



## Regulation of Chicken FABP4 Transcription by Toll-Like Receptor 3 Activation in DF-1 Cells

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**ABSTRACT** Long-chain fatty acids (LCFAs) are vital in cellular compartments, primarily regulating lipid metabolism. Fatty Acid-Binding Proteins (FABPs) facilitate LCFA transport, lipid synthesis, storage, and act as signaling molecules influencing various pathways, including inflammation. FABP4, in particular, is linked to vascular and cardio-related diseases, and it plays a role in macrophage-mediated inflammatory responses. Previous studies have identified FABP4 as not only a representative biomarker for lipogenesis but also as having correlations with immune responses. This study aims to investigate the regulation of the chicken *FABP4* (*chFABP4*) gene by toll-like receptor 3 (TLR3) activation and determine the signaling pathways that are involved in *chFABP4* transcriptional regulation. We analyzed the transcriptional regulation of *chFABP4* in TLR3-stimulated DF-1 cells. The results showed that *chFABP4* was up-regulated upon stimulation with polyinosinic-polycytidylic acid (PIC), a TLR3 ligand. Notably, *chFABP4* transcription was independently regulated in the NF- $\kappa$ B signaling pathway. It was up-regulated in p38 inhibition, demonstrating that the p38 signaling pathway might suppress the transcription of *chFABP4* within TLR3-activated DF-1 cells. In contrast, *chFABP4* expression was down-regulated in JNK signaling pathway inhibition, suggesting the positive regulation of JNK signaling pathway for *chFABP4* transcription in DF-1 cells in response to TLR3 activation, consistent with findings in macrophages. MEK pathway inhibition resulted in a similar regulation to NF- $\kappa$ B signaling. These results suggest that each MAPK contributes differentially to the transcriptional regulation of *chFABP4* by in DF-1 cells in response to TLR3 activation.

(Key words: chicken, fatty acid binding protein 4, DF-1 cells, toll-like receptor 3 signaling, transcriptional regulation)

## INTRODUCTION

Fatty Acid Binding Proteins (FABPs) are vital for fatty acid transport and metabolism, influencing fat storage and distribution (Zhang et al., 2020). They not only metabolize long-chain fatty acids (LCFAs) but also act as signaling molecules, impacting signaling pathways and gene expression (Yang et al., 2011). In vertebrates, there are 12 FABP types, with the chicken genome containing nine (Chmurzyńska, 2006; Zhang et al., 2020). FABP genes are expressed in various tissues, including muscle, adipose tissue, liver, intestine, myelin, and epidermis (Chmurzyńska, 2006; Cahyadi et al., 2013). Fatty Acid-Binding Protein 4 (FABP4) is an adipocyte-specific protein, highly expressed in subcutaneous adipose tissues (Gardan et al., 2007; Cahyadi

et al., 2013). It aids in fatty acid transport and oxidation and is associated with adipose tissue inflammation, synergizing with leptin (Gan et al., 2015). FABP4 enhances LCFA solubility and facilitates their transport between cellular membranes and organelles (Coburn et al., 2000; Gan et al., 2015). Its activity can be influenced by factors like LCFAs, oxidized low-density lipoprotein, peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), and insulin (Lapsys et al., 2000; Gan et al., 2015). Previous studies report the role of FABP4 in chicken physiology, impacting body weight (Cahyadi et al., 2013), fat mass expandability (Berger and Gélóën, 2023), and leptin regulation (Gan et al., 2015). It serves as a biomarker of lipogenesis and has links to inflammation and immunity. In macrophages, FABP4 regulates inflammatory responses through the IKK

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—nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway (Amiri et al., 2018). Suppressing FABP4 reduces inflammation, oxidative stress, and apoptosis by inhibiting NF- $\kappa$ B and activating PPAR $\gamma$  (Mao et al., 2021). Additionally, in acute kidney injury, increased FABP4 exacerbates inflammation and apoptosis via TLR4/c-Jun signaling activation (Wang et al., 2022).

