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Deoxynivalenol induces inflammation by activating NF-κB and inflammasome pathway

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However, I am the only person responsible for errors in the thesis.

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PART 01

Deoxynivalenol increases the expression of pro-inflammatory genes and mediators by activating the NF-κB pathway

Abstract

The tricothecene toxin deoxynivalenol (DON) is one of the most prevalent and hazardous fungal secondary metabolites which contaminates serial based foods and causing toxicity to human. Many previous studies reported that the amounts of DON only existed in foods; however, whether DON influences on deleterious effects such as the pro-inflammatory responses in the cellular levels has not been understood. Therefore, we evaluated whether DON gives any effects on the pro-inflammatory responses *in vitro* in murine macrophage RAW264.7 cell. In the current study, we observed that low concentrations of DON (below 1000 nM) exhibited no substantial influence on cell viability and cellular morphological modification. Nevertheless, over at 400 nM DON slightly increased sub-G_1 populations (4.65 ± 0.26% at 400 nM, 7.37 ± 0.22% at 800 nM and 7.61 ± 0.61% at 1000 nM DON, respectively) compared to the untreated control (1.66 ± 0.14%); however, the populations were lower than those of LPS-treated group (10.61 ± 0.08%). Then, we found that DON upregulated inducible nitric oxide (iNOS) and cyclooxygenase-2 (COX-2) at the transcription and translation levels, which consequently increased nitric oxide (NO) and prostaglandin E_2 production. In addition, DON dose-dependently enhanced the expression of pro-inflammatory cytokines including interleukin-6 (IL-6), IL-12 and tumor necrosis factor-α (TNF-α). DON also remarkably upregulated the nuclear translocation of NF-κB in a concentration-dependent way. Therefore, we suggest that DON could causes pro-inflammatory conditions in macrophages and thereby may lead to inflammatory disorders in human.

Keywords: deoxynivalenol, macrophages, NF-κB, iNOS, COX-2, TNF-α, IL-6, IL-12

1. Introduction

Deoxynivalenol (DON) belongs to a trichothecene mycotoxin family which are structurally related molecules produced by fungi of *Fusarium* and *Stachybotrys* species [1]. They are small molecules which usually bind to ribosomes and occur a mechanism known as ribotoxic stress, causing to the activation of various protein kinases, modulation of gene expression, the inhibition of protein synthesis and cell toxicity [2, 3]. According to the food and feed matrices demonstrations, DON is one of the most prevalent and most hazardous food-associated mycotoxins, particularly in cereals and cereal-derived products [4-6]. The ingestion of DON has been associated with alterations of the intestinal, immune and nervous systems, thereby leading, in cases of acute exposure, to illnesses characterized by vomiting, anorexia, abdominal pain, diarrhea, malnutrition, headache and dizziness [7].

Inflammation is a non-specific immune responding process by which the body's white blood cells and substances to protect the higher organisms from infection with foreign organisms, such as bacteria and viruses [8]. The cardinal signs of inflammation can be explained by increased blood flow, elevated cellular metabolism, vasodilatation, release of soluble mediators, extravasation of fluids and cellular influx. The nuclear factor kappa B (NF-κB) signaling pathway plays an essential role in regulating immune responses. The p50/p65 heterodimer are the most predominately characterized NF-κB complex, which is kept inactive in the cytoplasm of resting cells [10]. Once stimulated by some stimuli, the NF-κB activation results p50/p65 separation from IκB (inhibitor of NF-κB) and then translocate into the nucleus where NF-κB can regulate the transcription of several inflammatory cytokine genes, such as *TNF-α*, *IL-6*, and *IL-12* [9, 10].

DON has been reported to target the immune system after it cross the epithelium while some of the studies have demonstrated that immune cells including macrophages, B and T lymphocytes and natural killer cells are very sensitive to DON exposer [11]. Exposure to the toxin leading either to immune stimulatory /inflammatory or immunosuppressive effects depending of the dose [12]. Here we used RAW264.7 macrophages to assess the effect low concentrations (nM) of DON on inflammatory responses. We found that RAW264.7 macrophages are highly sensitive to DON exposure while simulation of macrophages with low concentrations of DON (nM range) causes their activation, the secretion of inflammatory cytokines such as IL-6, IL-12 and TNF-α and the expression of intracellular proteins involved in the innate immunity such as PGE_2 and NO by activating the NF-κB pathway.

2. Material and methods

2.1. Reagents and antibodies

Rabbit anti-mouse antibodies against iNOS, COX-2, β-actin, and LPS, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide (MTT) were purchased from Sigma (St.Louis, MO). Dulbecco´s modified Eagle´s medium (DMEM), fetal bovine serum (FBS), and antibiotic mixtures were obtained from WelGENE Inc. (Daegu, Republic of Korea). Other chemicals were purchased as Sigma grades.

2.2. Cell culture and MTT assay

Murine RAW264.7 macrophage cells were cultured in DMEM supplemented with 5% FBS, in a CO2 incubator with a humidified atmosphere containing 5% CO_2 in air at 37°C. Mitochondrial activities were determined by colorimetric MTT assay based on the reduction of MTT to formazan by cellular dehydrogenase. Briefly, RAW264.7 macrophage cells (1×10^5 cells/ml) were treated with various concentrations (0-1000 nM) of DON. After 24 h-incubation, the cells were incubated with MTT solution (0.5 mg/ml) for 30 min at 37°C. Supernatant was removed and the formation of insoluble formazan was dissolved in DMSO and observed by monitoring the absorbance at 540 nm using a microplate reader (Thermo Electron Corporation, Marietta, OH).

2.3. Reverse transcriptase polymerase chain reactions (RTPCR).

Total RNA was extracted using easy-BLUE™ total RNA extraction kit (iNtRON Biotechnology, Sungnam, Republic of Korea) according to the manufacturer´s instruction. Two microgram RNA was reverse-transcribed using MMLV reverse transcriptase (Promega, Madison, WI). The cDNA was amplified by PCR using specific primer of *iNOS* (forward 5´-CCT CCT CCA CCC TAC CAA GT-3´ and

reverse 5´-CAC CCA AAG TGC TTC AGT CA-3´), *COX-2* (forward 5´-TGC TGT ACC AGC AGT GGC AA-3´ and reverse 5´-GCA GCC ATT TCC TTC TCT CC-3´), *TNF-α* (forward 5´-ATG AGC ACA GAA AGC ATG AT-3´ and reverse 5´-TAC AGG CTT GTC ACT GA AT-3´), *IL-6* (forward 5´-AAG TGC ATC ATC GTT GTT TTC A-3´ and reverse 5´-GAG GAT ACC ACT CCC AAC AG-3'), *IL-12* (forward 5´-AAG ACA TCA CAC GGG ACCC AA-3´ and reverse 5´- GAG GAT ACC ACT TCC CAA CAG-3´) and *GAPDH* (forward 5´- AGG TCG GTG TGA ACG GAT TTG-3´ and reverse 5´-TGT AGA CCA TGT AGT TGA GGT CA-3´). The following PCR conditions were applied for PCR amplification: *COX-2, iNOS* and *IL-6*: 25 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 45 s, and extended at 72°C for 1 min, *TNF-α* 25 cycles of denaturation at 95°C for 45 s, annealing at 53°C for 45 s, and extended at 72°C for 1 min, *IL-12*: 25 cycles of denaturation at 95°C for 45 s, annealing at 61°C for 45 s, and extended at 72°C for 1 min. The *GAPDH* was used as an internal control to evaluate relative expression of *COX-2, iNOS* and *TNF-α, IL-6, IL-12*.

2.4. Western blotting assay

RAW264.7 macrophage cells were seeded at the density of 1×10^5 cells/ml and treated with the DON. After 24 h-incubation, total cell extracts were prepared using a PROPREP protein extraction solution (iNtRON Biotechnology, Sungnam, Republic of Korea). Briefly, the PROPREP solution was treated to the cells on the ice for 30 min and lysates were centrifuged at 14,000 × g at 4°C for 10 min to have the supernatants. In a parallel experiment, cytoplasmic and nuclear extracts were prepared from the cells using NE-PER nuclear and cytosolic extraction reagents (Pierce, Rockford, IL). Protein concentrations of collected samples were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). The samples were stored at −

80°C or immediately used for Western blot analysis after the extraction. The proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Finally, the transferred proteins were detected using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL).

2.5. Flow cytometric analysis

Cell cycle distribution was analyzed by BD cycletest^{TM} plus DNA reagent kit (USA). Briefly, cells (1×10^5 cells/ml) were treated with various concentrations of DON for 24 h.The cells were washed in phosphate-buffered saline (PBS) and the staining was done according to the manufacturer protocol. Cells were analyzed using Muse^{TM} cell cycle analyzer (EMD Millipore Corporation Hayward, CA). The levels of apoptotic cells with sub-G1 DNA were determined as a percentage of the total number of cells using Flowing software 2.5.1 (Turku centre for biotechnology, University of Turku, Finland).

2.6. ELISA

The cell free supernatants were collected from cultures and measured for concentration of mouse IL-6, IL-12, TNF-α and PGE_2 by the ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, RAW264.7 cells (1×10^5 cells/ml) were plated in 24-well plates and incubate with the indicated concentrations of DON in the presence or absence of 500 ng/ml LPS for 24 h. One hundred microliters of culture-medium supernatants were collected for determination of PGE_2 and TNF-α concentration by ELISA.

2.7. NO assay

RAW264.7 macrophage cells (1×10^5 cells/ml) were plated onto 24-well plates and incubated with the indicated concentrations of DON and 500 ng/ml LPS for 24 h. Supernatants were collected and measured for NO production using Griess reagent. Briefly, the samples were mixed with equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride) and then incubated at room temperature for 15 min. The absorbance was measured at 540 nm on a microplate reader. A standard concentration of sodium nitrite was used to determine the nitrite concentration.

2.8. Statistical analysis

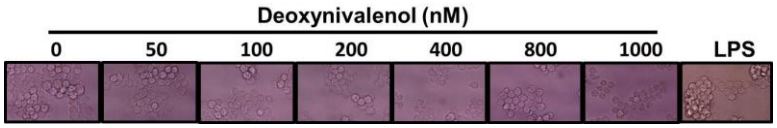
All data were derived from at least three independent experiments. The images were visualized with Chemi-Smart 2000 (Vilber Lourmat, Cedex, France). Images were captured using Chemi-Capt (Vilber Lourmat) and transported into Adobe Photoshop (version 8.0). All bands were quantified by Image J software (Wayne Rasband, National Institute of Health). All data of RT-PCR and western blots were statistically analyzed by Sigma plot 12 software. All data are presented as mean ± standard error (SE). Significant differences between the groups were determined with one-way analysis of variance (ANOVA). Values of $***p < 0.001$, $**p < 0.01$, $*p < 0.05$ were accepted as an indication of statistical significance.

