9-POHSA Reduces LPS-Stimulated TNF-α Expression in Rat Hepatocyte: The Involvement of NF-KB-p65 Subunit

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Abstract. Metabolic (dysfunction) associated fatty liver disease (MAFLD) is phenotypically displayed by a buildup of fat in the liver. Over time, a patient with MAFLD tends to develop the disease into worse states like liver fibrosis and cirrhosis. The transition from simple steatosis into steatohepatitis is marked by inflammation. Several reports have suggested that endotoxemia is one factor that activates hepatic inflammation in MAFLD. Elevated lipopolysaccharide (LPS) localization and tumor necrosis factor-α (TNF-α) upregulation are found in the liver of patients and animal models with MAFLD. Fatty acid ester of hydroxy fatty acids (FAHFAs), a new group of lipids, is beneficial for metabolic diseases such as diabetes. The most pronounced effects of FAHFAs are their antiinflammation activity due to their ability to prevent upregulation of proinflammatory cytokines upon infection. The present study was undertaken to examine the anti-inflammatory activity of palmitoleic acid ester of 9-hydroxystearic acid (9-POHSA), one of the major endogenous FAHFA species in rat hepatocytes stimulated by LPS. Clone9 rat hepatocytes were cultured in DMEM without serum for 12 h and treated with LPS for 24 h with various concentration ranging from 0.1 to 10 μg/mL. TNF-α, p65, and phospho-p65 expression was assessed by immunofluorescence and Western blot. With or without LPS, the Clone9 cells were pretreated with 9-POHSA for 1 h. LPS, in a concentration-dependent manner, increased TNF-α expression and time-dependently increased the nuclear translocation of p65. Pretreatment of 9-POHSA inhibited the p65 translocation into the nucleus, TNF-α, and phospho-p65 expression. The results suggest that 9-POHSA exerts its anti-inflammatory activity by inhibiting p65 NF-kB related pathway.

Keywords: FAHFA · Fatty liver · LPS · MAFLD · TNF-α · 9-POHSA

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1 Introduction

There is no precise mechanism of how simple steatosis develops into the inflammation stage in steatohepatitis of metabolic (dysfunction) associated with fatty liver disease (MAFLD). However, the involvement of inflammatory mediators such as lipopolysaccharide (LPS), tumor necrosis-α (TNF-α), IL-1β, IL-6, adiponectin, and inflammatory cells such as neutrophils and macrophages has been reported [1]. At the early stage of MAFLD, persistent infiltration of neutrophils triggered by the bacteria is considered the first event to initiate the inflammation [2]. At the latter stage, infiltration macrophages and the resident macrophages of the liver are considered to play a role in causing inflammation in MAFLD. The oxidative, nitrosative stress, and proinflammatory cytokines activated by the macrophages harm and damage the hepatocytes [3]. Research aimed to target LPS-induced pro-inflammatory macrophages and their secreted cytokines such as TNFα IL-1β, IL-6, and adiponectin has attenuated if not stopped the progression of MAFLD [4].

The anti-inflammatory and antidiabetic activity of a novel class of lipid, fatty acid esters of hydroxy fatty acids (FAHFAs) are recently discovered as a new class of lipids which highly expressed in the adipocyte tissue and liver tissue [5]. Since then, researches on the biological and mechanical activity of FAHFAs have undergone attention from researchers. The most pronounced effects of FAHFAs are their antidiabetic and antiinflammation activity due to their ability to potentiate insulin release and prevent upregulation of proinflammatory cytokines upon infection [6]. Interestingly, different FAHFAs and their regioisomer may pose different antidiabetic and antiinflammation potentials. One type of FAHFAs may possess both antidiabetic and antiinflammation activity, while another FAHFAs may possess antidiabetics alone or antiinflammation alone. The FAHFAs have been reported to inhibit LPS-stimulated cytokines such as TNF-α, IL-1β, and IL-6 and increase the phagocytosis activity of macrophages inhibited by LPS [6]. However, the detailed mechanisms of these activities remain unknown. Due to the unique and diverse structure of FAHFAs, each FAHFA may pose a different mechanistic function. This study investigates the TNF-α and p65 expressions following LPS with or without 9-POHSA treatment in rat hepatocyte.

2 Materials and Methods

2.1 Materials

Clone 9 rat hepatocyte was purchased from ATCC (Manassas, VA, USA). Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F-12), phosphate buffered saline (PBS), and 100X penicillin-streptomycin (10,000 U/mL) were purchased from Life Technologies Corporation (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). LPS from *Escherichia coli* O111:B4 (L2630) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 9-POHSA was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Antibody against

phospho-NF-κB p65 (Ser536) (#3033) was purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody against NF-kB p65 (ab32536) was purchased from Abcam (Waltham, MA, USA). Antibodies against GAPDH (sc-32233), TNF-α (sc-52746), mouse and rabbit secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Immobilon Western Chemiluminescent HRP Substrate was purchased from Millipore (Burlington, MA, USA).