The immune system responds to the invasion of bacteria, viruses, or external substances by activating innate immune receptors, such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs), which recognize characteristic molecular patterns of the microbes or derivatives. This leads to the production of inflammatory cytokines and the regulation of the transcription of innate immune receptor genes, thereby initiating innate immune responses. Toll-like receptors are transmembrane proteins that are activated upon recognizing pathogen-associated molecular patterns (PAMPs), including bacterial DNA, viral double/single-stranded RNA, lipopolysaccharides, peptidoglycans, and flagellin (Nawab et al., 2019).

RNA viruses like the avian influenza virus (AIV) trigger a host immune response through the TLR3 receptor within endosomes. This activation leads to NF- $\kappa$ B transcription factor activation, which, under normal conditions, is inhibited by I $\kappa$ B in the cytoplasm. Once activated, NF- $\kappa$ B regulates gene transcription in the nucleus. TLR3's activation initiates a cascade, resulting in cytokine and chemokine production, attracting immune cells to the infection site, and sparking inflammation. This response is vital for controlling viral infections and preventing their spread. Elevated TLR3 and cytokine levels during viral infection signify the initiation of a protective immune response. Studying TLR3 regulation in chickens can inform strategies to combat viral infections in poultry (Cheng et al., 2014; Zhang et al., 2015).

Recent studies have recognized FABP4 as a biomarker for chicken lipogenesis and chronic inflammation. However, Research on the signaling pathways affecting *chFABP4* expression in chickens is limited. Therefore, our investigation focused on *chFABP4* expression when exposed to TLR3 signals. We also assessed the transcriptional regulation of *chFABP4* using inhibitors for nuclear factor  $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein kinases (MAPKs), namely, p38, c-Jun-N-terminal kinase (JNK), and mitogen-activated protein

kinase kinase (MEK), in chicken DF-1 cells.

## MATERIALS AND METHODS

### 1. Cell Culture

The chicken cell line DF-1 (ATCC, CRL-12203) was purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). DF-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM), purchased from Biowest (Riverside, MO, USA), supplemented with 10% fetal bovine serum (FBS), 2 M L-glutamine and 100 U/mL each of penicillin and streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

### 2. Treatment of Poly (I:C) and Transcription Factor Inhibitors

Bay 11-7085 (Bay) was procured from Sigma-Aldrich (St. Louis, MO, USA). Polyinosinic:polycytidylic acid (PIC), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor) were acquired from Invivogen (San Diego, CA, USA), and PD-98059 (MEK inhibitor) was obtained from Medchem Express (Monmouth Junction, NJ, USA). These inhibitors were stored in accordance with the manufacturer's instructions. The inhibitors were applied to DF-1 cells at the following doses: 5  $\mu$ M for Bay, 10  $\mu$ M/mL for p38, 25  $\mu$ M/mL for JNK, and 10  $\mu$ M/mL for MEK. Prior to PIC treatment, Bay was treated for of 3 h, while p38 and JNK inhibitors were applied for 1 h, and MEK inhibitor was treated for 18 h.

### 3. RNA Extraction and cDNA Synthesis

RNA from DF-1 cells was isolated using the Pure-link MiniRNA Extraction Kit (Invitrogen, Carlsbad, CA, USA). In preparation for real-time quantitative polymerase chain reaction (RT-qPCR), 1  $\mu$ g of total RNA was utilized for cDNA synthesis, which was carried out with the Rever Tra Ace- $\alpha$ -first strand cDNA synthesis kit from Toyobo (Osaka, Japan).