3. RESULTS

3.1. Low concentrations of DON exhibit no influence on cell viability of RAW264.7 macrophages

To addresses whether DON influences on cellular morphological change and cell viability, we treated RAW264.7 cells with the indicated concentrations of DON (0-1000 nM) for 24 h. No morphological change was seen at all concentrations of DON under microscopic observation (Fig. 1A). Mitochondrial activity measured by MTT assay also revealed that low doses of DON do not influence on the normal cellular activities of the cells (Fig. 1B). Flow cytometric analysis data showed that DON slightly increased the sub-G_1 populations to 4.65 ± 0.26% at 400 nM, 7.37 ± 0.22% at 800 nM and 7.61 ± 0.61% at 800 nM DON, respectively. Taken together, these data suggest that low doses of DON do not exhibits substantial cytotoxicity or alteration of mitochondrial activity in RAW264.7 macrophages.

A



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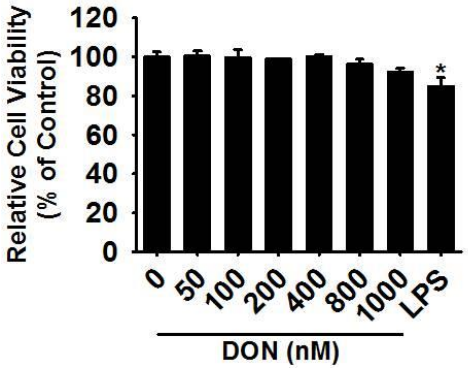

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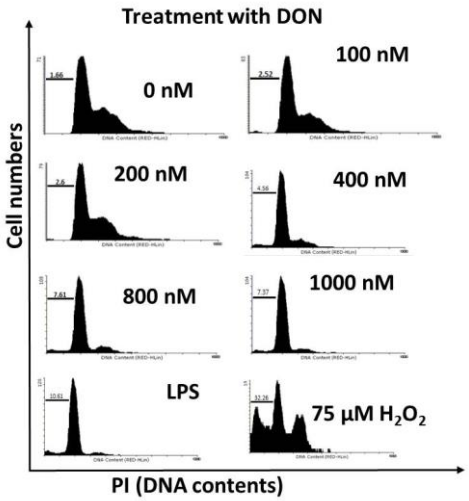
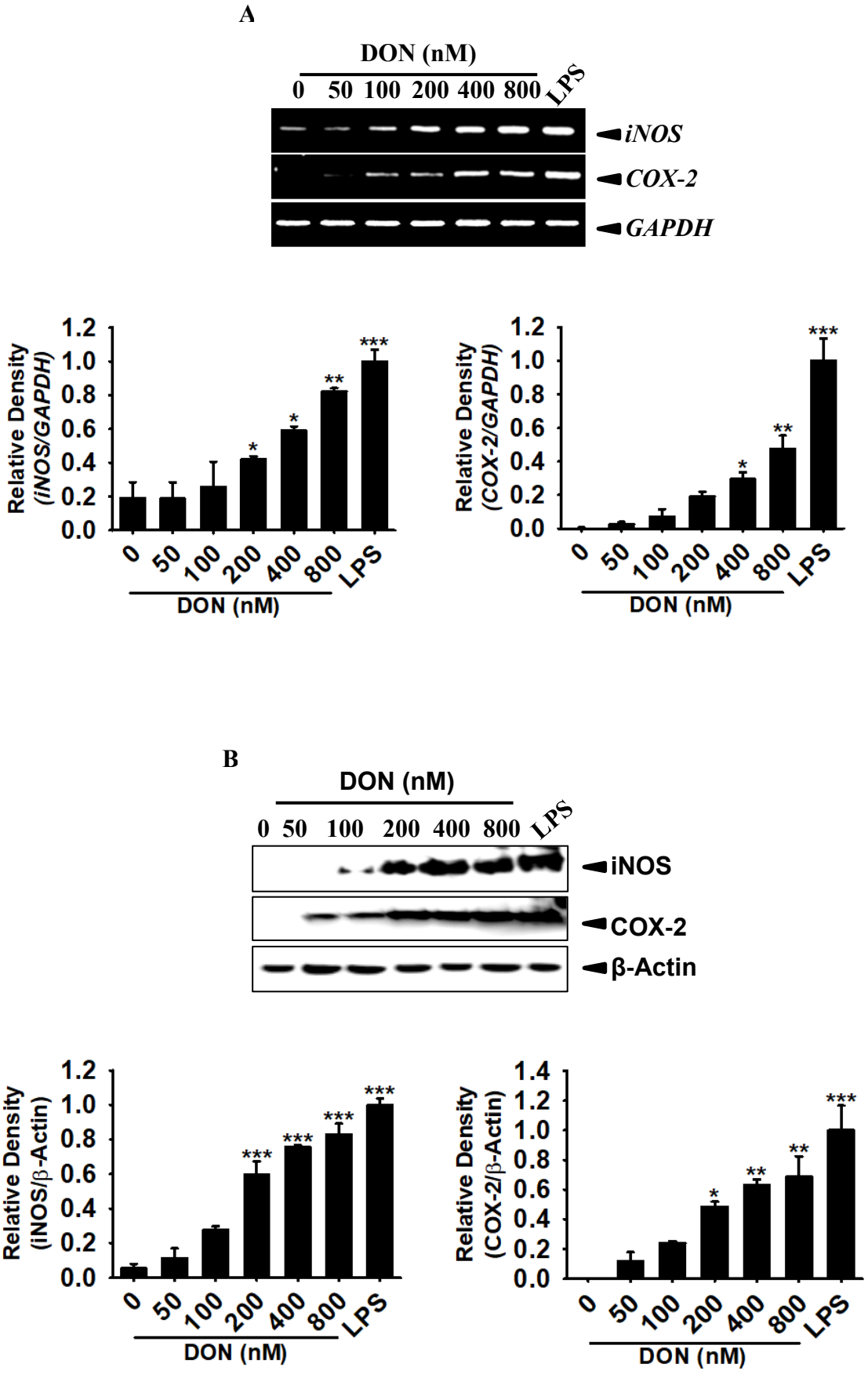


Treatment (for 24 h)		Sub-G1 (%)
DON (nM)	0	1.66 ± 0.14
	100	2.63 ± 0.30
	200	2.52 ± 0.05
	400	4.65 ± 0.26***
	800	7.37 ± 0.22***
	1000	7.61 ± 0.61***
LPS (ng/ml)	100	10.61± 0.08***
H_2O_2 (μM)	50	33.26 ± 0.4***

Figure 1. Deoxynivalenol (DON) exhibits no cytotoxicity on RAW264.7 macrophages. RAW264.7 macrophages were seeded at the density of 1×10^5 cells/ml and the indicated concentrations of DON were treated for 24 h. (A) Images of the cells at 24 h was taken by light microscope with toupview software. (B) Relative mitochondrial activity was measured by MTT assay. (C) DNA content of DON treated cells were measured by flow cytometry. Effect of DON on cell viability was measured by calculating the sub-G_1 population. Data from three independent experiments are expressed as the overall mean ± S.E. Statistical significance was determined by one-way ANOVA (***, $p < 0.001$ vs. untreated control).

3.2. DON increases iNOS and COX-2 expression, leading to NO and PGE2 production

DON markedly upregulated the *iNOS* and *COX-2* gene expression in RAW264.7 macrophages from at 100 nM and the expression at 800 nM DON was as much as same to those of 500 ng/ml LPS treatment (Fig. 2A). Consistent with gene expression of *iNOS* and *COX-2*, DON increased the expression of iNOS and COX-2 protein expression in a dose-dependent manner (Fig. 2B). In addition, DON enhanced extracellular NO production in a concentration-dependent manner (Fig. 2C) and it was significant 200 nM onward aligning with RT-PCR and western blot data. However, the effect was a little weaker compared to that of 500 ng/ml LPS treatment. Extracellular PGE_2 was also calculated by analyzing cell culture media at 24 h. PGE_2 secretion was gradually upregulated by treatment with DON (Fig. 2D). These results suggest that low concentrations of DON increase the inflammatory mediators such as NO and PGE_2, resulting from decrease of their regulatory genes such as iNOS and COX-2.



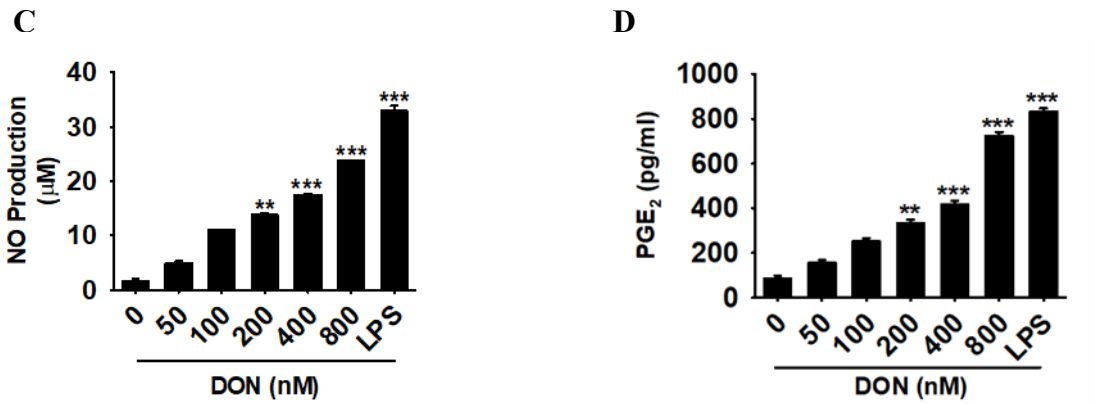


Figure 2. DON induces the expression of intracellular proteins involved in the innate immunity. RAW264.7 macrophages were seeded at the density of 1×10^5 cells/ml and the indicated concentrations of DON were treated. In brief, RT-PCR was used to assess mRNA expression of iNOS 9 h after treatment with 500 ng/ml LPS and COX-2 whereas western blot and ELISA were used to detect the protein expression at 24 h after treatment with 500 ng/ml LPS. (A) Cells were seeded at the density of 1×10^5 cells/ml and treated with the indicated concentrations of DON for 9 h, and samples were collected and performed the RT-PCR for *iNOS* and *COX-2*. LPS-treated group was used as the positive control. GAPDH was used as the internal control. (B) In a parallel experiment, the cells were exposed with LPS for 24 h and cell lysates were used for western blot analysis. β-Actin was used as an internal control. (C) The amount of NO production was determined at 24 h by Griess assay using the culture media. (D) Extracellular PGE_2 was measured at 24 h using cell culture media by ELISA assay. Data from three independent experiments are expressed as the overall mean ± S.E. Statistical significance was determined by one-way ANOVA ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ vs. untreated control).

