RNA-seq analysis reveals that oleic acid exerts anti-inflammatory effects via modulating cell cycle in Raw264.7 cells

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ABSTRACT

Background: Lipopolysaccharide (LPS), a structural and protective compound primarily found in ordinary benign bacteria, could induce pro-inflammatory effect in a macrophage cell line Raw264.7 cells. Additionally, we previously showed that oleic acid (OA) possessed apoptotic effect in mouse originated Raw264.7 cells as well.

Methods: Cellular and molecular methods including flowcytometry and Western blot as well as bioinformatic methods including RNAseq and analysis.

Results: We demonstrated that OA could alleviate LPS-activated inflammatory effects, including apoptosis and secretion of cytokines via the modulation of cell cycle process. Further analysis revealed that OA reduced the LPS-elevated expression of p21, but not p16.

Conclusion: Our investigation has provided detailed information on LPS stimulation and OA remission in Raw264.7 cells, and laid solid foundation for the potential pharmaceutical application of OA as an antiinflammatory agent.

Keywords: Oleic acid · Lipopolysaccharide · Cell cycle control · p21Cip1/Waf1 · Transcriptome

Introduction

Our immune system is made up of at least two parts: the faster but more general innate immunity, and the slower but more specific adaptive immunity. The innate immunity is important for us to defend against microbial pathogens, and stands as the first line of defense in our body. Macrophages are a group of innate immune cells playing essential roles in the host defense process and maintenance of tissue homeostasis (1). Macrophages are capable of engulfing particles to engage as a primary function in phagocytosis (2). In addition, our immune system could also be altered by macrophages through the production and secretion of various cellular factors including interferons, interleukins, growth factors and lipid mediators (3). Meanwhile, macrophages is also important for tissue repair processes as well (4). Due to its importance in the immunity, the functions and regulations of macrophages have been extensively studied.

Lipopolysaccharide (LPS), made up of lipids and sugar, is a structural and protective compound primarily found in ordinary benign bacteria, which, once presented in the bloodstream after microbial invasion, could drive sudden activation of macrophages and subsequent release of inflammatory mediators (5). It has been well established that, upon the treatment of LPS, macrophages could elicit pathogenic inflammatory responses (6) by the production and release of immune regulated enzymes such as nitric oxide synthases (iNOS) and cyclooxygenase-2 (COX-2),

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as well as cytokines and chemokines such as interleukin-1β (IL-1β), tumor necrosis factor (TNF-α), and IL-6 (7). Due to the observations of dysregulated inflammatory responses in many inflammatory disorders including asthma, rheumatoid arthritis and other autoimmune diseases (8), it would be quite beneficial to study the regulation of LPS-activated inflammatory response in macrophages.

Various investigations indicated that fatty acids exhibited inhibitory effect on cytokine production in LPS-stimulated human monocytes and Raw264.7 cells (9-12). Additional study showed that fatty acids exerted anti-inflammatory effects via blocking transcriptional factors including nuclear factor κB (NF-κB) (13), and suppressing the function of mitogen-activated protein kinases (MAPKs) (14). Meanwhile, we previously reported that unsaturated fatty acids extracted from Chrysanthemum, including linoleic acid and linolenic acid, induced apoptosis of Kupffer cells, a type of specific tissue-resided macrophages in the liver (15).

Therefore, we intended to investigate the detailed mechanism of oleic acid (OA) in suppressing both LPS-induced apoptotic and pro-inflammatory effects in Raw264.7 macrophages in this study by combining biochemistry and bio-informatics approaches.

Materials and Methods

Reagents

The DMEM (10569-044) basic medium supplemented with fetal bovine serum (12484-010) was obtained from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). The cell counting kit-8 (CCK-8; BestBio, BB4202) was used for cell survival assay. Antibodies were provided either by CST or Abcam. LPS (L8880) and bovine serum albumin (BSA; A8010) were obtained from Solarbio (Beijing, China) and OA (HY-N1446) were from MedChemExpress. The OA storage solution was prepared by dissolving OA in a BSA solution (15 mM) at the concentration of 100 mM. OA working solution was delivered from the China Center for Type Culture Collection (Wuhan, China). Normally macrophages were obtained via a dilution of the storage solution in a ratio of 1:50. Cell cycle analysis kit (40301ES60) and apoptosis detection kit (40302ES60) were obtained from Yeasen.