### 4. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

For the analysis of *chFABP4* expression, quantitative re-

al-time-PCR (qRT-PCR) was performed with SYBR green supermix using CFX96™ IVD Real-time PCR System (Bio-rad, Hercules, CA, USA). The sequences of the *chFABP4* primers were 5'-CTG GCC TGA CAA AAT GTG CG-3' for the forward primer and 5'-GTG TGC CAC TCC TGT CTA GGG TT-3' for the reverse primer. Chicken interleukin-8 like 2 (*chIL8L2*) primers were 5'-CCA AGC ACA CCT CTC TTC CA-3' (forward) and 5'-GCA AGG TAG GAC GCT GGT AA-3' (reverse). The PCR conditions were as follows: an initial step at 94°C for 3 minutes; 39 cycles at 94°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; and a final step at 72°C for 10 minutes. Dissociation was performed at 0.5°C increments from 55°C to 95°C for over 25 minutes. Relative quantification analysis was performed using the comparative Ct ( $2^{-\Delta\Delta CT}$ ) method (Livak and Schmittgen, 2001). The expression of *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was used as endogenous control for the detection of mRNA expression levels. The sequences of *GAPDH* primers are: 5'-TGC TGC CCA GAA CAT CAT CC -3' for forward primer and 5'-ACG GCA GGT CAG GTC AAC AA -3' for reverse primer.

## 5. Statistical Analysis

The results were presented as the mean±standard deviation of at least three independent experiments. Statistical significance was evaluated using Student's t-test. Compared to the vehicle control,  $P < 0.05$  were considered significant. The difference among each value was determined by Tukey's test with  $\alpha < 0.001$ .

# RESULTS

## 1. Transcription of the *chFABP4* in DF-1 Cells in Response to a TLR3 Ligand

In this study, our primary objective was to investigate the influence of TLR3 signal activation on the transcription of the *chFABP4* gene in chicken DF-1 cells. To achieve this, we conducted two sets of experiments. First, we assessed the impact of various doses of PIC (0.1 µg/mL, 1 µg/mL, 5 µg/mL, and 10 µg/mL) on DF-1 cells over a 24 h period. We observed that *IL8L2* expression increased in a dose-dependent manner up to 10 µg/mL (Fig. 1A) as in our previous study

(Jang and Song, 2020). We observed a dose-dependent increase in *chFABP4* mRNA expression up to 5 µg/mL, with a slight decrease at 10 µg/mL, as depicted in Fig. 1C ( $P < 0.001$ ).

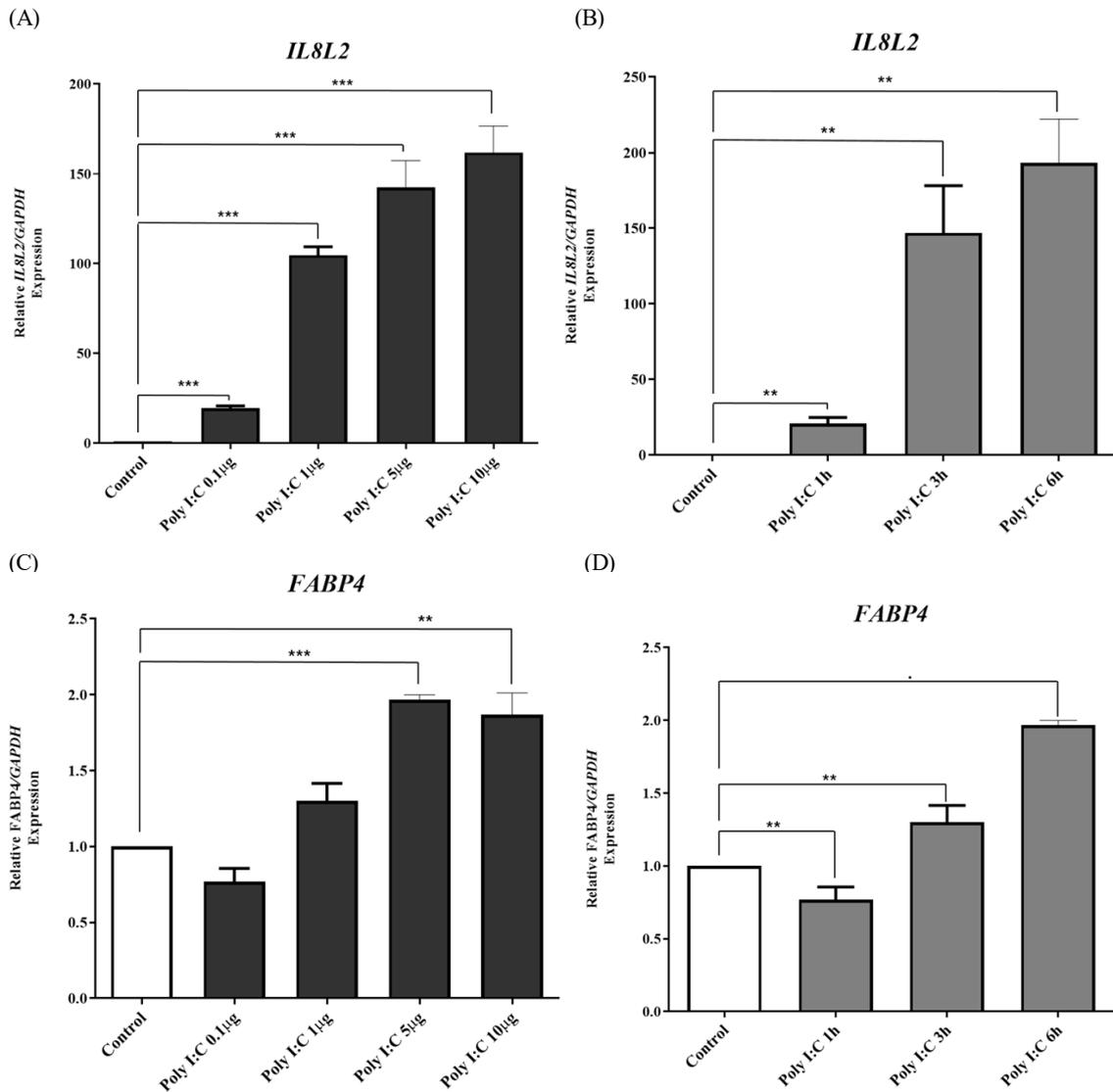
Subsequently, we treated DF-1 cells with 5 µg/mL of PIC for different durations, specifically 1 h, 3 h, and 6 h. Notably, both *chIL8L2* and *chFABP4* mRNA expressions increased in a time-dependent manner, as illustrated in Fig. 1B and Fig. 1D. These findings collectively indicate that *chFABP4* mRNA levels are responsive to TLR3 activation via PIC, suggesting its potential involvement in the TLR3 signaling pathway or other associated cellular responses.