3.3. Stimulation of RAW264.7 cells with low concentrations of DON results the upregulation of inflammatory cytokines such as IL-6, IL-12 and TNF-α

To evaluate whether DON upregulates the expression of proinflammatory cytokines such as IL-6, IL-12 and TNF-α, RAW264.7 cells were treated with the indicated concentrations of DON. As expected, DON treatment dose-dependently increased the expression of *IL-6*, *IL-12p35* and *TNF-α* at 6 h (Fig. 3A-3C). However, time points of those gene expression were a little different. *TNF-α* (Fig. 3A) and *IL-12* (Fig. 3B) were started to strongly express at 200 nM DON whereas *IL-6* expression was week at all concentrations of DON used in this study (Fig. 3C). The IL-12(p70) cytokine is a heterodimeric cytokine that is expressed by IL-12A (p35) and IL-12B (p40), separately. Low concentrations of DON did not increase the expression of *IL-12 (p40)* gene (data not shown). The cytokine concentration in cell culture media was measured by ELISA and both IL-6 and IL-12(p70) was increased upon DON treatment. The secretion of IL-6 increased from at 400 nM DON (Fig. 3D), even though the concentration induced a slight increase of IL-6. *IL-12(p35)* gene was highly expressed from at 200 nM DON, but IL-12 secretion was significantly expressed at 400 nM DON (Fig. 3E). Therefore, these results indicate that DON significantly increases the secretion of TNF-α, IL-6 and IL-12.

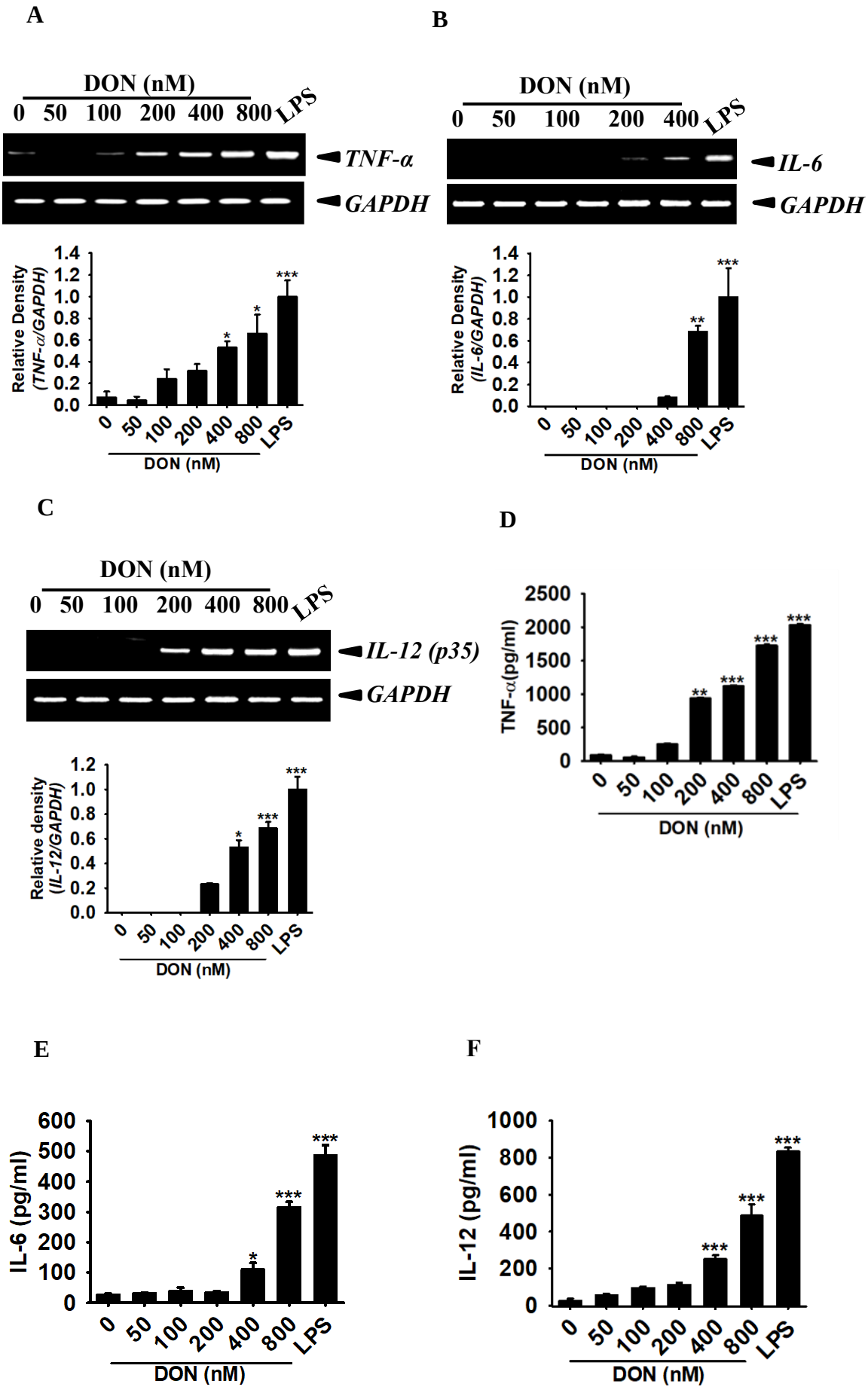


Figure 3. DON upregulates the expression of inflammatory cytokines such as IL-6, IL-12 and TNF-α. Gene expression was analyzed by RT-PCR and ELISA was performed to detect cytokine secretion upon DON exposure. RAW264.7 cells were seeded at the density of 1×10^5 cells/ml and treated with the indicated concentrations of DON for 9 h and RT-PCR was performed for *TNF-α* (A), *IL-6* (B) and *IL-12p35* (C). *GAPDH* was used as an internal control. Under the same experimental conditions at 24 h, cell culture media was used to measure the cytokine secretion TNF-α (D), IL-6 (E) and IL-12p35 (F). Data from three independent experiments are expressed as the overall mean ± S.E. Statistical significance was determined by one-way ANOVA ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ vs. untreated control).

3.4. DON stimulates the NF-kB pathway

The NF-κB pathway is shown to be involved in the upregulation of a broad range of cytokines and inflammatory mediators including iNOS and COX-2 [13], the main regulatory enzymes of NO and PGE_2 production. We next investigated whether DON upregulates pro-inflammatory genes and proteins such as iNOS and COX-2. Since the NF-κB regulated inflammatory mediators were upregulated upon low concentrations of DON incubation, then we hypothesized that low dose of DON would upregulated the NF-κB related cytokine expression. Then we checked the expression of NF-κB heterodimers upon DON incubation and as expected low concentrations of DON clearly increased the expression of p50 and p65 heterodimers in nuclear fraction of the cell lysate (Fig. 4).

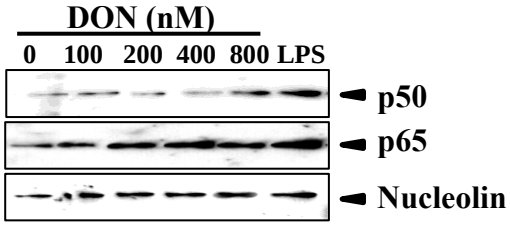


Figure 4. DON upregulates nuclear p50 and p65. RAW264.7 cells were seeded at the density of 1×10^5 cells/ml and treated with the indicated concentrations of DON for 30 min. Nuclear fraction of the cell lysate was used to check the effect of DON on NF-κB nuclear translocation. LPS (500 ng/ml) was used as a positive control.

4. Discussion

DON is regarded as one of the most prevalent food-associated mycotoxins in serials and serial-based products in worldwide, specially in US and Europe [14, 15]. DON is strong resistance to high heat (up to 350ºC), which ensures the stability and persistency during processing and cooking [16]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has proposed 1 μg/kg body weight as the maximum tolerable daily intake per person since it is associated with alterations of the intestinal, immune and nervous systems, thus leading, in cases of acute exposure, to illnesses characterized by vomiting, anorexia, abdominal pain, diarrhea, malnutrition, headache and dizziness [6, 7].

It is still unclear how does DON enter into the cells exactly [17]. One possible way is that epoxide motif of DON binds with the nucleotide forming ribosomal RNA (rRNA) of the ribosomes since the nucleotides containing amino acids are potentially able to react with epoxides. However, precise interactions of the molecules have still remained understood. The second possibility is entering into the cells via passive diffusion mechanism but -097 of log*D* value prevents the entering via lipid diffusion leaving only two possible passive entries. It could be diffuse through unknown membrane-associated passive transporter or enter in to the cell via bulk phase endocytosis process.

Even though we could not still understand how DON enters into the cells, DON ensures activation of various cell signaling pathways affecting cellular and molecular functions. Low doses (nM) of DON have been reported to activate ERK, which cause cell survival and gene expression, whereas high doses (μM) have been found to activate p38, leading to apoptosis by activating rRNA cleavage and protein synthesis inhibition in mouse macrophages. Therefore, we, in the current study, used low

concentrations (nM) of DON to assess its effect on pro-inflammatory mediators such PGE_2 and NO, pro-inflammatory cytokines such as TNF-α, IL-6 and IL-12 through the NF-κB pathway. In the current study, we found that RAW264.7 macrophages are highly sensitive to DON exposure and ensures the upregulation of NF-κB signaling pathway. Nevertheless, we couldn't identify any of cytotoxic effect up to 1000 nM on cell morphology (Fig. 1A). The sub-G_1 population was increase from 400 nM onward whereas mitochondrial functions were slightly decreased 800 nM onward (Fig. 1B).

Upregulation of NF-κB upon DON exposure ensured the expression of *iNOS* and *COX-2* genes, which consequently upregulated the secretion of pro-inflammatory mediators, NO and PGE_2. NO is responsible for vasodilation, neural communication and host defense. PGE_2 is a lipid mediator that is not stored by cells and it is synthesized from arachidonic acid via the actions of COX enzymes [18]. PGE2 can regulate key responses in the major systems including, gastrointestinal, neuroendocrine, reproductive and immune by mediating inflammation, pain and fever [19]. Over expression of these two regulatory enzymes are associated with a variety of inflammatory diseases including autoimmunity and several human cancers [20-23].

Since low dose of DON was associated with the upregulation of intracellular proteins involved in the pro-inflammatory genes such as COX-2 and iNOS, we, next, focused on its effect on pro-inflammatory cytokine expression. We observed that DON exposure markedly increased the expression *IL-6*, *IL-12* and *TNF-α* gene at 9 h. Not only the genes but also the cytokines in the cell culture media dose-dependently increased upon DON exposure. Overexpression of IL-6 is involve in not only the chronic inflammatory diseases but also bone degeneration and alteration of neural functions [24] whereas IL-12 is associated with autoimmune disorders such as rheumatoid arthritis, inflammatory bowel, and graft versus-host disease [25]. In

addition, overexpression of TNF-α leads to the conditions like rheumatoid arthritis (RA), crohn's disease, AIDS, septic shock, cancer, transplantation rejection, multiple sclerosis, diabetes, trauma, malaria, meningitis, ischemia-reperfusion injury, and adult respiratory distress syndrome [26].

