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Original Contribution

Glucocorticoids inhibit lipopolysaccharide-mediated inflammatory response by downregulating microRNA-155: a novel anti-inflammation mechanism

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ABSTRACT

Glucocorticoids (GCs) are among the most widely used and effective therapies for many chronic inflammatory diseases. Although attempts have been made to identify important protein-coding genes and pathways involved in the anti-inflammatory effect of GCs, knowledge of genomic aberrations associated with noncoding genes, such as micro-RNAs (miRNAs), and their contributions is relatively limited. In this study, a systematic screening of the miRNA expression profile by microarray showed that GCs inhibited the expression of miR-155 in lipopolysaccharide (LPS)-induced macrophage inflammatory responses. Overexpression of miR-155 markedly reversed the suppressive action of GCs, whereas inhibition of miR-155 exhibited an effect similar to that of GCs on LPS-treated RAW264.7 cells, indicating miR-155 to be a functional regulator in the anti-inflammatory effect of GCs. Furthermore, GCs inhibited miR-155 expression in a GC receptor- and NF-κB-dependent manner. Bioinformatics analysis and luciferase assay revealed that the NF-κB binding site located in the promoter region of the B-cell integration cluster was important in mediating the GC-driven suppression of miR-155 in response to LPS stimulation. In addition, the combination of treatment with GCs and inhibition of miR-155 enhanced the anti-inflammatory effect of GCs on LPS-stimulated RAW264.7 cells. Therefore, we identify miR-155 to be a novel target through which GCs exert their anti-inflammatory effect on the LPS-induced macrophage inflammatory response. These findings may provide a basic rationale for new approaches in the effort to develop anti-inflammatory therapeutics.

Because of the recognition of their anti-inflammatory effects, GCs have been at the forefront of therapies to control inflammatory and autoimmune diseases for the past few decades [1]. Natural and synthetic GCs are effective at dampening the inflammatory response to pathogen invasion and protecting the host from excessive inflammation [2]. Thus, they are widely used to treat both acute and chronic inflammation, such as severe shock, asthma, inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis [1,3–6]. However, their clinical efficacy is compromised by the metabolic effects of long-term treatment and resistance [7,8]. For the treatment to remain effective, greater insights are required into the mechanisms by which GCs exert their anti-inflammatory and immunosuppressive actions.

One possible mechanism whereby GCs mediate their effects is through the glucocorticoid receptor (GR), a ligand-activated transcription factor of the nuclear receptor superfamily [9]. The GR–corticosteroid complex may bind to specific DNA response elements in the promoter region or interact with other transcription factors and thereby directly or indirectly regulate the transcription of target genes. A large number of protein-encoding genes regulated by GCs have been identified in the immune inflammatory response, especially some proinflammatory molecules downstream of Toll-like receptor (TLR) signaling [2,8,10–13].

Recently, a novel family of noncoding RNAs, referred to as miRNAs, has been found to provide additional controls in the complicated regulation of gene expression [14]. They are produced from actual genes within the genomic loci of cells and then processed by Drosha and Dicer into mature miRNAs [15,16]. Mature miRNAs complementarily bind to the 3′ untranslated region of the mRNA transcripts of their targeted genes, containing seed sequences, leading to either mRNA degradation or translational repression [17,18]. Increasing evidence has indicated miRNAs to be key regulators in various biological processes, including innate and adaptive immune responses [19–23]. By regulating multiple target genes, miRNAs profoundly control a wide variety of pathways. Indeed, a number of miRNAs, such as miR-146a, miR-147, let-7e, let-7i, and miR-155, were shown to

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become induced in response to TLR4 signaling and functionally promote or inhibit inflammation [24–27]. MiR-146a targets TRAF6, IRAK1, and IRAK2, three upstream signaling components within the TLR4 pathway, and then inhibits NF-κB activation [28–30]. On the other hand, miR-155 transgenic mice produce more proinflammatory cytokines in response to LPS [31]. Despite advances in our understanding of miRNA biology, little is known about the regulation of miRNAs by GCs in the inflammatory process or their role in the anti-inflammatory effects of GCs.

