1 antibody was purchased from abcam (Cambridge, UK).

4. Cell death analysis

Cell were plated at 24 well plate. After appropriate treatment, supernatant was removed and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added to all wells of an assay, and incubated at 37°C for 2 hr. Then the sup was removed and add DMSO for dissolving the formazan. After few minutes, OD value of the plates were read at 570 nm.

5. Immunoblot analysis

Cells were lysed in 20 mM HEPES (pH 7.5) buffer supplemented with 1.5 mM MgCl2, 150 mM NaCl, 50 mM KCl, 1 mM EGTA, 0.5% Non-idet P-40, and protease inhibitors. Cell culture supernatants were obtained after inflammasome stimulation.

Proteins of the supernatants were precipitated by extraction method using methanol/chloroform mixture. The Soluble lysates and proteins of supernatants were seperated by SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 3% skim-milk in PBS contained 0.1% Tween 20 for 30 min. After blocking, the membranes were incubated with appropriate antibodies at 4°C.

6. Assay of inflammasome activation

To stimulate NLRP3 inflammasome activation, mouse BMDM were treated with LPS (100 ng/ml, or 0.25 ug/ml, for 3 hr) followed by the treatment of ATP (3 mM, 1 hr), nigericin (5 μM, 1 hr). To activate NLRC4 inflammasome, BMDMs were transfected with poly(dA:dT) (1 μg/ml, 2 hr), and to activate AIM2 inflammasome, BMDMs were transfected with Flagellin(250 ng/ml, 2 hr) using lipofectamine. Inflammasome activation was quantified by evaluating extracellular IL-1β using a Quantikine IL-1β ELISA Kit (R&D Systems) and detected by the presence of bands of active caspase-1 (p20) and active IL-1β in immunoblots.

7. Intracellular NAD^+ quantification

To measure intracellular NAD^+ level, the cells were plated at 96-well pate. After appropriate treat, the medium was removed and replaced with PBS. NAD/NADH-Glo™ Detection Reagent (Promega) was added to each well, and shake the plate

carefully for lysing cells. The plates were incubated at room temperature for 60 mins, and luminescence was recorded using a luminometer.

8. Determination of inflammasome oligomerization

To determine ASC oligomerization, treated cells were lysed in a 20 mM HEPES (pH 7.5) buffer supplemented with 0.5% Non-idet P-40, 50 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA and protease inhibitors and centrifuged at 12,000 rpm for 10 min. and the cells were syringed 10 times through a G26 needle on ice. The cell lysates were centrifuged at 6000 rpm at 4℃ for 10 min. Pellets were resuspended in PBS and crosslinked with disuccinimidyl suberate (DSS) (2 mM) (Thermo Scientific-Pierce, Rockford, IL, USA). The cross-linked pellets were centrifuged at 13000 rpm for 15 min and dissolved directly in SDS sample buffer.

9. Immunofluorescence

Cells were plated on coverslip in a 12-well or 24-well plates. The cells were fixed with 4% formaldehyde and permeabilized using 0.2% Triton X-100. Then, blocking with 4% BSA was performed. Cells were then observed by confocal microscopy (Zeiss, LSM700).

10. Quantification of mRNA production

To quantification of mRNA production, total mRNA was extracted by TRIzol regent (Invivogen), and reverse transcription was performed using PrimScript RT Master Mix (Takara) according to the manufacturer's protocol. Template cDNA was

amplified using SYBR Premix Ex Taq (TaKaRa) by quantitative real-time PCR. All primers were obtained from Genotech (Daejeon, Korea).

11. Fractionation

Cells were plated on 60π dish. After adequate treatment, cells were lysed in buffer A including 10 mM KCl, 2 mM MgCl2, 250 mM sucrose, 10mM Hepes (pH 7.8), 1 mM DTT, and protease inhibitors. The lysates were centrifuged at 700 g for 10 min. Following this, supernatants were centrifuged at 12,500 g for 10 min. Then, the supernatants were used as cytosolic fraction. The pellets were washed and re-suspended in NL buffer containing composition of cell lysis buffer from above and 0.06% NP-40, and used for membrane-enriched fraction.

12. Statistical analysis

All values were expressed as the mean SEM of individual samples. Data were analyzed using t-test or one-way ANOVA with a bonferroni post-test. The p values $\leq$ 0.05 were considered significant. Analyses were performed using GraphPad Prism.

Table 1. Primers used for PCR

Gene	Primer sequence
Mouse IL-6	Forward: 5′ - AGT TGC CTT CTT GGG ACT GA -3′
	Reverse: 5′ - TCC ACG ATT TCC CAG AGA AC -3′
Mouse IL-1β	Forward: 5’- GCC CAT CCT CTG TGA CTC AT -3’
	Reverse: 5’- AGG CCA CAG GTA TTT TGT CG -3’
Mouse TNF-α	Forward: 5’-CGT CAG CCG ATT TGC TAT CT-3’
	Reverse: 5’-CGG ACT CCG CAA AGT CTA AG-3’
Mouse Rn18s	Forward: 5′- CGC GGT TCT ATT TTG TTG GT -3′
	Reverse: 5′- AGT CGG CAT CGT TTA TGG TC -3′

III. RESULT

1. Intracellular NAD^+ reduction does not affect PRR-mediated cytokine production

Previous studies demonstrated that nicotinamide phosphoribosyl transferase (NAMPT) is the rate limiting enzyme for salvage pathway of NAD^+ biosynthesis[50]. FK866 is known as a potent chemical inhibitor of NAMPT[51]. To induce reduction of intracellular NAD^+ level in bone marrow-derived macrophages (BMDMs), BMDMs were treated with FK866 for 4, 8, 12, and 21 hr (Fig 1A). Inhibition of NAMPT activity with FK866 significantly induces time-dependent depletion of intracellular NAD^+ level and about 8% of intracellular NAD^+ level was observed after 21 hr in FK866-treated BMDMs.