In conclusion, low dose of DON markedly upregulates the NF-κB signaling pathway and thereby ensures the upregulation of inflammatory cytokine secretion and the expression of protein that are involved in the pro-inflammatory genes such as COX-2 and iNOS. Since the overexpression of the pro-inflammatory mediators are responsible for the initiation of various inflammatory disorders and thereby represent a risk to human health, food agencies should take this fact into account and make sure to avoid the contamination of products from DON.

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PART 02

Deoxynivalenol enhances IL-1β expression by activating the NF-κB and ASC/NLRP3-mediated inflammasome pathway

Abstract

Deoxynivalenol (DON) is one of the most common fungal toxins which contaminate food grains and cereal-derived products. However, whether DON induces inflammasome-mediated inflammation is still unknown. In the current study, we examined whether DON stimulates IL-1β secretion in BV2 microglia cells by activating NLRP3/ACS-mediated inflammasome. Treatment with high doses of DON (over 800 nM) decreased cell proliferation; however, no significant apoptotic sub-G_1 was observed, which indicates that DON induces BV2 cells at a stagnant stage. We also found that DON significantly upregulated *IL-1β* expression in a dose-dependent manner from 0.5 h to 6 h as much as comparing to treatment with LPS and ATP, which was regulated by nuclear factor-κB (NF-κB) activation. In addition, IL-1β was remarkably secreted in the presence of DON at 24 h and a caspase-I inhibitor significantly prohibited DON-mediated IL-1β secretion, which suggest that caspase-1, which is an effector molecule of inflammasome, is an important upregulator of IL-1β. Thus, components of inflammasome such as ASC and NLRP3 substantially increased by DON treatment and transition knockdown of ASC and NLRP3 significantly downregulated DON-mediated IL-1β expression and secretion, which means that DON stimulates NLRP3/ASC-mediated inflammasome, which consequently promotes IL-1β expression and secretion in BV2 microglia cells. Taken together, these data suggest that DON induces IL-1β expression in BV2 microglial cells by activating the NF-κB signaling pathway and subsequently upregulated NLRP3/ASC-mediated inflammasome-mediated IL-1β secretion. Therefore, DON could induce inflammatory diseases or disorders by activating inflammasome-mediated IL- 1β.

Keywords: Deoxynivalenol; Inflammasome; NLR family pyrin domain containing 3 (NLRP3); Nuclear factor-κB (NF-κB)

1. Introduction

Inflammation is regarded as an ordinary and essential component of responses to the infection and injury [1]. It includes sequence of complex, interrelated events that leads to the recruitment of phagocytes, the elimination of harmful particles and the initiation of tissue repair in due course [2, 3]. This particular sequence of events is basically governed by the network of cellular components including the inflammatory cytokines. Among these inflammatory cytokines, interleukin-1β (IL-1β) is known as the major inflammatory mediator [3, 4]. Secretion of IL-1β leads to the induction of the activation of not only the various cell types including phagocytes, epithelial and endothelial cells but also helps the activation and polarization of T lymphocytes, and enhances the expression of further pro-inflammatory cytokines such as IL-6 and TNF-α [5]. IL-1β is also considered as an important pro-inflammatory cytokine in the brain and plays a critical role in the progression of neuroinflammation which is a well-known factor of the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis [6].

Microglial cells are the resident macrophages of the brain and their secretion of IL-1β is greatly depending on the multiprotein complex called inflammasome [7]. Inflammasomes are multiprotein complexes that play an important role in regulating innate immunity and inflammatory response [8]. To the date, four distinct inflammasomes (NLRP1, NLRP3, NLRP4 and AIM) have been identified and they sense intracellular danger signals via NOD-like receptors that recognize pathogen-associated molecular patterns [4]. Among the inflammasomes, one of the most intensively study is the NOD-like receptor family pyrin domain-containing3 (NLRP3) inflammasome [8] which contains a NLRP3 sensor, an apoptosis-associated speck-

like protein containing a caspase recruitment domain (ASC) adaptor and a caspase-1 enzyme [9].

Substantial evidences have indicated that the NLRP3 inflammasome is primed by two main signals named as primary and secondary signals. The primary signals prime the synthesis of pro-IL-1β and NLRP3 by transcriptional induction of NF-κB [10-13], meanwhile, the secondary signals lead to oligomerization of caspase-1-dependent cleavage and the subsequent release of the biologically active IL-1β [8, 14, 15]. The secondary signals are composing of a group of chemically and biologically unrelated molecules including pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) [9, 16]. For instance, ATP induces NLRP3 activation through stimulation of the purinergic receptor, P2X ligand–gated ion channel 7 (P2X7), which induces K^+ efflux [10]. The concentration of ATP required for P2X7-mediated caspase-1 activation is in the millimolar range. This much of high concentrations of ATP are not normally found in the *in vivo* extracellular environment, even though they could possibly be reached under the circumstance of cell lysis or injury.

DON is a member of family of trichothecene mycotoxins that is primarily found in cereal grains such as wheat, barley and maize [17]. It is a secondary metabolite product of several fungi including *Fusarium, Mycothecium, Trichorderma, Trichothecium, Stachybotrys, Verticinosporium* and *Chephalosporium sp* [18-20]. DON affects the gastrointestinal, reproductive, and neuroendocrine systems, particularly the immune system and it can induce emesis, diarrhea, hemorrhaging, and immunosuppression, as well as reduce reproductive capacity of both humans and animals [21]. It has been demonstrated that DON rapidly activates mitogen-activated protein kinases [22] under *in vitro* and *in vivo* conditions and the activated MAPKs

drive upregulated expression of mRNAs and proteins for inflammation-related genes such as the cytokines, chemokines, and cyclooxygenase-2 [23].

Nevertheless, the involvement in DON in the field of the upregulation of inflammasome related genes are not studied in depth. Therefore, in this current study we tried to figure out the involvement of DON in the upregulation of inflammasome related genes, proteins and transcription factors in BV2 microglial cells.

2. Material and Methods

2.1. Reagents and antibodies

Rabbit anti-mouse antibodies against IL-1β, caspase-1, β-actin and P2X7, and LPS and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). The antibodies against NLRP3 and ASC were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Dulbecco´s modified Eagle´s medium (DMEM), fetal bovine serum (FBS) and antibiotic mixtures were obtained from WelGENE Inc. (Daegu, Republic of Korea). Other chemicals were purchased as Sigma grades.

2.2. Cell culture and viability

Murine BV2 microglial cells were cultured in DMEM supplemented with 10% FBS in a CO_2 incubator with a humidified atmosphere containing 5% CO_2 in air at 37°C. Cell viability was determined by colorimetric MTT assay based on the reduction of MTT to formazan by cellular dehydrogenase. Briefly, BV2 microglial cells (1×10^5 cells/ml) were treated with various concentrations (0-2000 nM) of DON. After 24-h incubation, the cells were incubated with MTT solution (0.5 mg/ml) for 30 min at 37°C. Supernatant was removed and the insoluble formazan was dissolved in DMSO and observed by monitoring the signal at 540 nm using a microplate reader (Thermo Electron Corporation, Marietta, OH).

2.3. Reverse transcriptase polymerase chain reactions (RT-PCR).

Total RNA was extracted using Easy-blue reagent (iNtRON Biotechnology, Sungnam, Republic of Korea) according to the manufacturer's instructions. Genes of interest were amplified from cDNA that was reverse-transcribed from 1 μg total RNA

using the One-Step RT-PCR Premix (iNtRON Biotechnology). The specific primers for *caspase-1* (forward 5'-CTG ACT GGG ACC CTC AAG-3' and reverse 5'-CCT CTT CAG AGT CTC TTA CTG-3'), *IL-1β* (forward 5'- GCC CAT CCT CTG TGA CTC AT-3' and reverse 5'- AGG CCA CAG GTA TTT TGT CG-3'), *NLRP3* (forward 5'- TCG CAG CAA AGA TCC ACA CAG-3' and reverse 5'- ATT ACC CGC CCG AGA AAG G-3'), *ASC* (forward 5'- GCA ACT GCG AGA AGG CTA T-3' and reverse 5'- CTG GTC CAC AAA GTG TCC TG-3'), *GAPDH* (forward 5'- AGG TCG GTG TGA ACG GAT TTG-3' and reverse 5'-TGT AGA CCA TGT AGT TGA GGT CA-3'). The following PCR conditions were applied: for *NLRP3*: 30 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s and extended at 72°C for 30 s; for *IL-1β*: 30 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s and extended at 72°C for 30 s; for *caspase-1*: 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extended at 72°C for 30 s; for *ASC*: 59 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s and extended at 72°C for 30 s; for *GAPDH*: 27 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extended at 72°C for 30 s. GAPDH was used as an internal controller to evaluate the relative expression of *IL-1β, caspase-1, NLRP3 and ASC*.

2.4. Western blot assay

BV2 microglial cells were seeded at the density of 1×10^5 cells/ml and treated with the indicated concentrations of DON in the presence or absence of LPS/ATP. After 6 h-incubation, total cell extracts were prepared using a PROPREP protein extraction solution (iNtRON Biotechnology). Briefly, the PROPREP solution was treated to the cells on the ice for 30 min and lysates were centrifuged at 14,000 × g at 4°C for 10 min to have the supernatants. In a parallel experiment, cytoplasmic and

nuclear extracts were prepared from the cells using NE-PER nuclear and cytosolic extraction reagents (Pierce, Rockford, IL). With the use of collected supernatants, protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). The samples were stored at -80°C or immediately used for Western blot analysis after the extraction. The proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Finally, the transferred proteins were detected using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL).

2.5. Flow Cytometric Analysis

Cell cycle distribution was analyzed by BD cycletest^{TM} plus DNA reagent kit. Briefly, cells (1×10^5 cells/ml) were treated with various concentrations of fisetin for 24 h. The cells were washed in phosphate-buffered saline (PBS) and the staining was done according to the manufacturer protocol. Cells were analyzed using Muse^{TM} cell cycle analyzer (EMD Millipore Corporation Hayward, CA). The levels of apoptotic cells with sub-G_1 DNA were determined as a percentage of the total number of cells using Flowing software 2.5.1 (Turku centre for biotechnology, University of Turku, Finland).

2.6. ELISA

The cell free supernatants were collected from cultures and assayed for concentration of human IL-1β using Ready-set-go ELISA kit (Affymetrix, eBioscience). The test was performed according to the ready-set-go protocol.

2.7. siRNA transfection

BV2 microglial cells were seeded on a 12-well plate at a density of 1×10^5cells/ml and transfected *ASC* and *NLRP3*-specific silencing RNA (siRNA, Santa Cruz Biotechnology) for 24 h. For each transfection, 450 μl growth medium was added to 20 nM siRNA duplex with the transfection reagent G-Fectin (Genolution Pharmaceuticals Inc., Seoul, Republic of Korea) and the entire mixture was added gently to the cells.