2.2 Culture and Treatment of Clone 9 Cells

Clone 9 was maintained in DMEM/F-12 supplemented with 10% FBS and 1X penicillin-streptomycin at 37 °C in a humidified 5% CO2 incubator. Once the cells reached around 80% confluence, they were seeded in 6-well or 12-well culture plates for experiments. Before LPS and 9-POHSA treatments, the growth media was replaced with DMEM/F-12 without serum for 12 h to make the cells quiescent for overnight. Experiments were done in triplicate with similar results.

2.3 Immunofluorescence Staining

The cells were fixed with 4% paraformaldehyde after treatment for 20 min, followed by washing with PBS thrice. Next, 1% BSA was added to block the unspecific binding of antibodies for one hour at RT. After blocking, the cells were incubated with primary antibody overnight at 4 °C. The following day, the primary antibody solution was removed, and the cells were washed with PBS thrice. Finally, a secondary antibody was added for one hour at RT. The cells were mounted with DAPI solution and covered with coverslips. The images were taken using fluorescence microscopy (Olympus), with the excitation and emission wavelength in the range of 358/461, 498/517, 590/617 nm for DAPI, p65, and TNF-α, respectively. The images were quantified using ImageJ (NIH).

2.4 Western Blot Analysis

After media removal, the cells were washed with PBS. The lysis buffer was added to collect the cells. The total protein was measured using Bradford assay kit, separated in 10% SDS-PAGE gel, and then transferred to a nitrocellulose membrane. The membrane was cut according to the desired protein size, and then incubated in primary antibody (1:1000) diluted in 5% BSA at 4 °C for overnight. The following day, the primary antibody solution was removed, and the membrane was washed thrice for 5 min each with tris buffered saline containing 1% Tween 20. Thereafter, the membrane was incubated with appropriate secondary antibody (1:2000) diluted in 5% BSA at RT for 1 h. Finally, chemiluminescent substrate was used to visualize the proteins. The signal was captured by using UVP ChemStudio Plus touch (Analytik Jena; Jena, Germany). The images were quantified using ImageJ (NIH).

2.5 Statistical Analysis

Data were shown as mean ± S.E.M. and were plotted and analyzed statistically with GraphPad Prism version 7.0.0.

3 Results

3.1 LPS Upregulates TNF-α in Hepatocytes and Induces Nuclear Translocation of P65

See Fig. 1.

3.2 9-POHSA Hampers TNF-α Upregulation and P65 Nuclear Translocation Induced by LPS in Rat Hepatocyte

See Fig. 2

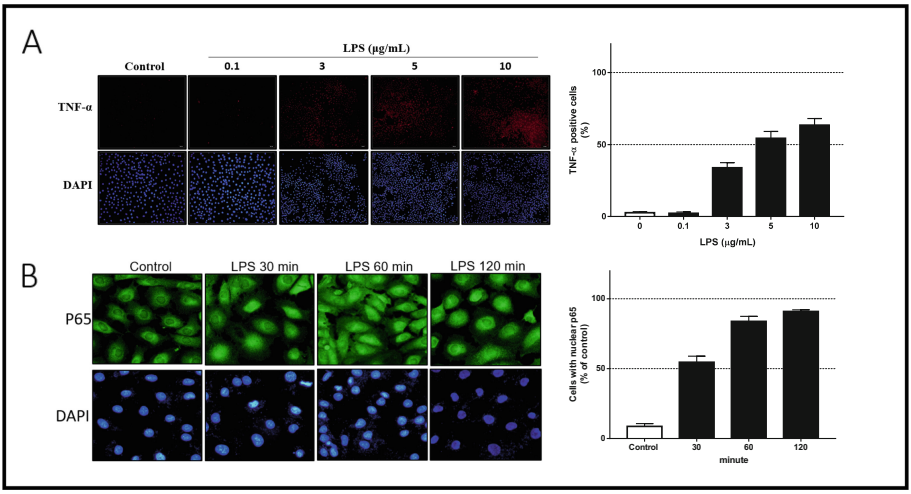

Fig. 1. **A.** Clone9 were cultured in DMEM/F-12 without serum for 12 h and treated with LPS for 24 h with various concentration ranging from 0.1 to 10 μg/mL. Representative fluorescence images are shown, n = 3. The images were taken using fluorescence microscope at 4X magnification. TNF-α expression was quantified by ImageJ software. **B.** Clone9 were cultured in DMEM/F-12 without serum for 12 h and treated with LPS (3 μg/mL) for 30-, 60-, and 120-min. Representative fluorescence images are shown, n = 3. The images were taken using fluorescence microscope at 20X magnification. P65 expression was quantified by ImageJ software.