To investigate the effect of intracellular NAD^+ depletion on TLR-dependent cytokines production and secretion in BMDMs, I pre-treated BMDMs with FK866 for 18 hr followed by LPS, a TLR4 agonist, treatment for 3 hr. To assess the mRNA expression level of pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α, (Fig 1B, C and D). The data showed that NAD^+ depletion with FK866 failed to affect the production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. Next, I further explored whether the reduction of intracellular NAD^+ level affect secretion of pro-inflammatory cytokines including IL-6 and TNF-α (Fig 1E, F). The results demonstrated that NAD^+ depletion by FK866 did not trigger the secretion of IL-6 and TNF-α. These findings clearly showed that NAD^+ depletion with FK866 does not affect TLR-mediated cytokine production in BMDMs.

Next, to verify the effect of intracellular NAD^+ depletion on production and secretion of RLR-mediated cytokines in BMDMs. BMDMs were pre-treated with

FK866 for 21 hr, followed by transfection of poly(I:C) for 3 hr. FK866-mediated NAD^+ depletion impaired both the mRNA expression of IL-6 (Fig. 2A) and secretion of IL-6 (Fig. 2B) in BMDMs. In accordance with these data, FK866 treatment does not influence on poly(I:C) transfection-triggered inflammasome activation mediated by RIG-I in BMDMs.

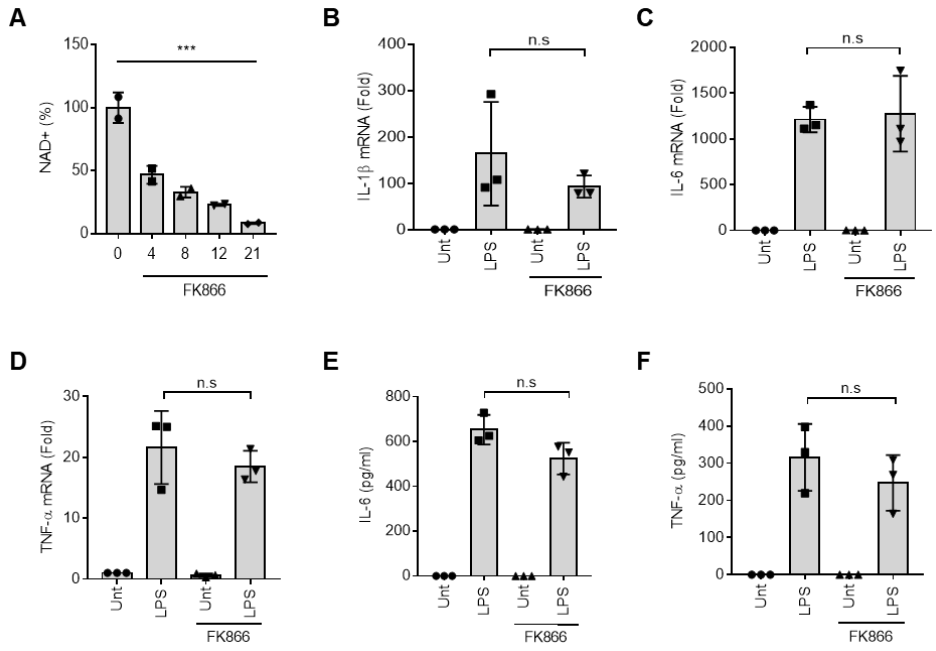

Figure 1. NAD$^+$ depletion does not influence Toll-like receptor induced inflammatory cytokines production. (A) Intracellular NAD$^+$ level was measured using the NAD$^+$ quantification kit in BMDMs treated with FK866 (100 nM) for 4, 8, 12, 21 hr (n=2). BMDMs were pre-treated with FK866 (100 nM) for 18hr, followed by LPS (100 ng/ml) treatment for 3 hr. (B) IL-1β, (C) IL-6, and (D) TNF-α mRNA levels were measured by quantitative polymerase chain reaction (qPCR) in treated BMDMs. The changes in the levels of each mRNA were normalized to the Rn18s and then fold-changes were calculated in comparison to corresponding untreated cells (n=3). (E) IL-6, (F) TNF-α levels were measured by enzyme-linked immunosorbent assay (ELISA) in the culture supernatants of treated BMDMs (n=3). Statistical significance was analyzed by one-way ANOVA with a bonferroni post-test (n.s., not significant, ***P<0.001).