2.8. Statistical analysis

All data were derived from at least three independent experiments. The images were visualized with Chemi-Smart 2000 (Vilber Lourmat, Cedex, France). Images were captured using Chemi-Capt (Vilber Lourmat) and transported into Adobe Photoshop (version 8.0). All bands were quantified by Image J software (Wayne Rasband, National Institute of Health, USA). All data of RT-PCR, western blot were statistically analyzed by Sigma plot 12 software. All data are presented as mean ± standard error (SE). Significant differences between the groups were determined with one-way analysis of variance (ANOVA). Values of ***$p < 0.001$, **$p<0.01$, *$p<0.05$ were accepted as an indication of statistical significance.

3. Results

3.1. DON exhibits no cytotoxic effect on BV2 microglia cells at low concentrations

To assess the effect of DON on the proliferation of BV2 cells, we treated the cells with the indicated concentrations of DON for 24 h and then performed an MTT assay as an indirect measurement of cellular proliferation, since it measured the mitochondrial activity. MTT assay showed that a significant reduction of cell proliferation was observed from at 1000 nM and below at 400 nM DON appeared no any significant effect (Fig. 5A). Eight hundred nM DON moderately decreased cell proliferation. Next, direct effect of cytotoxicity of DON was measured by analyzing the morphological changes of the cells and the sub-G_1 population of flow cytometric data. We could not find remarkable morphological changes at 800 nM DON and higher concentrations of over at 1000 nM DON induced a little of shrinking cells (Fig. 5B). Based on the amount of sub-G_1 population measured by flow cytometry, no apoptotic cell death was seen in the presence of DON up to 1500 nM compared to that of the H_2O_2-treated group and 2000 nM DON induced a significant increase of sub-G_1 population; however, highest concentration of DON occurred 6.6 ± 0.9% of sub-G_1 populations (Fig. 5C). Taken together, these data indicate that low doses (≤ 800 nM) of DON are not cytotoxic to BV2 microglial cells.

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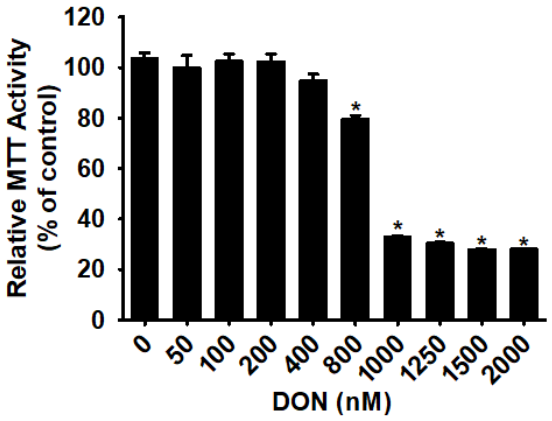

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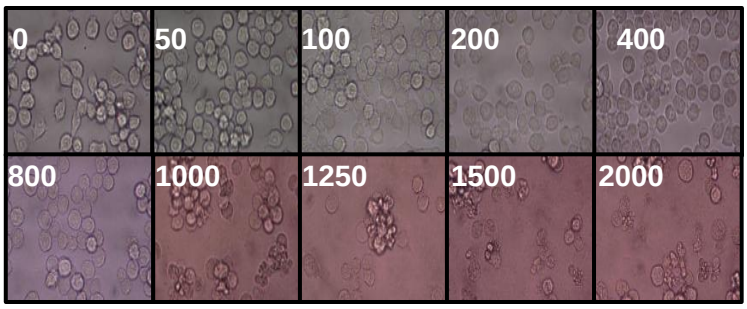

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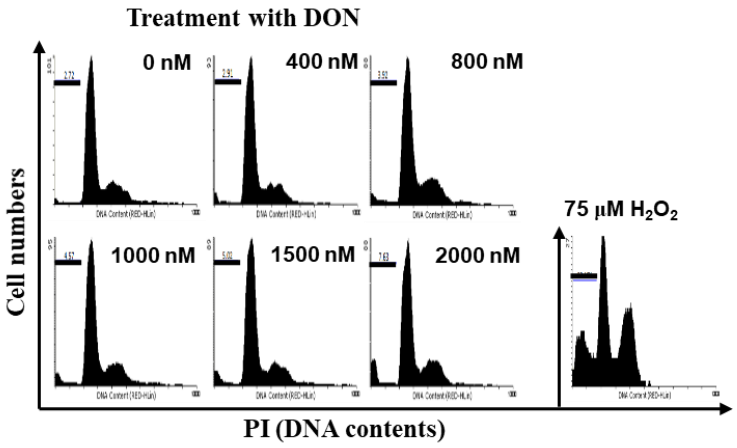

Treatment (for 24 h)		Sub-G1 (%)
DON	0 nM	2.5 ± 0.4
	400 nM	3.7 ± 1.5
	800 nM	4.8 ± 0.9
	1000 nM	3.7 ± 1.0
	1500 nM	3.6 ± 1.2
	2000 nM	6.6 ± 0.9*
H_2O_2	75 μM	27.5 ± 2.5*

Figure 5. Deoxynivalenol (DON) exhibits no influence on the viability of BV2 microglial cells. BV2 microglial cells were seeded at a density of 1×10^5 cells/ml and incubated with the various concentrations of DON for 24 h. (A) Cell viability was determined with an MTT assay. (B) The morphology of cells was examined under light microscopy (×400) with the use of toupview software. (C) DNA content was analyzed by flow cytometry. Percentages of sub-G_1 DNA contents are represented in each panel. Data from three independent experiments are expressed as the overall mean ± S.E. Statistical significance was determined by one-way ANOVA $*p < 0.05$ vs. untreated control).

3.2. DON induces the expression of IL-1β gene in BV2 microglia cells by activating the NF-κB signaling pathway

With the intention of finding the time points of *IL-1β* gene expression, 800 nM DON-treated samples were collected at the indicated time points and performed the RT-PCR. Fig. 6A showed that *IL-1β* gene was highly expressed at 0.5-3 h after the DON treatment. To assess dose-dependent function of DON on *IL-1β* gene expression, we treated the cells with the indicated concentrations of DON for 1 h. As shown in Fig. 6B, *IL-1β* gene was markedly upregulated upon the incubation of the cells with the indicated concentrations of DON as much as treatment with 1 mM ATP and 100 ng/ml LPS. Then, we measured the effect of DON on NF-κB activation. We found that NF-κB heterodimers, p50 and p65, were markedly upregulated in the nuclear compartments at 30 min upon DON incubation (Fig. 6C). Next, to find out the function of NF-κB on DON-mediated *IL-1β* gene expression, we pretreated with NF-κB inhibitors, PDTC and PS1145 1 h before DON treatment. Pre-incubation of 10 μM PDTC and PS1145 significantly inhibited the *IL-1β* gene upregulation by induced by DON or ATP/LPS, which confirm that DON increases NF-κB-dependent *IL-1β* gene expression (Fig. 6D). For further confirmation of the upregulation of NF-κB pathway upon DON incubation, we transfected the cells with the NF-κB promoter-containing reporter vector and treated with the indicated concentrations of DON in the presence or absence of NF-κB inhibitors (no data). Taken together, DON induces the expression of *IL-1β* gene in BV2 microglia cells by activating the NF-κB signaling pathway.