## 2. Regulation of Chicken *chFABP4* Transcription through the NF-κB Signaling Pathways in TLR3-Stimulated DF-1 Cells

To test whether the NF-κβ transcription factor pathway is involved in *chFABP4* transcription during TLR3 activation, we treated DF-1 cells with Bay 11-7085, an NF-κβ inhibitor, and analyzed mRNA expression of *chFABP4* as well as *chIL8L2* (Fig. 2B). As a result, inhibition of NF-κβ transcription factor pathway did not alter the transcription of *chFABP4* whereas *chIL8L2* transcription was suppressed significantly, suggesting NF-κβ transcription factor pathways are dispensable for *chFABP4* transcription (Fig. 2B), whereas this pathway is indispensable for *chIL8L2* transcription (Fig. 2A).

## 3. Regulation of Chicken *chFABP4* Transcription through the MAPK Signaling Pathways in TLR3-Stimulated DF-1 Cells

To explore the potential involvement of MAPK pathways in *chFABP4* transcription during TLR3 activation, DF-1 cells were subjected to MAPK inhibitors, specifically SB203580 (a p38 inhibitor), SP600125 (a JNK inhibitor), and PD98059 (a MEK inhibitor), as previously described in our study (Park et al., 2023). Subsequently, we analyzed the mRNA expression of both *chFABP4* and *chIL8L2* as shown in Figs. 3A and 3B. In line with our previous study, the transcription of *chIL8L2* exhibited significant downregulation when each of the MAPK signaling pathways was inhibited (Fig. 3A). Indeed, the regulation of *chFABP4* transcription by MAPKs exhibited a unique pattern in the study. When JNK was inhibited in DF-1