Cell culture and cell survival assay

Mouse macrophages (Raw264.7, GDC0143) were delivered from the China Center for Type Culture Collection (Wuhan, China). Normally macrophages were maintained in a CO2 incubator at 37 °C. Cell morphology was monitored under a microscope (Eclipse Ti, Nikon Inc) and imaged with a DI-RS2 camera (Nikon Inc).

The CCK-8 assay was used to monitor the cytotoxicity of LPS and OA to Raw264.7 cells. Briefly, Raw264.7 cells with a density of approximately 1 × 10^5 cells/mL were placed into 96-well plates, and then treated separately for 24 h. Next, CCK-8 was added for an incubation of 2 h. Absorbance at 450 nm were monitored using an Infinite 200 PRO system (Tecan Group Ltd., Maennedorf, Switzerland). All experiments were done in triplicates and conducted independently for three times at least. A set of control wells without cells was included in each plate.

Apoptosis monitoring via flow cytometry

Raw264.7 cells with a density of 1 × 10^5 cells were placed into a well of the six-well plate. After incubated with indicated treatments, cells were washed and aspirated in 1 mL PBS. Later 5 μL Annexin-V-FITC were suspended in 400 μL binding buffer and directly added to a well for 15 mins at 4 °C. Finally, 10 μL PI buffer was incubated for 5 mins. Flow cytometry (Guava System; Merck, Germany) was used for the detection.

qPCR performance and analysis

Total RNA was extracted from indicated treatment of Raw264.7 cells using TRIzol reagent (Invitrogen) and reversely transcribed with a PrimeScript RT reagent kit (Takara, Dalian, China). The transcriptional expression levels were analyzed by qPCR using Thermal Cycler Dice Real Time System (Takara, Shiga, Japan). Ordinary conditions with ∆∆Ct method were used during the performance of qPCR. Each data were represented from triplicates. GAPDH for each experiment was monitored and employed as an internal control.

The primer sets used in mouse-sourced Raw264.7 cells were:
For COX-2 (ptgs2), forward, 5'-GCGCATACTCAAGCAGGGA-3' and reverse, 5'-AGTGGTAACCGCTCAGGTGTG-3';
For iNOS (nos2), forward, 5'-GAGACAGGGAAGTCTGAAGCC-3' and reverse, 5'-CCACGAGTAGTGTGCTCTTTC-3';
For β-catenin (ctnnb1), forward, 5'-GGTGCGCTCATTATGGACCTGC-3' and reverse, 5'-ATAGACACCTGTTGCAAGAAG-3';
For F4/80 (agrl), forward, 5'-CGTGTGGTGGTCGAGCCCTGTA-3' and reverse, 5'-CCACATCAGTGTCCAGGAGAC-3';
For Cd68 (cd68), forward, 5'-GGCGTGTAACATAATGTGTCG-3' and reverse, 5'-AGCAGGCAAGGTGAACAGCTG-3';
For Mrc1 (mrc1), forward, 5'-GTTCACCTGGAGTGAGGTTTC-3' and reverse, 5'-AGGACATGCCAGGCTACCTT-3'.

Detection of protein expressions

After treatments as indicated, cells were harvested and the total protein concentration was determined. After that, lysates of cells were fractionated with either 10% or 12.5% SDS-PAGE gels and transferred to PVDF membranes (IPVH00010; Millipore Corporation, MA, USA). After blocking with 5% non-fat milk, the membranes were blotted with antibodies against the following proteins: caspase-3 (1:1000, 9662S, CST), PARP (1:500, 9542S, CST), iNOS (1:500, ab15323, Abcam), COX-2 (1:1000, ab169782, Abcam), p16 (1:1000, 18769S, CST), p21 (1:500, 2947S, CST)
and β-actin (1:1000, 4970S, CST) at 4 °C overnight. After that, the blot was washed thrice with TBST solution and followed by the incubation with a secondary antibody (1:2000, goat anti-rabbit or goat anti-mouse, A21010 or A21020, Abbkine, USA) for 1 h at room temperature. A developer and fixing solution were used for visualization of protein bands. ImageJ and Microsoft Excel were used for quantification and analysis of the protein bands.