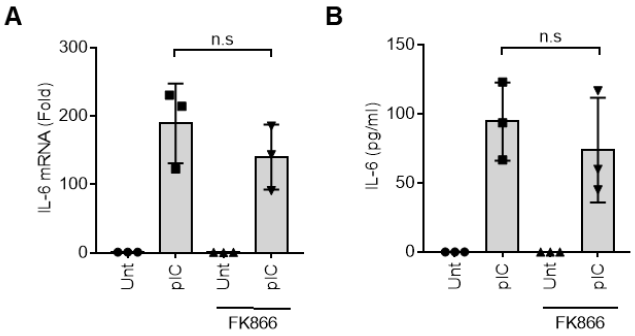

Figure 2. IL-6 production via RIG-I-like receptor pathway does not change in response to NAD^+ depletion. BMDMs were pre-treated with FK866 (100 nM) for 21 hr, followed by transfection of poly(I:C) (1 μg/ml) for 3 hr. (A) mRNA expression level of IL-6 was measured by qPCR in treated BMDMs (n=3), (B) IL-6 secretion level was determined by ELISA in the culture supernatants of treated BMDMs (n=3). The changes in the levels of each mRNA were normalized to the Rn18s and then fold-changes were calculated in comparison to corresponding untreated cells. Statistical significance was analyzed by one-way ANOVA with a bonferroni post-test (n.s., not significant).

2. NAD^+ depletion functions as a priming signal for the NLRP3 inflammasome activation

To ascertain whether the intracellular NAD^+ reduction affect NLR-mediated inflammasome activation and caspase-1 processing, I use various known inflammasome activators including LPS primed BMDM followed by ATP (NLRP3 activator), poly(dA:dT) (AIM2 activator), and Flagellin (NLRC4 activator) [52-54]. First, I evaluated the potential implication of the NAD^+ decline with FK866 on NLRP3 inflammasome activation in BMDMs. BMDMs were pre-treated FK866 for 21hr, followed by LPS, ATP treatment. When LPS and ATP were treated in FK866 treated BMDMs, there was no difference in activation of caspase-1 and processing of IL-1β in supernatant (Fig. 3A). Next, I pre-treated FK866 for 21 hr, followed by transfection with poly(dA:dT) and Flagellin to activate NLRC4, AIM2 inflammasome in BMDMs. There were no differences in processing cleavage of caspase-1 in culture sup and cleavage of GSDMD of cell lysate in BMDMs (Fig. 3B). It suggested that the NAD^+ depletion did not affect the NLRC4 and AIM2 inflammasome.

Even if the exact mechanism of NLRP3 inflammasome activation is still unclear, it is well-established that priming step (signal 1) and activation step (signal 2) is required for NLRP3 inflammasome activation[37,42]. Therefore, whether the intracellular NAD^+ depletion promotes the activation of priming signal or activation step. First, to examine the effect of the NAD^+ depletion on priming step, I treated BMDMs with FK866, followed by the stimulation of ATP and Nigericin, stimuli of signal 2 (Fig. 4A, B). The data showed an active form of caspase-1 in supernatant, and cleavage of GSDMD in lysates in FK866/ATP or Nig-treated BMDMs. However, the Pro-IL-1β expression and quantitative increase of NLRP3 expression were not detected in the following BMDMs. It demonstrated that the intracellular NAD^+

depletion is associated with the priming signal of NLRP3 inflammasome activation, but not the transcription of inflammatory genes in priming signal.

Next, to investigate whether the intracellular NAD^+ reduction could affect signal 2 step of NLRP3 inflammasome activation. BMDMs were stimulated with LPS followed by FK866 (Fig. 5A). The data showed that cytosolic NAD^+ depletion failed to affect the NLRP3 inflammasome activation in primed BMDMs. These findings collectively indicated that intracellular NAD^+ depletion with FK866 might have a role of priming step for NLRP3 inflammasome activation.

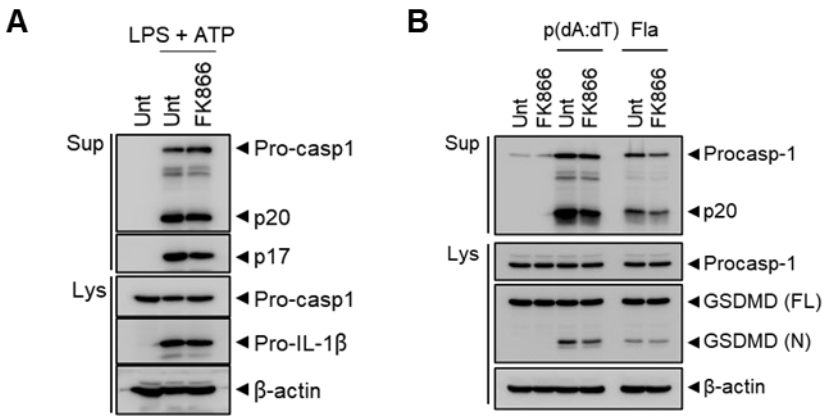

Figure 3. NAD$^+$ depletion does not increase the amount of cleaved caspase-1. (A) Immunoblots of BMDMs pre-treated with FK866 (100 nM) for 21 hr. Then the cells were treated with LPS (100 ng/ml) for 3 hr followed by ATP (3 mM) stimulation for 1 hr. (B) Immunoblots of BMDMs pre-treated with FK866 (100 nM) for 21 hr. Then the cells were transfected with poly(dA:dT) (1 μg/ml) and *P. aeruginosa* flagellin (PA-FLA, 0.25 μg/ml). Cell culture Supernatants(sup) and Cell lysates (Lys) were immunoblotted with the indicated antibodies. (Unt: untreated, FK: FK866, ATP: Adenosine triphosphate, Pro-casp1: pro-caspase-1, p20: active caspase-1, p17: mature IL-β)