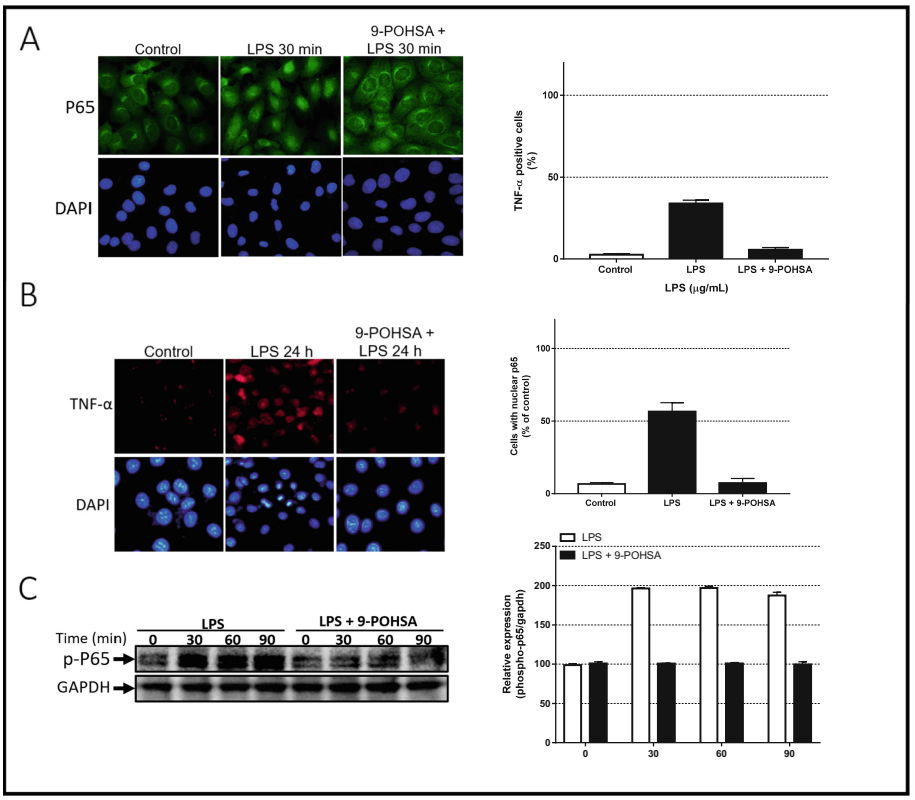

Fig. 2. **A.** Clone9 were cultured in DMEM/F-12 without serum for 12 h and pretreated with or without 9-POHSA for 1 h followed by LPS (3 μg/mL) for 24 h. Representative fluorescence images are shown, n = 3. The images were taken using fluorescence microscope at 20X magnification. TNF-α expression was quantified by ImageJ software. **B.** Clone9 were cultured in DMEM/F-12 without serum for 12 h and pretreated with or without 9-POHSA for 1 h followed by LPS (3 μg/mL) for 30-min. The images were taken using fluorescence microscope at 20X magnification. P65 expression was quantified by ImageJ software. **C.** Clone9 were cultured in DMEM/F-12 without serum for 12 h and pretreated with or without 9-POHSA for 1 h followed by LPS (3 μg/mL) for 30-, 60-, 90-min. Representative blot images are shown, n = 3. Phospho-p65 expression was assessed by Western blot and quantified by ImageJ software.

4 Discussion

MAFLD is characterized as hepatic steatosis accompanied by metabolic conditions such as diabetes, overweight, and abnormal lipid profile. Over time, a patient with MAFLD tends to develop the disease into worse states like liver fibrosis and cirrhosis. The transition from simple steatosis into steatohepatitis is marked by inflammation. Elevated LPS localization is found in the liver of patients and animal models with steatohepatitis [7]. Moreover, LPS accelerates the progression of fatty liver disease and increases the signaling and gene expression related to fibrosis [8, 9]. In response to LPS accumulation, liver cells secrete many cytokines and interleukins, hence magnifying the inflammation stage

[10]. TNF-α is one of the upregulated inflammatory cytokines following LPS accumulation which mediates inflammation and fibrosis progression in fatty liver disease [11, 12]. In addition, the amount of TNF-α in the serum reflects the pathogenicity of fatty liver disease [13]. In this study, we investigated the TNF-α secretion of hepatocytes to various concentrations of LPS. The activation of p65 is needed to initiate the expression of TNF-α [14]. Our results concur with the previous finding, hence the TNF-α upregulation in clone-9 hepatocyte is likely mediated by p65 activity. The recent findings of new endogenous lipid groups, FAHFAs, have opened new potential strategies to combat metabolic disease due to their anti-inflammation and anti-diabetes properties [5]. 9-POHSA, one of FAHFAs, is one of major FAHFAs in the serum [15]. Pretreatment with 9-POHSA hampers the TNF-α expression induced by LPS. As expected, TNF-α inhibition is accompanied by the blunted p65 activity, as shown by immunofluorescence staining and Western blot results.

5 Conclusion

Although this study lacks of detailed mechanism, we provide evidences that 9-POHSA indeed inhibited TNF-α upregulation possibly achieved through NF-κB inhibition. However, it does not rule out the chance that other cytokines and interleukins are involved in the hepatocyte response to LPS challenge; hence, further studies are needed to investigate these in *in vitro* and *in vivo* models. Moreover, a study on the mechanistic signaling pathway is warranted to open further insight into the 9-POHSA role in inflammation, particularly in the MAFLD.

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