RNA-sequencing and analysis
RNA samples were collected and used for the construction of cDNA libraries by an Illumina TruseqTM RNA sample prep kit (Illumina, CA, USA). To summarize, cDNA was prepared and reversely transcribed in a double-stranded manner. After that, they were ligated and amplified before the assessment and quantitation of the library. Cluster generation of loads of a single mRNA-sequencing library was performed on the Cluster Station. Sequence-by-synthesis single reads of 54-base-length using the Hiseq2000 Truseq SBS Kit (v3-HS, Illumina) were generated on the HiSeq X system. TPM and FPKM matrix were calculated from the read count matrix. FPKMs were mainly used for the analysis among samples while less biased TPMs were used for further analysis on gene expressions. Gene expression between compared groups with log2FC > 1 and p < 0.05 was considered as significantly different. The Venn diagram was composed at the following website: https://www.venn.diagram.net/venn-diagram-and-sets.html. The David resources version 6.8 was utilized for the performance of GO enrichment at the following website: https://david.ncifcrf.gov.

Cell cycle phase identification
Raw264.7 cells were plated at the density of 105 in a 35-mm dish and treated with either LPS or OA for 24 h. Later on, cells were detached and washed twice with PBS, and subsequently fixed overnight at 4 °C in 75% ethanol. After removal of ethanol by centrifugation, cells were re-suspended and incubated for 30 min in PBS with 10 µg/mL of RNase A at 37 °C, and then stained with PI (50 μg/mL). The fluorescence was measured by a flow cytometric Guava System (Merck, Germany). Cellular multiplets were excluded. Histograms of DNA content were determined by the fractions of the population in cell cycle.

Statistical analysis
Results were normally indicated as means ± standard error of the mean (SEM). One-way or two-way ANOVA followed by a Tukey’s post-hoc test or a Bonferroni post-test as indicated were performed using Microsoft Excel software. Statistical significance was identified with a p value < 0.05.

Results
OA treatment alleviates inflammatory responses to LPS in Raw264.7 cells
To test the effect of OA treatment on LPS-induced
inflammatory responses in Raw264.7 cells, 1 mM OA was added to the culture medium in the presence or absence of LPS treatment (100 and 200 ng/mL for 24 h). As shown in Figure 1, the overall morphology and cell viability were greatly alleviated in the OA-treated groups, comparing with LPS treatment alone, suggesting that OA treatment could tranquilize the inflammatory response of Raw264.7 cells to LPS treatment. Comparatively, it is also worth noting that OA treatment alone possessed neither morphological effects nor reduction in viability of Raw264.7 cells. Further flow cytometry analysis confirmed the effect of OA treatment on LPS-induced apoptosis in Raw264.7 cells as demonstrated in Figure 2. Interestingly, 1 mM OA treatment was sufficient to nearly abolish the apoptotic effect of 200 ng/mL LPS treatment. However, since 100 ng/mL LPS treatment was far more than enough to illustrate the effect of OA treatment, we therefore used only 100 ng/mL LPS throughout the rest of our investigation.

Additionally, further analysis on both the mRNA and protein levels also revealed the same trends, as illustrated in Figure 3. OA treatment greatly reduced the elevated levels of inflammatory effectors and markers, COX-2 (ptgs2) and iNOS (nos2), by the treatment of LPS. Moreover, OA was also effective in decreasing the elevated expression level of β-catenin (ctnnb1), the regulatory protein in the Wnt signaling. Meanwhile, inflammation-related surface markers of Raw264.7 cells during LPS treatment, such as F4/80, CD68 and CD206, were all sensitive to OA treatment. Western blot also demonstrated that apoptosis-related proteins caspase-3 and PARP were sensitive to OA treatment as well. These evidences further strengthened the notion that OA treatment could alleviate inflammatory responses in LPS-stimulated Raw264.7 cells.

RNA-seq analysis reveals a drastic mRNA expression profile change
To comprehensively study the effects of OA treatment on LPS-treated Raw264.7 cells, transcriptome analysis was conducted for the Control group, the LPS (LPS treatment alone) group, and the LPS/OA (LPS plus OA treatment) group. Figure 4 showed the general correlation and component analysis results. As could be seen, gene profiles with changed expression levels were quite different among the three groups. However, the pattern change of the LPS group was the most significant (with a correlation of 0.92), while the correlation increased to 0.94 between the LPS/OA group and either the LPS or the Control group, suggesting that OA treatment restored, at least, part of the gene expression profiles from the LPS group to the Control group.