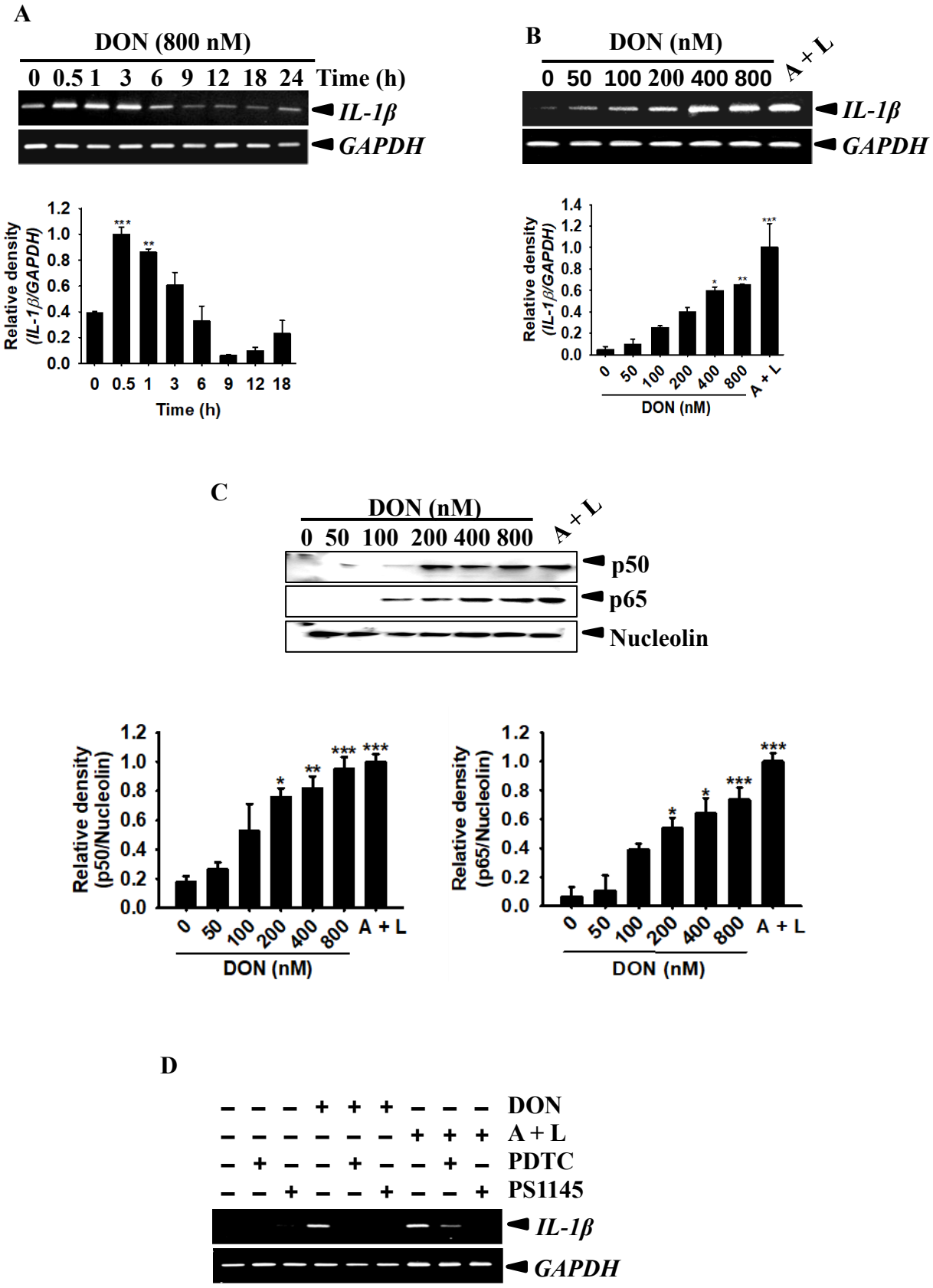

Figure 6. DON induces the expression of IL-1β gene in BV2 microglia cells via the NF-κB signaling pathway. RT-PCR was performed to assess mRNA expression of *IL-1β* and western blot analysis and NF-κB luciferase binding assay were subjected for NF-κB activity. (A) BV2 microglial cells (1×10^5 cells/ml) were treated with 800 nM DON and harvested at the indicated time points. Total cellular RNAs was subjected to RT-PCR and the PCR products were resolved with use of 2% agarose gel. (B) BV2 microglia cells were treated with the indicate concentrations of DON and with 1 mM ATP and 100 ng/ml LPS as the positive control. Then, the extracted mRNA was subjected to RT-PCR and the PCR products were resolved in the 2% agarose. (C) In a parallel experiment, the cells were treated with indicated concentrations of DON or ATP/LPS for 30 min and the nuclear compartment was purified, which performed for western blot analysis for p50 and p65. (D) For the functional analysis of NF-κB, BV2 microglial cells were pre-incubated with 10 μM PDTC and 10 μM PS1145 for 2 h and then treated with 800 nM DON or 1 mM ATP and 100 ng/ml LPS. Total cellular RNAs was subjected to RT-PCR for *IL-1β* gene expression. Data from three independent experiments are expressed as the overall mean ± S.E. Statistical significance was determined by one-way ANOVA ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ vs. untreated control).

3.3. DON increases the secretion of active IL-1β protein in BV2 microglia cells

Since DON upregulated *IL-1β* gene expression, we, next, focused on the secretion of active IL-1β which is regulated by the cleavage of pro-IL-1β. Active IL-1β in culture media was assessed by western blot analysis and ELISA 24 h after treatment with DON. As shown in Fig. 7A and Fig. 7B, DON dose-dependently upregulated IL-1β secretion. However, NF-κB inhibitors, PDTC and PS1145, decreased DON-induced IL-1β secretion, even LPS-induced (Fig. 7C). All results suggest that DON upregulates the cleavage of pro-IL-1β in BV2 microglia cells and promotes the secretion of IL-1β.

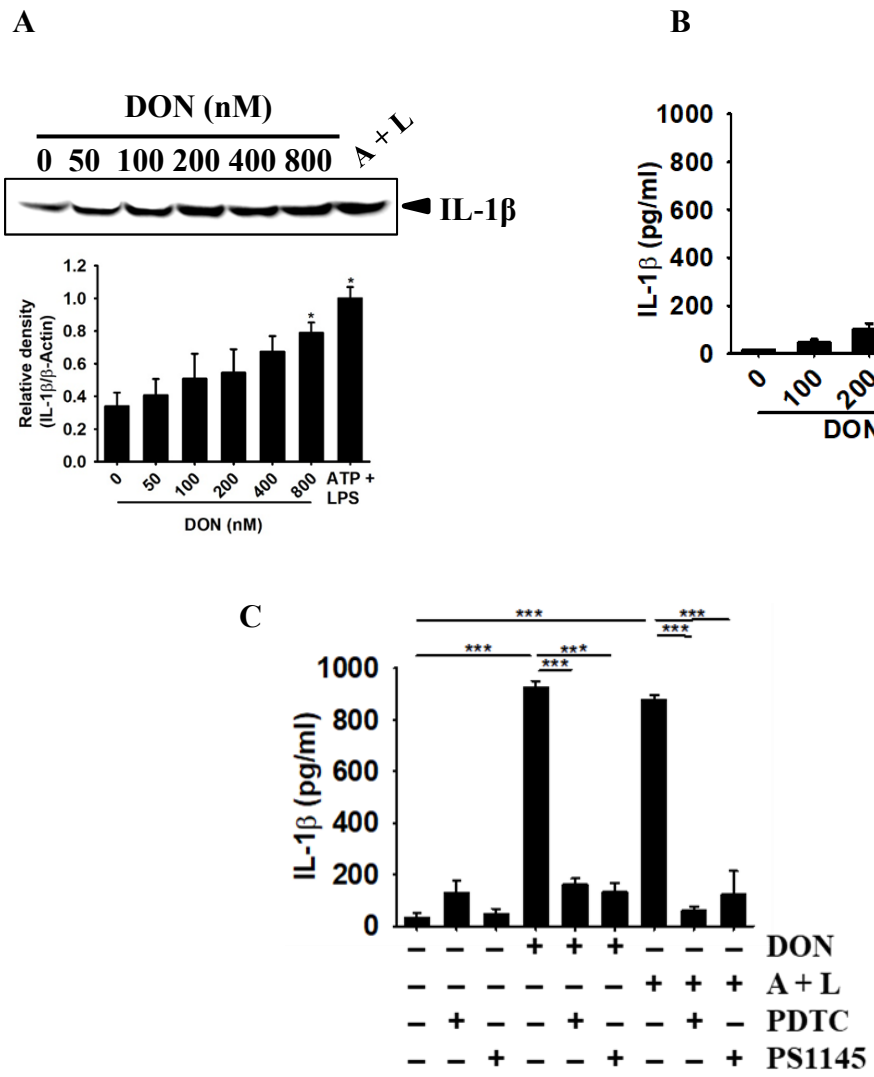

Figure 7. DON increases the secretion of active IL-1β in BV2 microglia cells. (A and B) BV2 microglial cells were treated with the indicated concentrations of DON and ATP/LPS for 24 h and then, culture media was collected. (A) Western blot analysis (A) and ELISA (B) were performed to measure active IL-1β secretion. (C) In a parallel experiment, BV2 microglial cells were pre-treated with the NF-κB inhibitors, PDTC (40 μM) and PS1145 (20 μM), for 2 h and then treated with 800 nM DON and ATP/LPS as a positive control. Active IL-1β secretion was quantified by ELISA assay. Data from three independent experiments are expressed as the overall mean ± S.E. Statistical significance was determined by one-way ANOVA ***p < 0.001 and *p < 0.05 vs. untreated control).

3.4. DON enhances IL-1β secretion by activating caspase-1

Cleavage of pro-IL-1β into its active form requires the active caspase-1 manipulation. Therefore, we evaluated the effect of DON on caspase-1 expression in BV2 microglial cells. DON (800 nM) triggered the expression of *caspase-1* from at 0.5 h and sustained by at 24 h (Fig. 8). DON (≥100 nM) also increased the expression of *caspase-1* gene in a dose-dependent manner (Fig. 8B). Intracellular active caspase-1 protein was significantly upregulated upon DON incubation in a concentration-dependent manner (Fig. 8C). When the cells were pre-treated with a caspase-1 inhibitor, z-YVAD-fmk, the expression of *IL-1β* gene still remained in the presence of DON or ATP/LPS (Fig. 8D); however, extracellular active IL-1β protein was downregulated (Fig. 8E), which indicate that DON-induced caspase-1 cleaves pro-IL-1β for the secretion of active IL-1β. Taking these results into account, it suggests that DON increases active caspase-1 in BV2 microglial cells, which subsequently cleave pro-IL-1β into active IL-1β.

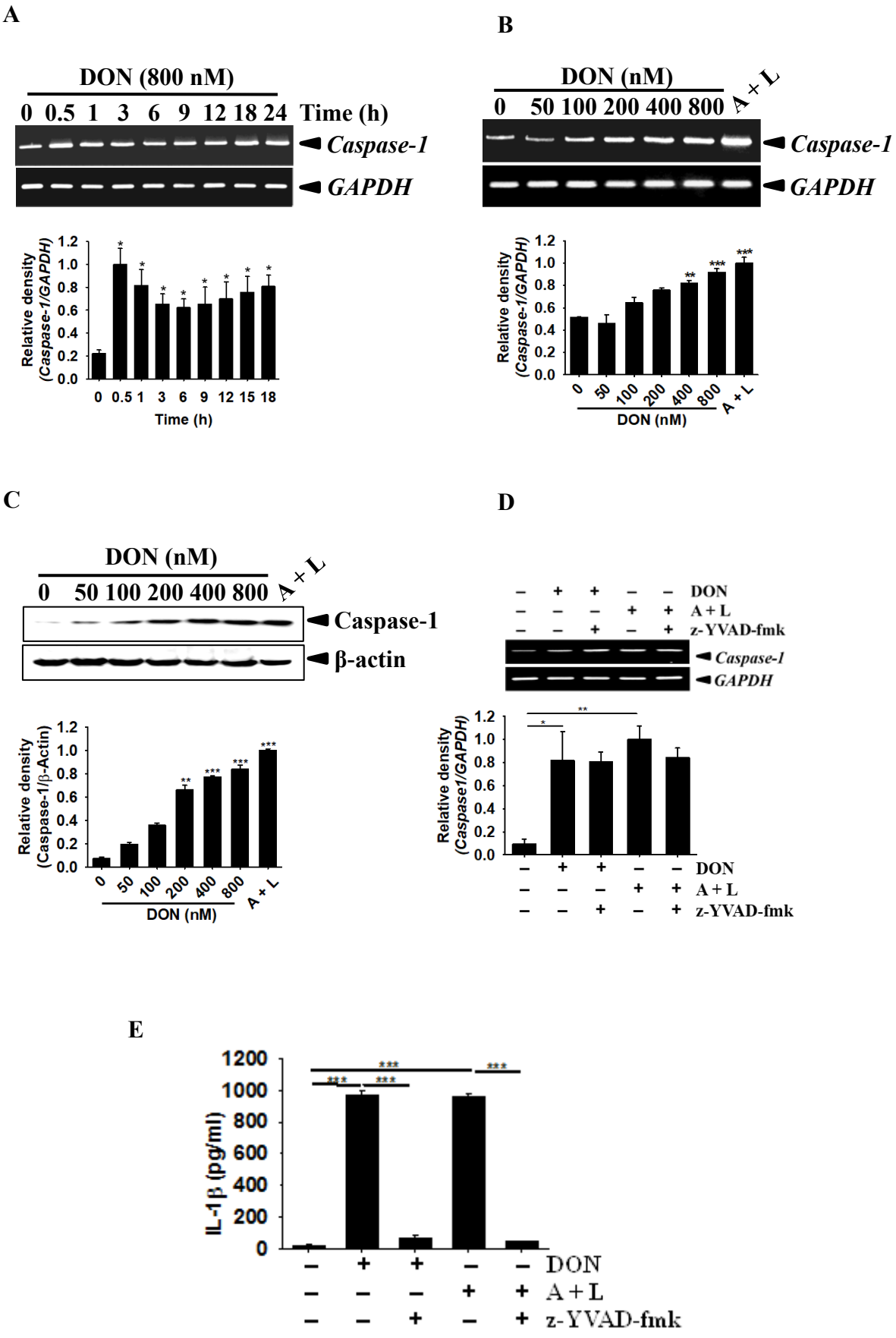

Figure 8. DON increases the expression of caspase-1 in BV2 microglial cells, leading to the secretion of active IL-1β. (A) BV2 microglial cells were seeded at the density of 1×10^5 cells/ml, treated with 800 nM DON and harvested at the indicated time points. RT-PCR was conducted to assess the optimum gene expression time points in DON-treated BV2 microglial cells. (B) Then, the cells were treated with indicted concentrations of DON, or 1 mM ATP and 100 ng/ml LPS. Effect of DON on *caspase-1* gene expression in BV2 microglial cells was assessed by RT-PCR. (C) In a parallel experiment, western bolt analysis was performed to assess caspase-1 expression. (E) z-YVAD-fmk (10 μM) was treated the cells 2 h before treatment with DON or ATP/LPS and RT-PCR was conducted to assess *caspase-1* expression. (D) Caspase-1-dependent IL-1β secretion was elucidated using a caspase-1 inhibitor, z-YVAD-fmk and ELISA was performed using cell culture supernatant. Data from three independent experiments are expressed as the overall mean ± S.E. Statistical significance was determined by one-way ANOVA ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ vs. untreated control).