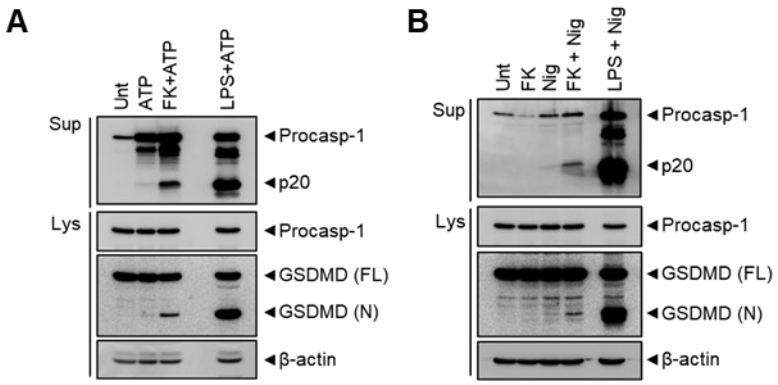

Figure 4. NAD^+ depletion promotes the activation of NLRP3 inflammasome. (A) Immunoblots of BMDMs pre-treated with FK866 (100 nM) for 24 hr. Then the cells were treated with ATP (3 mM) for 1 hr. (B) Immunoblots of BMDMs pre-treated with FK866 (100 nM) for 21 hr. Then the cells were stimulated with Nig (5 μM) for 1 hr. Cell culture Supernatants(sup) and Cell lysates (Lys) were immunoblotted with the indicated antibodies. (Nig: nigericin)

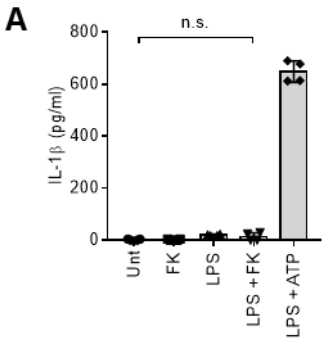

Figure 5. NAD$^+$ depletion does not stimulate activation step of NLRP3 inflammasome. (A) BMDMs were pre-treated with LPS (0.25 μg/ml) for 3 hr. Then the cell was stimulated with FK866 for 21 hr. BMDMs primed with LPS (0.25 μg/ml, 3 hr), followed by treatment with ATP (3 mM) for 1 hr were used as a positive control. Secreted IL-1β levels were measured by ELISA in the culture supernatants of treated BMDMs (n=4). Statistical significance was analyzed by one-way ANOVA with a bonferroni post-test

3. NAD^+ depletion in macrophages leads to caspase-1 activation in NLRP3-dependent manner

To determine whether the observed events were specific on a NLRP3-depenent manner, BMDM isolated from *Nlrp3*$^{+/+}$ or *Nlrp3*-deficient mice were used. First, I checked that intracellular NAD^+ level in *Nlrp3*$^{+/+}$ and *Nlrp3*-deficient BMDMs were properly depleted by FK866 (Fig 6A). As a result, the depletion of NAD^+ by FK866 is not related to *Nlrp3*-deficient conditions. To determine whether the observed events were specific on a NLRP3-depenent manner, BMDMs were treated with FK866, followed by ATP/Nig (Fig. 6B, C) As expected, Intracellular NAD^+ depletion triggered active caspase-1 and cleaved GSDMD in WT BMDMs, but not in NLRP3-deficient BMDMs.