Figure 2. OA treatment alleviates LPS-induced apoptosis in Raw264.7 cells. (A) Flow cytometry analysis of annexin-V / PI staining in Raw264.7 cells after various treatments. The proportions of cells were included in each quadrant. (B) Summary of (A) on double staining.
After a closer look at the expression profiles of the known factors and effectors involved in the inflammation process, many of them increased after LPS treatment, but restored in the presence of OA, as shown in Figure 5A.

To better separate the expression profiles, genes were categorized into four groups by their behavior: LPS up-regulated, LPS down-regulated, LPS/OA up-regulated and LPS/OA down-regulated genes. Venn diagram was drawn based on the above-mentioned categorization, as illustrated in Figure 5B. Primarily, 748 genes located in the LPS up-regulated and LPS/OA down-regulated regions, while 716 genes located in the LPS down-regulated and LPS/OA up-regulated regions (Figure 5C). We assumed that most of genes potentially involved should fall into these two categories due to the pattern of changes we observed previously. It was also important to note that 0 could be seen in the Venn diagram since one gene could not be categorized as both LPS down-regulated and up-regulated in the LPS group or likewise in the LPS/OA group. Otherwise, non-zero value occupied these zero would be a sign of defect in the RNA-seq result.

**OA treatment modulates cell-cycle related genes to counteract LPS’s effect**

To further clarify involved genes in OA treatment, genes from the aforementioned four categories were collected for GO enrichment analysis. Results were illustrated in Figure 6A. genes that are involved in inflammation-related biological processes fell into three of four categories. While in LPS down-regulated and LPS/OA up-regulated category (with 716 genes), cell cycle and mitosis were the most enriched biological processes, suggesting a close relationship between these genes and cell cycle control. More importantly, as depicted in Figure 6B, 70 out of 119 genes that were related with any of three processes including cell cycle, cell division and mitotic nuclear division, fell into the cross point, suggesting that OA treatment indeed affected the cell cycle control of Raw264.7 cells. Some of the genes and their expression levels were shown in Figure 6C, and among them aurora kinase a (aurka, mitotic serine/threonine kinase), cyclin A2 (ccna2, controls both checkpoint at the G1/S and the G2/M transition), cyclin B2 (ccnb2, essential for G2/M transition) and cyclin-dependent kinase 1 (cdk1, promotes G1/S and G2/M transition) were known key modulators of the cell cycle.

**OA treatment affects cell cycle control via p21 but not p16 in Raw264.7 cells**

To confirm our observations in the RNA-seq analysis, cell cycle states of Raw264.7 cells were monitored by flow cytometry. As demonstrated in Figure 7A and B, LPS treatment significantly decreased the G2 phase population while increased the G0/G1 phase population of Raw264.7 cells. However, OA treatment restored these changes, suggesting that OA treatment indeed recovered the phase population. Further Western blot results demonstrated that p21, instead of p16, were the potential upstream regulator of cell cycle control in the effect of OA on LPS treatment, as shown in Figure 7C. This further strengthened the
notion that OA could restore LPS-induced inflammatory effect via cell cycle control in Raw264.7 cells.

Discussion
In this study, cellular and molecular as well as bioinformatic methods including flow cytometry, qPCR and Western blot were used to verify the detailed mechanism of OA’s inhibitory effect on LPS-activated Raw264.7 cells. But before any conclusion could be drawn, several points need to be discussed. Firstly, Raw264.7 cells are a well-established cell model for macrophages. Hence our results here might possess the potential to be extrapolated into other types of macrophages, for instance, microglia in the central nervous system, sinus histiocytes at lymph nodes, pulmonary alveolus, and intestine macrophages in the gastrointestinal tract, which could widen the potential application of OA, or even other fatty acids, in our body, as being proposed previously (16). However, as mentioned previously (17), the polymorphic feature of Raw264.7 cells, which contain more than one phenotype individually within this cell lineage, would probably compromise the significance of the current study. Meanwhile, the low apoptotic rate by LPS treatment revealed in Figures 1 and 2, and the low inhibitory rate of cell cycle in Figure 7 might be explained largely by this specific feature of Raw264.7 cells as well. Therefore, more investigations on other in vitro and in vivo systems and/or on other types of fatty acids should be conducted to confirm the phenomenon uncovered in the current study.