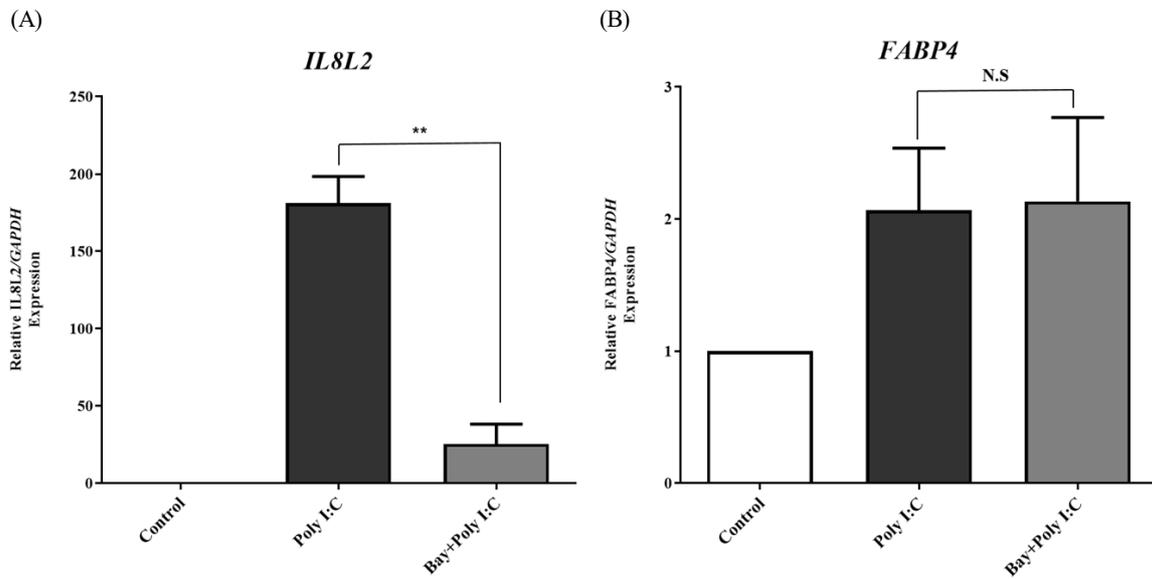
**Fig. 1.** Optimum time- and dose of poly (I:C) in DF-1 cell. Poly I:C time- and dose of *IL8L2* (A), (B) and *FABP4* (C), (D) to select the optimum condition during poly I:C treatment in DF-1 cell. Poly I:C 10 µg/mL considered to be the optimal dose but we used 5 µg/mL dose of poly I:C because of cell death. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  calculated using unpaired two-tailed Student's t-test. NS, not significant.

cells, it resulted in a decrease in *chFABP4* expression. In contrast, inhibition of p38 led to a significant upregulation of *chFABP4* expression, indicating that p38 normally suppresses the transcription of *chFABP4* during TLR3 activation. Notably, MEK inhibition did not have a substantial impact on the expression of *chFABP4*.