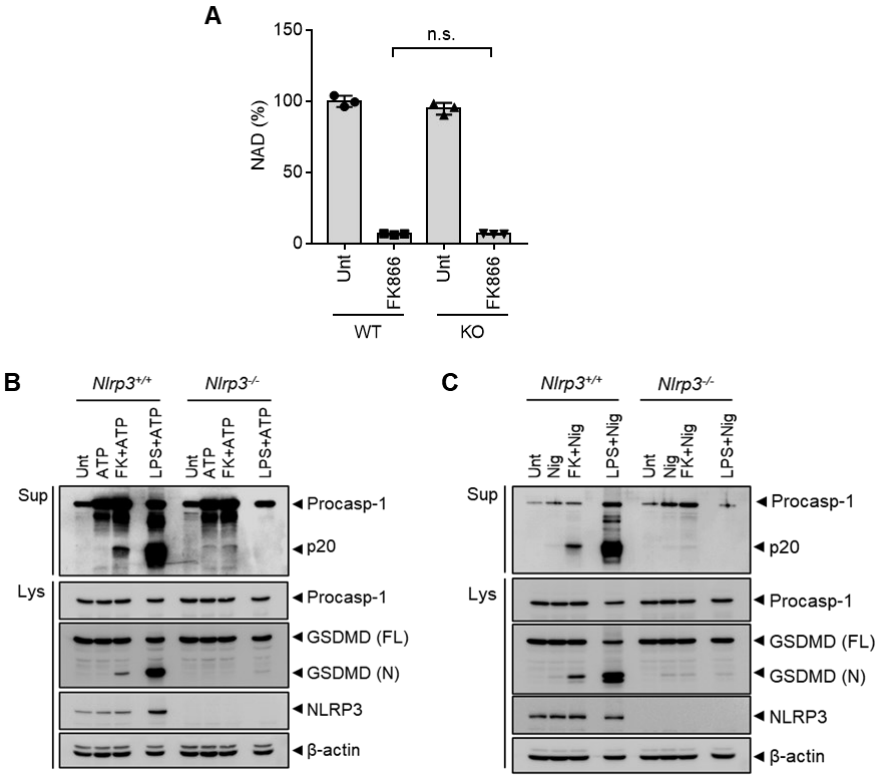

Figure 6. NAD^+ depletion mediates NLRP3-dependent caspasae-1 activation. (A) Intracellular NAD^+ level was measured using the NAD^+ quantification kit in BMDMs isolated from *Nlrp3*$^{+/+}$ or *Nlrp3*$^{-/-}$ mice treated with FK866 (100 nM, 21 hr) (n=3). (B) BMDMs isolated from *Nlrp3*$^{+/+}$ or *Nlrp3*$^{-/-}$ mice were treated with FK866 for 21 hr, followed by stimulation of ATP (3 mM) for 1 hr. (C) *Nlrp3*$^{+/+}$ or *Nlrp3*$^{-/-}$ BMDMs were stimulated with FK866 for 21 hr, followed by treatment of Nig (5 μM) for 1 hr. Cell culture Supernatants(sup) and Cell lysates (Lys) were immunoblotted with the indicated antibodies.

4. Intracellular NAD^+ depletion promotes the assembly of the NLRP3 inflammasome

As previous studies reported that the activation NLRP3 inflammasome is required the oligomerization of NLRP3 and the subsequent recruitment of ASC (apoptosis-associated speck-like protein containing a CARD) to NLRP3[55]. Therefore, I confirmed whether the NAD^+ depleted BMDMs could induce the formation of ASC speck by ATP/Nigericin. BMDMs were pre-treated with FK866, followed by ATP/Nig (Fig. 7A, B). The results showed that FK866-treated BMDMs induce the ASC oligomerization by ATP/Nig. Also, I detected the formation NLRP3 speck-like aggregates in NLRP3-GFP expressing BMDMs followed by the FK866 and Nigericin (Fig. 8A). These results collectively suggest that NAD^+ depleted macrophage without signal 1 activator can induce the ASC oligomerization and NLRP3 assembly by just only signal 2 activator, and that might play a major role in the initiation of pro-inflammatory responses.

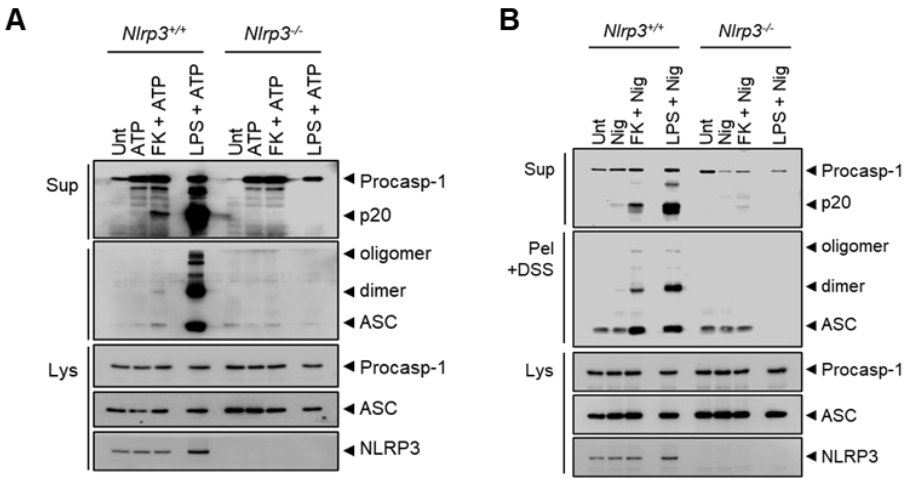

Figure 7. NAD$^+$ depletion induces ASC oligomerization. (A) BMDMs isolated from *Nlrp3*$^{+/+}$ or *Nlrp3*$^{-/-}$ mice were treated with FK866 (100 nM) for 21 hr, followed by the treatment with ATP (3 mM) for 1 hr. (B) BMDMs isolated from *Nlrp3*$^{+/+}$ or *Nlrp3*$^{-/-}$ mice were treated with FK866 (100 nM) for 21 hr, followed by the stimulation of Nig (5 μM) for 1 hr. Immunoblots of culture supernatants (Sup), disuccinimidyl suberate (DSS)-crosslinked pellets (DSS-pel), cellular lysates (Lys) from BMDMs with the indicated antibodies.