Secondly, it has been shown that OA is abundant in olive oil (809.53–1045.52 μg/g), which is available and recommended for everyday life (18). Hence, it would be quite meaningful to study the potential pharmacological usage of OA. Various proteins and signaling pathways, including matrix metalloproteinase-9 (19), focal adhesion kinase (20), free fatty acid receptor 1 and free fatty acid receptor 4 (21), mTOR signaling (22), PI3K/AKT

Figure 4. RNA-seq analysis reveals different gene expression profiles in the control, LPS, and LPS/OA groups. (A) Correlation analysis of RNA-seq results. The color of the block indicated the correlation co-efficient. (B) Principle component analysis of the RNA-seq results. (C) Volcano plot of the RNA-seq results. Blue dots indicated expression of down-regulated genes while red dots indicated expression of up-regulated genes. The correlation co-efficient (r) between compared groups was indicated in each image.
signaling (23), etc., have been implicated to be modulated upon the treatment of OA in many different cells and tissues. Meanwhile, as stated in the introduction, investigations also implicated the efficacy of fatty acid treatment on the inhibitory effect in the immune system or in macrophages in particular. However, investigation at the whole transcriptional scale was still insufficient in this very topic. In our study here, RNA-seq analysis was performed in the attempt to acquire more information, and results showed that OA restored the expressions of a large quantities of genes altered by LPS treatment, leaving only a small portion unchanged. Moreover, by combining the results in the LPS group and the LPS/OA group, we successfully narrowed down the target of change into cell cycle process. It was also interesting to note that the mitosis process was also altered and restored by LPS and OA, respectively. This piece of evidence further strengthened the notion that OA was capable of overturning the pro-inflammatory effect of LPS in macrophages.

Thirdly, p21, sometimes named as p21Cip1/Waf1, is also known as CDK-interacting protein 1, which not only participates in the control of cell cycle process but also is involved in tumor suppression (24). In addition, p21 has been proposed to be a biomarker for cancer stem cells due to their associations. As shown in the current study, OA could reduce the expression of p21 evoked by LPS treatment, which would further highlight the therapeutic potential of OA in cancer treatment. Multiple recent studies illustrated the anti-cancer ability of OA or other fatty acids (25-27), which were quite consistent with the current investigation. However, whether p21 was involved or modulated in this anti-cancer ability still needed to be illustrated. Furthermore, p21 has been shown to be one of the downstream factor of p53 signaling. Here in this study, we observed no significant change in the expression of
p53 mRNA in RNA-seq analysis. However, transcriptional change was not the only way for p53 to perform its physiological or pathological functions. Changes in other stages such as post-translational modification and subcellular localization might as well be important for the function of p53. Therefore, it would be plausible to anticipate changes of p53 function after the treatment of OA.

Last but not least, no obvious effect was seen in cells under the OA alone treatment. This suggested that the modulation of cell cycle by OA treatment was limited in the LPS treatment condition. This could be explained partly by the relative low expression levels of p21 before LPS treatment. Alternatively, the internalization and accumulation rate of OA in different states of cells might also be a contributing factor for the ineffectiveness of OA towards unstimulated macrophages.

**Figure 6.** Cell cycle related process is the most obvious in GO analysis of the RNA-seq results. (A) List of top 5 enriched processes in the GO analysis. Red color highlighted processes with the most p value in the analysis. (B) Venn diagram of genes related with the three most enriched processes including cell cycle, cell division, mitotic nuclear division. The percentage of gene enclosed in each catalog was enclosed in the bracket. (C) Expression profiles of checkpoint and/or mitosis related genes.

**Conclusion**

In a word, in murine macrophage Raw264.7 cells, we have demonstrated that OA could alleviate LPS-stimulated inflammatory effect, via the inhibition of p21 and modulation of the cell cycle.

**Conflict of interest**

The authors declare that they have no conflicts of interest to disclose.

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**Supplementary Information**

The supplemental material can be downloaded online at: https://stemedicine.org/index.php/stem/article/view/83

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Figure 7. p21 but not p16 controls the alteration of cell cycle process in Raw264.7 cells under LPS or OA treatments. (A) Flow cytometry analysis revealed the increase of G0/G1 phase and the reduction of G2 phase. (B) Summary of A. (C) Western blot analysis revealed that the expression of p16 remained steady while the expression of p21 changed significantly. The molecular weight of each protein was indicated at the left side of the blot.

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