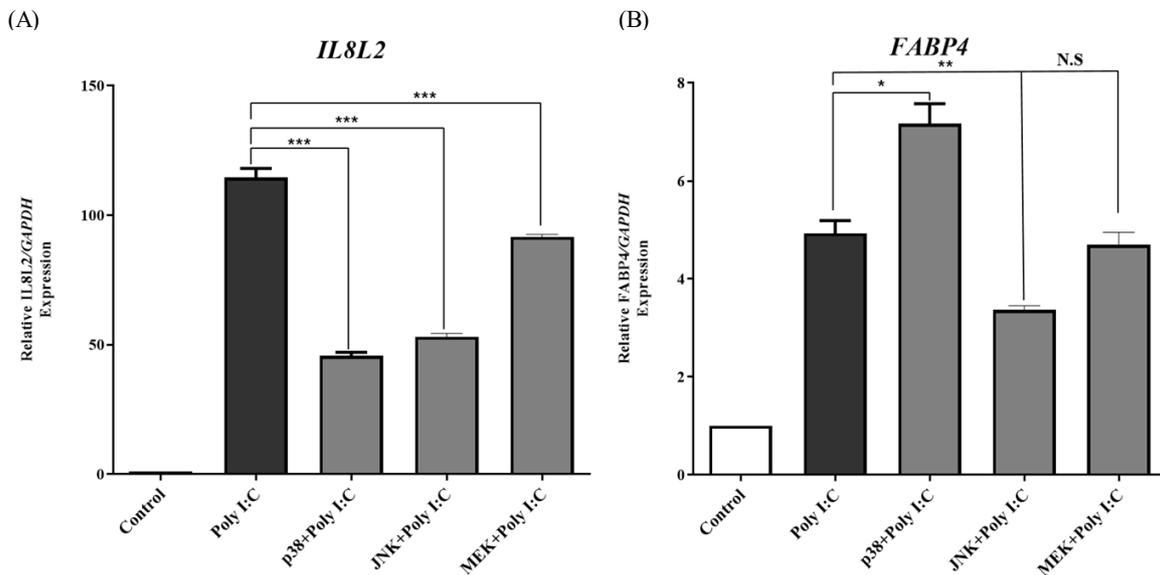
## DISCUSSION

Previous studies have emphasized the critical role of FABPs in lipid transport and metabolic responses across vari-

ous tissues, with distinct FABPs found in specific tissues and organs, such as liver FABP (L-FABP), intestinal FABP, heart FABP, adipocyte FABP, epidermal FABP, ileal FABP, brain FABP, and testicular FABP in mammals (Zimmerman and Veerkamp, 2002; Qigui et al., 2009). In avian species, the liver plays a significant role in de novo lipogenesis (Leveille et al., 1975; Shrago and Spennetta, 1976; Wang et al., 2017) and L-FABP has been identified as a genetic marker for fatness traits within the chicken QTL region (Wang et al., 2006). The role of FABP4 can extend beyond lipogenesis



**Fig. 2.** FABP4 transcription regulation by NF- $\kappa$ B inhibitor in DF-1 cell. Analysis of the expression of *IL8L2* and *FABP4* by QRT-PCR in DF-1 cells with suppressed NF- $\kappa$ B signaling pathways. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, \*\*\*\* $P$ <0.0001 calculated using unpaired two-tailed Student's *t*-test. NS, not significant.



**Fig. 3.** FABP4 transcription regulation by MAPK inhibitors in DF-1 cell. Analysis of the expression of *IL8L2* and *FABP4* by QRT-PCR in DF-1 cells with suppressed MAPK signaling pathways. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, \*\*\*\* $P$ <0.0001 calculated using unpaired two-tailed Student's *t*-test. NS, not significant.

and adipogenesis regulation to include its involvement in immune responses following diseases or inflammation. Elevated circulating FABP4 levels have been associated with metabolic and cardiac dysfunction (Furuhashi et al., 2014). Furthermore, a positive link between FABP4 and plaque instability in carotid atherosclerosis, connected to the presence of CD68,

CD163, and CD4, suggesting a vital role for FABP4 in the inflammatory lipid signaling process, potentially in conjunction with leukotrienes (Agardh et al., 2011). Additionally, gene expression profiles related to Wooden Breast disease (WBD) indicated an increase in *FABP4* expression during vascular disease (Papah et al., 2018). Given the close associa-

tion of *FABP4* with the inflammatory response, there is a strong need for studies exploring the connection between *FABP4* and signal transduction pathways involved in inflammatory responses in animals.

In our study, we investigated the transcriptional regulation of *FABP4* in TLR3-stimulated DF-1 cells. We identified the *chFABP4* gene through a transcriptome study in the kidneys of chickens exposed to varying calcium doses, as differentially expressed genes (DEGs) were identified in a previous study (Park et al., 2017). Activation of TLR3 in DF-1 cells with a synthetic ligand resulted in an up-regulation of *FABP4* mRNA expression, indicating that the TLR3 signaling pathway regulates the transcription of *chFABP4*. Importantly, the activation of both TLR3 and TLR4 led to an up-regulation of *FABP4* expression. However, it is particularly noteworthy that *FABP4* expression significantly increased in response to LPS stimulation (Kazemi et al., 2005).