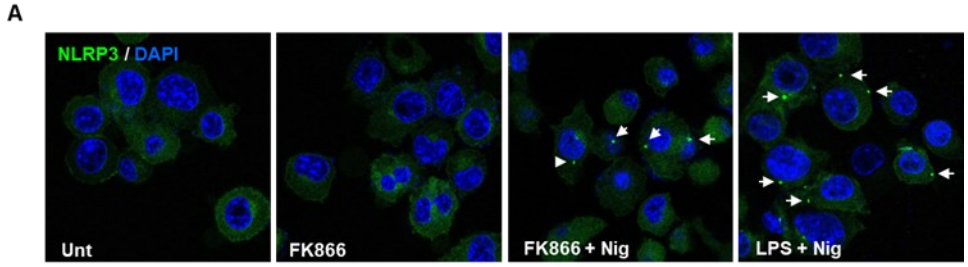

Figure 8. NAD^+ depletion generates NLRP3 specks in NLRP3-GFP expressing macrophages. (A) Representative immunofluorescence image of NLRP3-GFP-expressing BMDMs were treated with FK866 (10 nM, 21 hr), followed by the treatment of Nig (5 μM, 1 hr). The cells primed with LPS (100 ng/ml, 3 hr), followed by treatment with Nig (5 μM) for 1 hr were used as a positive control. DAPI represents the nuclear signal (blue).

5. Intracellular NAD^+ depletion mediates NLRP3 localization and perinuclear clustering of mitochondria

Accumulating evidence has suggested that the position of NLRP3 and mitochondria is critical factor for NLRP3 inflammasome activation[56-58]. Thus, I identified the change in position of NLRP3 and mitochondrial that occurs in NAD^+ depleted macrophages. The results showed that NLRP3 is translocated from cytosol to mitochondria upon NAD^+ depletion in macrophages (Fig 9A). Moreover, Mitochondrial movement is detected in NAD^+ depleted BMDMs, like as LPS-primed BMDMs (Fig 10A). Interestingly, the perinuclear clustering of mitochondria is formed (Fig 10B, C). These results collectively suggest that NAD^+ depleted macrophage induce both NLRP3 and mitochondria translocation, indicating a potential contribution of NAD^+ depletion to inflammasome activation.

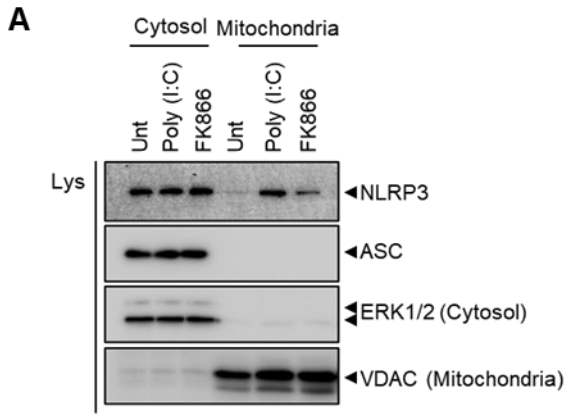

Figure 9. NAD^+ depletion promotes the co-localization of NLRP3 and mitochondria. (A) BMDMs were treated with FK866 (100 nM) for 4 hr or Poly (I:C) (100 μg/ml) for 4 hr. Cell lysates were fractionated into cytosolic or mitochondrial fraction, and Cell lysates (Lys) were immunoblotted with the indicated antibodies.