3.5. DON upregulates NLRP3/ASC-mediated inflammasome, which stimulates IL-1β secretion by activating caspase-1

Cleavage of pro-caspase-1 into active caspase-1 requires inflammasome complex formation. Therefore, we assessed the effect of DON on inflammasome complex formation in BV2 microglial cells. As we expected, DON increased the expression of ASC and NLRP3 genes (Fig. 9A) and proteins (Fig. 9B) in a dose-dependent manner. Transient knockdown of *ASC* and *NLRP3* genes significantly downregulated the expression of *ASC* and *NLRP3* genes stimulated by DON (Fig. 9C); however, DON-induced *caspase-1* (Fig. 9D) and *IL-1β* (Fig. 9E) expression was sustained, which suggest that the gene expression of *caspase-1* and *IL-1β* is not regulated by NLRP3/ASC inflammasome. Thus, active caspase-1 and IL-1β remarkably increased by treatment with DON; however, the strong expression was diminished in the presence of *ASC* and *NLRP3* siRNA (Fig. 9E and Fig. 9F). Collectively, these results suggest that DON upregulates the expression of NLRP3/ASC inflammasome complex, which subsequently induce active caspase-1, leading to the secretion of active IL-1β.

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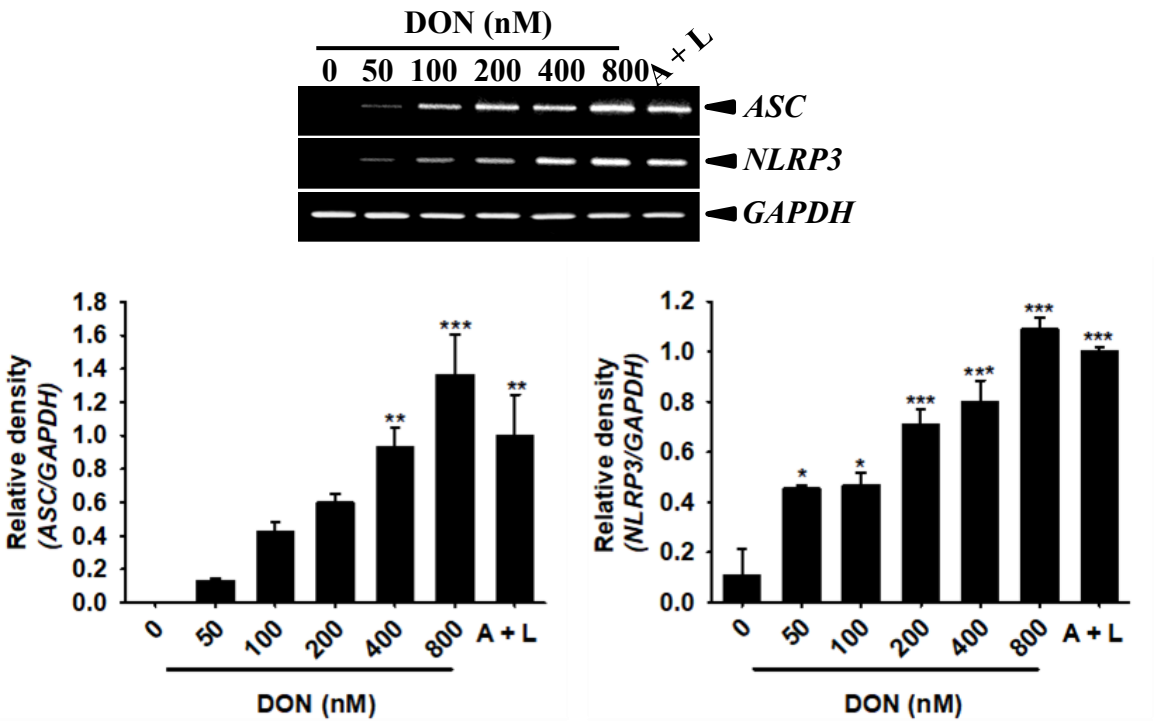

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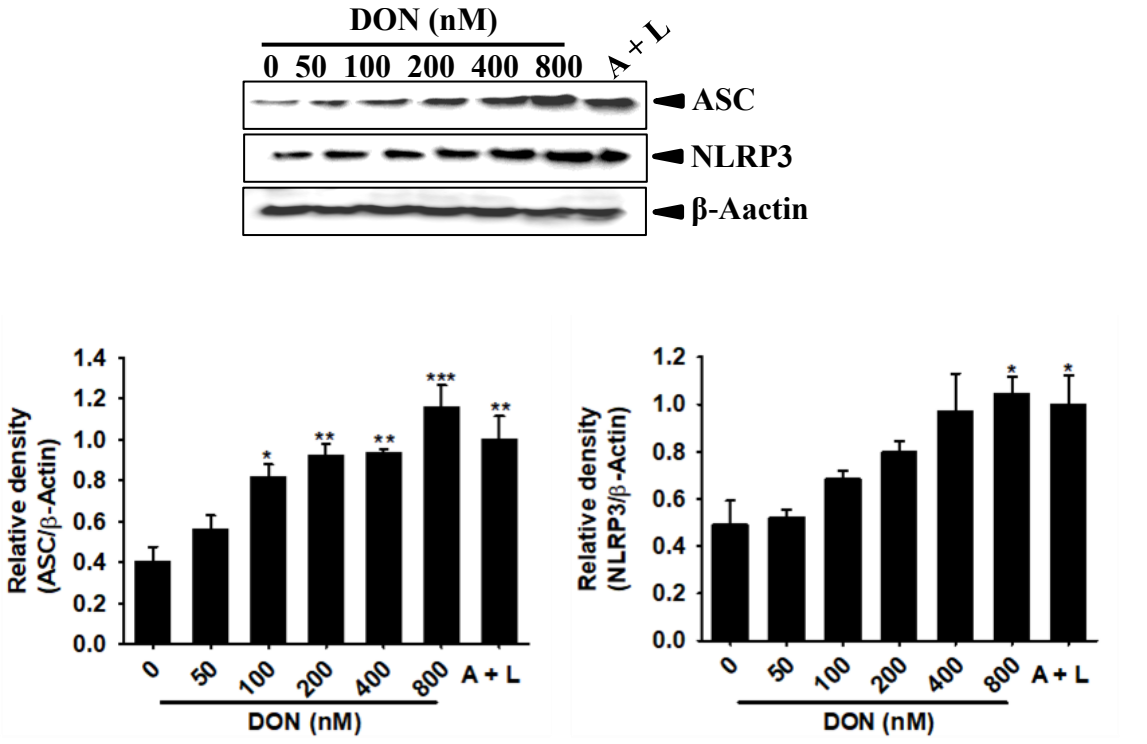

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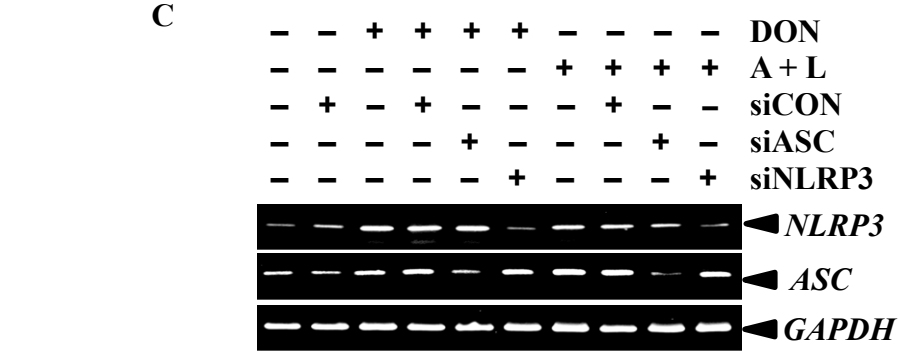

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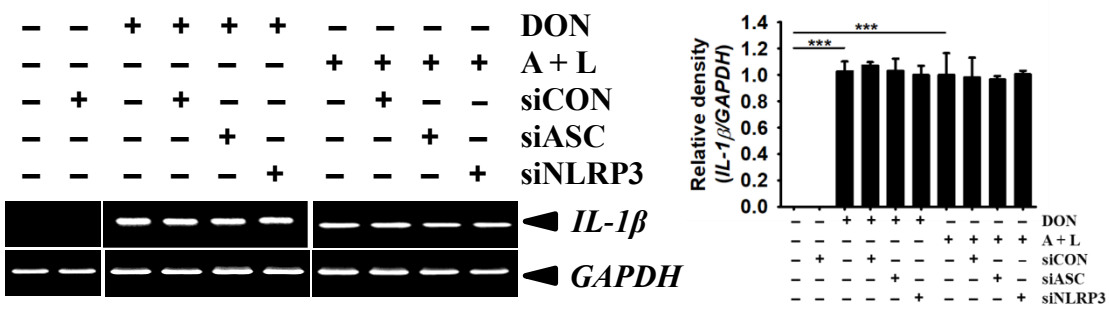

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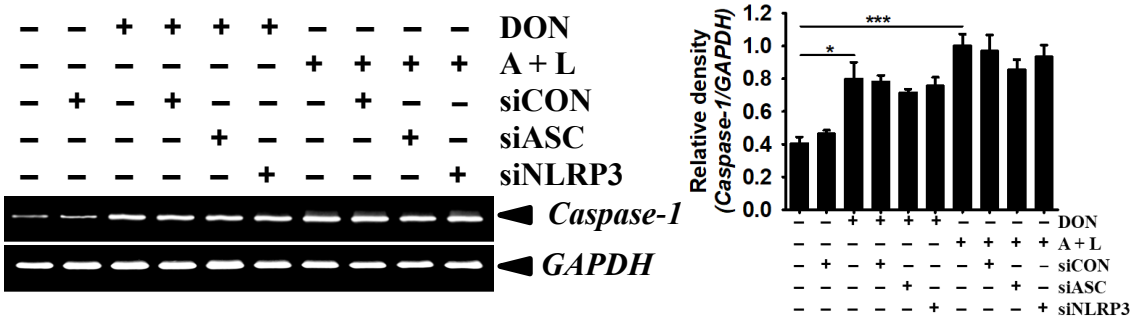