In the context of lipid metabolism, various signaling pathways, such as MAPK, TGF- $\beta$ , and Wnt signaling pathways, have been shown to support PPAR signaling, as reported by Ma et al. in 2021 (Ma et al., 2021). Therefore, we conducted an analysis of *FABP4* regulation by inhibiting NF- $\kappa$ B, p38, JNK, and MEK signaling pathways. Our findings revealed distinct expression patterns for *FABP4*. Specifically, inhibiting JNK resulted in a significant decrease in *FABP4* expression, while inhibiting the p38 signaling pathway led to an increase in *FABP4* expression. Conversely, *FABP4* expression seemed to be independent of NF- $\kappa$ B and MEK inhibition.

Concerning inflammatory responses, it was evident that *FABP4* transcription was reliant on the TLR4-JNK signaling pathway activation, especially during LPS stimulation in mouse microglial cells. The absence of *FABP4* in this context had a substantial impact on microglial energy metabolism, resulting in a reduction of the pro-inflammatory cascade and phosphorylation of JNK (Kagawa et al., 2023). In macrophage cells, a positive correlation was noted between *FABP4* and JNK, associated with the activation of the inflammatory response (Hui et al., 2010). Notably, our present study yielded different results in the case of p38 inhibition, where inhibiting the p38 signaling pathway unexpectedly up-regulated

*FABP4* expression. Further research is needed to comprehensively understand the regulation of *FABP4* expression by other signaling pathways, such as p38, and MEK.

In summary, these results underscore the intricate regulation of *chFABP4* expression during TLR3 activation, which is influenced by the interplay of various MAPKs pathways. Specifically, JNK and p38 exhibited opposing effects on *chFABP4* regulation, implying that the role of MAPKs in controlling *chFABP4* is complex and involves multiple pathways. The specific pathway activated may vary based on the cellular context and signaling cues. Additional studies are warranted to thoroughly investigate these complex interactions. Further studies are required to fully understand these relationships.

## 적 요

지방산 결합 단백질(FABP)은 LCFA 수송, 지질 합성, 저장을 용이하게 하고, 염증을 포함한 다양한 경로에 영향을 미치는 신호 분자로 작용한다. 특히 FABP4는 혈관 및 심장 관련 질환과 관련이 있으며, 대식세포 매개 염증 반응에서 역할을 한다. 이전의 연구들은 FABP4를 지방 생성을 위한 대표적인 바이오 마커일 뿐만 아니라, 면역 반응과도 상관 관계가 있는 것으로 확인하였다. 본 연구는 톨-유사 수용체 3(TLR3) 활성화에 의한 닭 *FABP4(chFABP4)* 유전자의 조절을 조사하고 *chFABP4* 전사 조절에 관여하는 신호 경로를 결정하는 것을 목표로 한다. 우리는 TLR3 자극 DF-1 세포에서 *chFABP4*의 전사 조절을 분석하였다. 결과는 TLR3 리간드인 폴리이노신-폴리시티딜산(PIC)으로 자극 시 *chFABP4*가 상향 조절되었음을 보여주었다. 특히 *chFABP4* 전사는 NF- $\kappa$ B 신호 경로에서 독립적으로 조절되었다. p38 억제에서 상향 조절되어 p38 신호 경로가 TLR3 활성화 DF-1 세포 내에서 *chFABP4* 전사를 억제할 수 있음을 보여주었다. 이와는 대조적으로, JNK 신호 경로 억제에서는 *chFABP4* 발현이 하향 조절되었으며, 이는 대식세포의 연구 결과와 일치하며, TLR3 활성화에 반응하여 DF-1 세포에서 *chFABP4* 전사를 위한 JNK 신호 전달 경로의 긍정적인 조절을 시사한다. MEK 경로 억제는 NF- $\kappa$ B 신호 전달과 유사한 조절을 초래하였다. 이러한 결과는 각 MAPK가 TLR3 활성화에 반응하여 DF-1 세포에서 *chFABP4*의 전사 조절에 차별적으로 기여함을 시사한다.

(색인어 : 닭, 지방산 결합 단백질 4, DF-1 세포, 톨 유사 수용체 3 신호, 전사 조절)

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