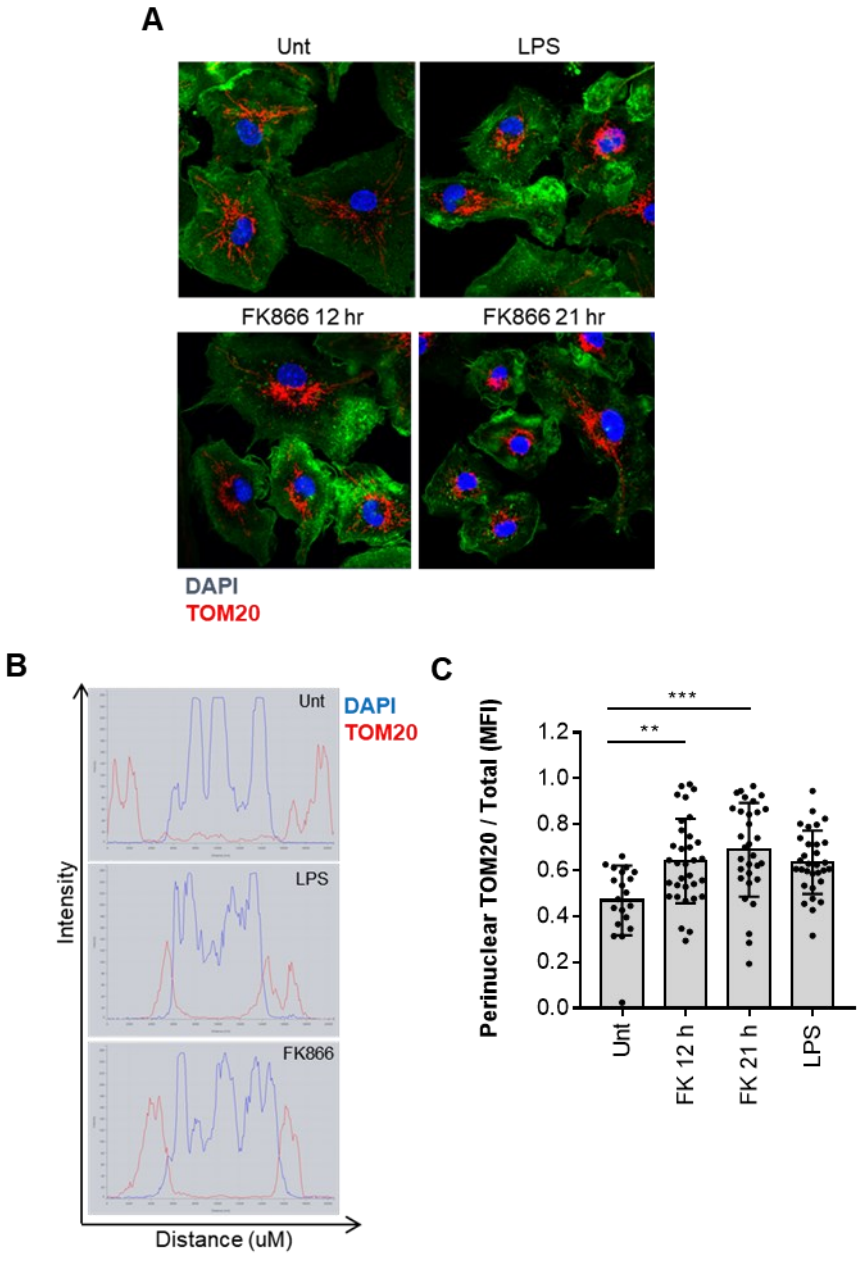

Figure 10. NAD^+ depletion induces perinuclear accumulation of mitochondria. BMDMs were stimulated with FK866 for 12, 21 hr or LPS (0.25 μg/ml) for 3 hr, after staining with TOM20 (red). DAPI represents the nuclear signal (blue). (A) Representative immunofluorescence images of BMDMs were observed by confocal microscopy. (B) Intensity profiles of nucleus and mitochondria along an ideal straight line crossing the nucleus in BMDMs. (C) Mean Fluorescence Intensity (MFI) of perinuclear clustering of mitochondria per total MFI of mitochondria (*p<0.00332, **p< 0.0021, ***p<0.00021, n=20-33, n.s., not significant.)

IV. DISCUSSION

Recent studies suggested that the decline of NAD^+ level has emerged as a multiple hallmark of aging[9]. Furthermore, mounting evidence reported that reduction of NAD^+ level is detected aged-related disease including Alzheimer's disease, cancer, atherosclerosis, and cognitive decline[44,59,60]. These diseases have been linked to excessive immune responses[38-41]. However, the mechanism underlying how NAD^+ affect immune response in immune cell is yet to be defined. In this study, I tried to find out potential role of NAD^+ depletion on immune response.

At first, I demonstrated that whether intracellular NAD^+ depletion with FK866 is involved in the TLR, RLR, and NLR signaling activation in BMDMs. Although some previous studies suggested that NAD^+ depletion impairs TLR signaling via inhibiting NF-kB signaling in response to Lipopolysaccharide[20], My data demonstrated that NAD^+ depletion does not influence the production of pro-inflammatory cytokines upon TLR and RLR signaling in macrophages. These opposing results could be explained by using different types of cells.

Previous studies reported that SIRT-deficient macrophages, NAD^+ consuming enzyme, induce NLRP3 inflammasome activation and secretion of pro-inflammatory cytokines[47,61]. Therefore, I confirmed whether intracellular NAD^+ depletion affects the activation of inflammasomes including NLRP3, AIM2, NLRC4 in macrophages. First of all, I focused on the effect that intracellular NAD^+ regulates activation of various types of inflammasomes in macrophages. My data indicates that regulation of inflammasome activation is not related to the reduction of NAD^+. Previous studies suggested that NLRP3 could be activated by various intracellular signal[45,46], thus, I confirmed that whether the NAD^+ depletion promotes activation of NLRP3 inflammasome. My data showed that active caspase-1 was detected in macrophages

upon FK866 plus ATP or nigericin stimulation. However, matured IL-1β is not detected upon FK866 in LPS-primed macrophages. It indicates that the intracellular NAD^+ depletion could play as an activator of priming step, not activation step. Although the NAD^+ depletion induces NLRP3 inflammasome activation, there is no difference in regulation of NLRP3 inflammasome activation. One possible explanation could be that the activation of NLRP3 inflammasome by NAD^+ depletion is not strong. Next, by using NLRP3-deficient macrophages, the activation of caspase-1 by NAD^+ depletion is induced in NLRP3-dependent manner was identified.

Recent studies reported that pyroptotic cell death is mediated by GSDMD pores, consisting of cleaved GSDMD-NT in caspase-1-dependent manner[31,33]. Emerging evidences have proposed putative association between pyroptosis and diseases, such sepsis and autoimmune diseases[62,63]. In this study, cleaved GSDMD were observed in macrophages following FK866 plus ATP or Nig. In this regard, my data proposed novel evidence that NAD^+ depletion in macrophage could be a potential factor in macrophage pyroptosis.