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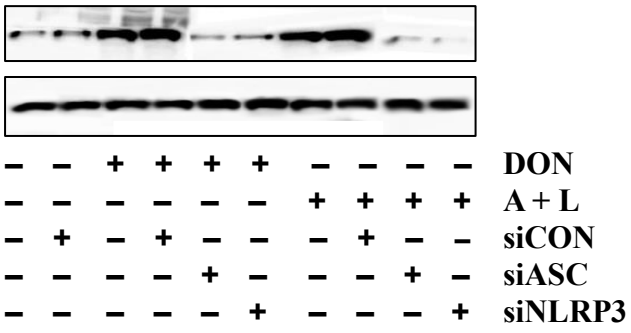

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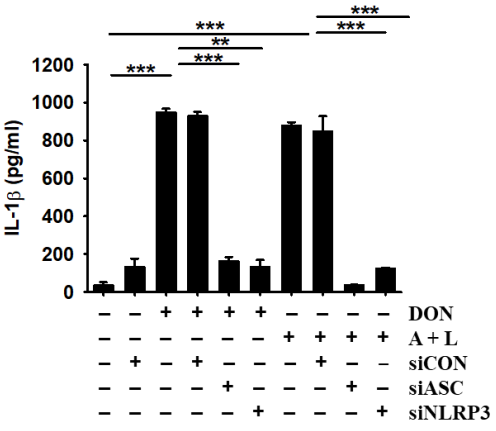

Figure 9. DON stimulates NLRP3/ASC inflammasome. (A) BV2 microglial cells were seeded at the density of 1×10^5 cells/ml and treated with the indicated concentrations of DON, and 1 mM ATP and 100 ng/ml LPS. Effect of DON on *ASC* and *NLRP3* inflammasome gene expression was assessed by RT-PCR. (B) Cytosolic fraction of the BV2 microglial cell lysate was used to assess the ASC and NLRP3 protein expression. (C-E) *ASC* and *NLRP3*-mediated capase-1 and IL-1β activation was assessed using transient knockdown of *ASC* and *NLRP3*. After pre-incubation of BV2 microglial cells with *siASC* and *siNLRP3* for 24 h, the cells were treated with 800 nM DON, and 1 mM ATP and 100 ng/ml LPS. RT-PCR was performed to confirm the effect of transient knockdown of *ASC* and *NLRP3* on *ASC, NLRP3, IL-1β* genes. (F and G) In a parallel experiment, active caspase-1 (F) and IL-1β (G) expression were detected by western blot analysis and ELISA, respectively. Data from three independent experiments are expressed as the overall mean ± S.E. Statistical significance was determined by one-way ANOVA ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ vs. untreated control).

4. Discussion

DON is one of the most common food-associated mycotoxins, particularly in cereals and cereal-derived products that possesses cell survival, activation and inflammation at low concentrations (nM) [24]. Since DON also contributes to brain tumour progression [25], pain hypersensitivity [26] and alterations in learning and memory consolidation [27], we focused on the pro-inflammatory effect of DON in BV2 microglial cells to elucidate its effect on creating brain disorders. Microglia cells are responsible for maintaining brain homeostasis and survival of neurones [28]. Chronic hyperactivation of microglia cells results in neuroinflammation while hypoactivation is associated with increased sensitivity of the brain to infections [29].

We found that low concentrations of DON exhibit no cytotoxic effect (up to 1000 nM) in BV2 microglia cells. However, DON drastically decreased the MTT activity with slight reduction of sub-G_1 phase compared to the untreated control and without any morphological apoptotic body, indicating that high concentrations of DON (≥ 1000 nM) stagnate the cell proliferation.

We observed that RAW264.7 macrophages are highly sensitive to DON exposure. Stimulation of macrophages with low concentrations of DON (nM range) causes their activation, the secretion of inflammatory cytokines such as IL-6, IL-12, TNF-α and the expression of intracellular proteins involved in the innate immunity such as COX-2 and iNOS via the NF-κB pathway (unpublished data). Therefore, we hypothesized that DON induces the brain disorders by stimulating the pro-inflammatory response. In current study, we found that DON induced the expression of *IL-1β* gene in BV2 microglia cells 0.5-1 h after DON exposure and extracellular IL-1β protein levels at 24 h. *IL-1β* gene is transcribed by activating the NF-κB signal pathway [30]. On the regards, DON exposure increased nuclear translocation of p50

and p65, and inhibition of NF-κB pathway drastically downregulated DON-induced *IL-1β* gene and active IL-1β protein expression. Extracellular IL-1β was used to assess the active form since it is secreted out of the cell after the cleavage via unknown process [31].

Cleavage of pro-IL-1β into active IL-1β is mediated by active caspase-1 which also upregulated upon DON exposure. Inhibition of caspase-1 lead to the downregulation of active IL-1β which is also induced by DON, confirming that caspase-1 directly upregulated the pro-IL-1β cleavage to active IL-1β. On the other hand, *caspase-1* gene also transcribed at the same time paralleled to the *IL-1β*, indicating the relationship between IL-1β and caspase-1. However, inhibition of caspase-1 has no influence on *IL-1β* gene transcription.

Activation of caspase-1 needs to undergo inflammasome complex formation. Combination of pro-caspase-1, ASC and NLRP3 is known as NLRP3 inflammasome complex which ultimately lead to the activation of pro-caspase-1 and thereby pro-IL-1β. Treatment of BV2 microglial cells with DON significantly induced the expression of *ASC* and *NLRP3* gene transcription and protein synthesis. However, transient knockdown of *ASC* and *NLRP3* has no influence on *caspase-1* or *IL-1β* gene transcription, which means that ASC and NLRP3 do not regulate *caspase-1* and *IL-1β* expression. Nevertheless, transient knockdown of ASC and NLRP3 significantly decreased the active caspase-1 and active IL-1β protein synthesis confirming the critical role of inflammasome complex formation for active form of caspase-1 and IL-1β.

In general, inflammasome-mediated mature IL-1β secretion is associated with two steps. The first signal is associated with NF-κB priming and the second is

associated with the inflammasome complex formation. Therefore, in the current experiment, we used 100 ng/ml LPS and 1 mM ATP as a positive control for the DON. LPS is a TLR4 ligand which activates the NF-κB pathway and thereby upregulates the IL-1β gene transcription [32]. Extracellular ATP mediates a wide range of effects by acting on P2 receptors which have been classified into P2X (ligand gated ion channels) and P2Y (G protein-coupled receptors [33]. After binding of extracellular ATP into ionotropic P2X7 receptor leads the cell to form NLRP3 inflammasome complex [34].

Taken together, our results demonstrate that DON upregulates *IL-1β* gene expression via NF-κB pathway and thereby upregulates the pro-IL-1β protein synthesis. The pro-IL-1β should be converted into its active and mature form to be a functional molecule. DON also upregulates the active caspase-1, which leads the cleavage of pro-IL-1β into active IL-1β and secreted it out of the cells. Low concentrations of DON exposure of BV2 cells upregulates the formation of inflammasome complex to cleave the pro-caspase-1 into active caspse-1 (Fig. 10). Although the brain permeability to DON could be differ from human to mouse, particularly DON exposure, could be a risk in terms of neurological diseases including brain in humans as well.

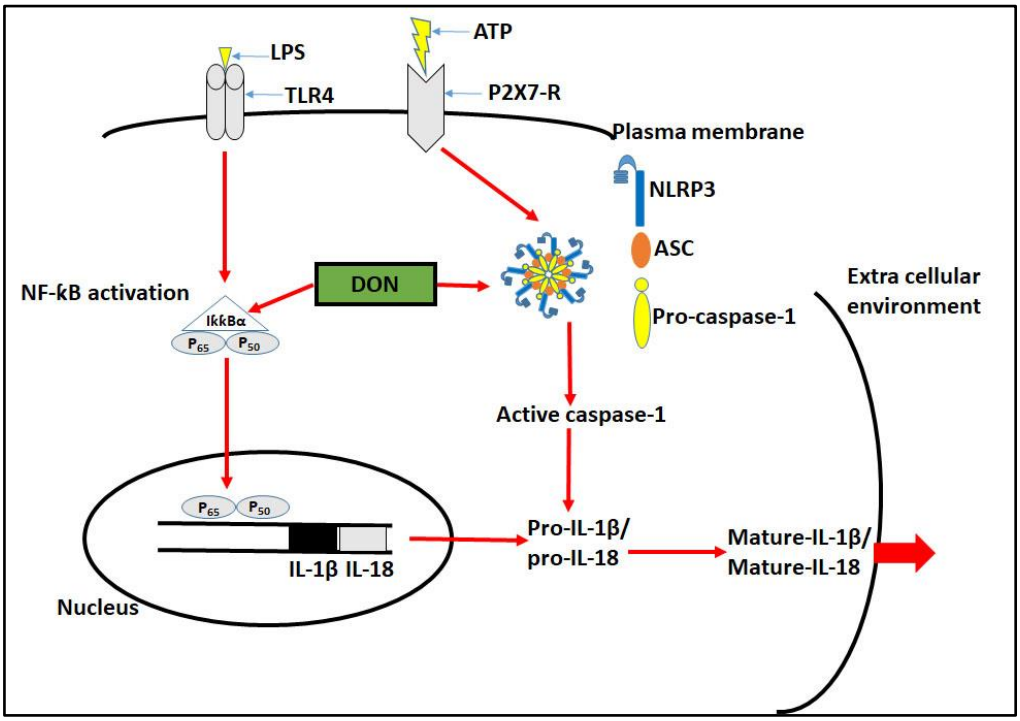

Figure 10. Graphical conclusion. Deoxynivalenol (DON) enters into the cell via either epoxide motif of DON binding with the nucleotide forming ribosomal RNA (rRNA) of the ribosomes or membrane-associated passive transporter/ bulk phase endocytosis process. After entering into the macrophages low dose of DON preferentially upregulates the NF-kb cell signaling pathway. Upregulation of NF-kb ensures the expression of *IL-1β* and thereby produces the pro-IL-1b cytokine which is cleaved by active caspase-1 into active IL-1β. Pro-caspase-1 binds to the cytosolic receptor complex called NLRP3 inflammasome in order to cleave pro-caspase-1 into active caspase-1. DON upregulates not only *IL-1β* but also the components of NLRP3 inflammasome complex including *caspase-1, ASC* and *NLRP3.*

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