It has been reported that NLPR3 is located in the cytosol or ER of THP-1 cells and BMDMs in resting conditions. However, upon activation of NLRP3 inflammasome, translocation of NLRP3 to the perinuclear space and NLRP3 inflammasome localization on mitochondria-associated ER membranes (MAMs) occurs[56-58]. In addition, previous studies suggested that intracellular NAD^+ plays as a substrate to SIRT2, SIRT3, related to microtube dynamics[48,64]. It is well-established that the mitochondria is translocated via microtubule[65], thus, I identified the mitochondrial movement and NLRP3 localization to check mechanism for NAD^+ depletion-mediated caspase-1 activation. My observation shows that the intracellular NAD^+ depletion promotes mitochondrial mobility and generates perinuclear clustering of

mitochondria as an LPS-primed macrophages. Moreover, it was observed that NLRP3 translocation from cytosol to mitochondria in NAD^+ depleted macrophages. The molecular mechanism underlying the mitochondrial movement and NLRP3 localization for NLRP3 inflammasome activation is not fully understood at present. However, me, and others previously observed that mitochondria move around the nucleus during NLRP3 inflammasome activation. Although it is currently difficult to investigate how the mitochondrial clustering in perinuclear region triggers the activation of NLRP3 inflammasome, further studies will be required to elucidate the mechanism of the association between mitochondria and NLRP3 inflammasome in detailed.

Numerous studies suggest that the aged individuals with low intracellular levels of NAD^+ have a high incidence rate of developing inflammatory disease. Considering that inflammasome activation occurs mostly in myeloid cells, the NAD^+ depletion in macrophages could be an adequate trigger on immune response involving NLRP3 inflammasome signaling. Collectively, this finding indicates that the declined level of NAD^+ as one ages plays a critical role in the NLRP3 inflammasome signaling in myeloid cells. With careful scientific evaluation, the investigation of maintenance NAD^+ level in aged people will provide appropriate clinical benefits for age-associated disorders.

V. CONCLUSION

Here, I demonstrated that the potential role of intracellular NAD^+ depletion on NLRP3 inflammasome signaling in macrophages. NAD^+ depleted macrophage is sufficient to trigger caspase-1 activation with signal 2 stimuli for NLRP3 inflammasome. Furthermore, I observed that the immunostimulant effect of intracellular NAD^+ depletion was specific to NLRP3 inflammasome by using the NLRP3-deficient macrophages. The NAD^+ depletion failed to induce the transcription of inflammatory genes, However, pyroptosis, a programmed lytic cell death, is induced by pores caused by cleaved GSDMD upon caspase-1 activation in NAD^+ depleted macrophages. Interestingly, it is observed that NLRP3 is translocated into mitochondria and mitochondria is move toward perinuclear region in NAD^+ depleted macrophages. Taken together, these observations propose that the intracellular NAD^+ depletion plays a crucial role in NLRP3 inflammasome signaling and boosting NAD^+ level could be a promising therapeutic strategy aging and age-related disease.

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ABSTRACT (IN KOREAN)

세포 내 NAD+ 결핍에 의한 선천면역반응 조절기전 규명

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조 효 정

NAD+는 전자 수용체로서 세포 내 다양한 산화환원반응에 관여한다. 대표적으로 해당작용, 지방산의 베타 산화, 그리고 ATP 합성에 관여한다고 알려져 있다. 이외에도 PARP1, CD38, SARM1 과 같은 여러 효소들이 NAD^+를 기질로 사용함으로써 다양한 세포의 생리활성 조절에 중요한 역할을 한다고 알려져 있다. 특히 이러한 효소들은 손상 DNA 의 수리, 인슐린 대사, 세포의 노화 조절기능을 담당하고 있다고 보고되었다. 체내에 존재하는 NAD^+의 양은 노화가 됨에 따라 감소하는 것으로 잘 알려져 있으며, 이러한 감소는 에너지 생산의 감소 및 효소의 활성의 저하로 이어져 알츠하이머나 당뇨병 등과 같은 퇴행성 질환의 원인이 될 수 있다고 알려져 있다. 이러한 질환들은 과도한 선천 면역반응에 의해서도 발병, 진행될 수 있다고 보고되었으며,

관련 질병을 가진 환자들에게서 선천 면역반응의 결과물인 사이토카인이 증가되어 있다는 결과가 밝혀졌다. 하지만, 생체 내 NAD^+의 불균형과 선천 면역반응 사이의 상관성에 대한 연구가 지속적으로 보고되었음에도 현재까지 면역반응에서 NAD^+의 역할이 명확하게 밝혀진 것이 없다. 본 연구에서는 선천 면역반응에서 NAD^+의 역할을 확인하고자 진행되었다. 마우스 골수 대식세포에서 NAD^+가 감소하였을 경우 NLRP3 인플라마좀 활성이 유도되었음을 확인하였으며, 그 결과 카스파제-1(caspase-1)이 활성화되는 것을 확인하였다. 또한 활성화된 카스파제-1 에 의해 gasdermin D 의 가수분해를 통해 염증성 세포사인 파이롭토시스(pyroptosis)가 유도되는 것을 확인하였다. 뿐만 아니라 NAD^+ 가 감소함에 따라 NLRP3 의 미토콘드리아로의 위치 변화가 관찰되었으며, 미토콘드리아들은 핵 주위로 모여 클러스터를 이루는 것을 확인되며, 이러한 변화들은 NLRP3 인플라마좀 활성에 기여할 것으로 추측된다. 위의 결과들을 토대로 본 연구에서는 골수 대식세포에서 NAD^+의 감소가 NLRP3 인플라마좀의 활성 유도 및 선천면역반응에 관여할 수 있다는 것을 제시한다.

핵심되는 말: 노화, NAD^+, NLRP3 인플라마좀, 대식세포, pyroptosis.

PUBLICATION LIST

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