In this study, we identified several miRNAs that are regulated by GCs in the LPS-induced macrophage inflammatory response. One of these miRNAs, miR-155, transcribed as a primary (pri-) transcript from BIC (B-cell integration cluster, the host gene of miR-155) and then sequentially processed by the enzymes Drosha and Dicer to form a precursor (pre-) and mature miR-155 [32,33], was further shown to be downregulated by GCs, and its downregulation was critical for the anti-inflammatory effect of GCs. In addition, GCs decreased miR-155 expression in a GR- and NF-κB-dependent manner. The NF-κB binding site located in the promoter region of BIC was important for mediating the GC-driven suppression of miR-155 in response to LPS stimulation. Together, these results suggest miR-155 to be a novel target through which GCs inhibit the LPS-induced macrophage inflammatory response.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). LPS (*Escherichia coli,* serotype) and dimethyl sulfoxide (DMSO) were from Sigma (St. Louis, MO, USA). RU486 was bought from Cayman Chemical (Ann Arbor, MI, USA). Phospho-specific and total ERK1/2, JNK, and p38 antibodies were from Cell Signaling Technology (Danvers, MA, USA), whereas BAY 11–7082 and SB203580 were obtained from Calbiochem (San Diego, CA, USA).

Mice and cell lines

Female C57BL/6 mice, age 6 to 8 weeks, were purchased from the Shanghai Experimental Center, Chinese Academy of Science. All mice were maintained under specific-pathogen-free conditions and all animal experiments were carried out in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996) and the guidelines of the Shanghai Medical Laboratory Animal Care and Use Committee. RAW264.7 and THP-1 cell lines were provided by the Institute of Biochemistry and Cell Biology of the Chinese Academy of Science and originated from the American Type Culture Collection. Peritoneal macrophages were isolated according to a previous report [34]. All the cells were grown in DMEM with 10% FBS, 2 μM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin sulfate.

RNA extraction and RT-PCR

Total RNA of cultured cells was extracted with TRIzol reagent (Invitrogen, San Diego, CA, USA) following the manufacturer's instructions. RNAs were then stored at −80 °C. For mature miR-155 expression analysis, approximately 20 ng of RNA was converted to cDNA using the High-Capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA) along with a miR-155-specific primer (Ribobio, Guangzhou, China). The U6 gene was used as a normalization control. For miRNA expression analysis, synthesis of cDNA was performed on 1 μg of total RNA per sample using the PrimerScript RT reagent kit (TaKaRa, Shiga, Japan) according to the manufacturer's protocol. After reverse transcription, real-time PCR was performed using a FastStart Universal SYBR Green Master Kit (Roche, Indianapolis, IN, USA) on the ABI 7500 thermocycler (Applied Biosystems) following the manual. Primers of pri-mmu-155 and pre-mmu-155 were from a previous report [35], whereas the others were designed using the PrimerExpress software. GAPDH was used as a housekeeping gene for normalization. The sequences of the primers were as follows: (1) GAPDH, 5′-TGCACCACCAACTGCTTAGC-3′ (forward) and 5′-GCATGGACTGTGGTCATGAG-3′ (reverse); (2) TNF-α, 5′-GGCAGGTCTACTTTGGAGTCATTG-3′ (forward) and 5′-ACATTCGAGGCTCCAGTGAATTCGG-3′ (reverse); (3) IL-6, 5′-ACAACCACGGCCTTCCCTACTT-3′ (forward) and 5′-CACGATTTCCCAGAGAACATGTG-3′ (reverse); and (4) iNOS, 5′-GGCAGCCTGTGAGACCTTTG-3′ and 5′-GCATTGGAAGTGAAGCGTTTC-3′.

MiRNA microarray

RAW264.7 cells were pretreated with dexamethasone (Dex; 10^{-7} M) or DMSO for 1 h and then stimulated with LPS for 24 h. The cells were harvested. Total RNA was extracted, and hybridization on a miRCURY LNA microRNA array (version 10.0; Exiqon, Denmark) was performed by KangChen Bio-Tech (Shanghai, China). The experiments were repeated in triplicate.

MiRNA transfection

The murine miR-155 duplex mimic (miR-155), miR-155 inhibitor, and negative control oligonucleotide duplex mimic (miR-NC) were from Ribobio. Thirty to fifty percent of confluent cells were transfected with miRNAs using the Amaxa Cell Line Nucleofector Kit V (Lonza, Switzerland) according to the manufacturer's protocol.

Enzyme-linked immunosorbent assay

Murine TNF-α and IL-6 expression was measured using an enzyme-linked immunosorbent assay kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Measurement of NO production

NO production was assessed by measuring nitrite/nitrate using the Griess reagent as previously described [36]. In brief, the Griess reagent was mixed with equal amounts of cell supernatants. After incubation at 37 °C for 10 min, the OD value was measured using a Bio-Rad microplate reader (Hercules, CA, USA) at 550 nm. Concentration of nitrite was assessed by reference to the sodium nitrite standard curve.

Immunofluorescence

Immunofluorescence was performed as per a previous report [37]. Anti-p65 antibody (Cell Signaling) was diluted at 1:100 and Cy5-conjugated secondary antibody (Cell Signaling) was diluted at 1:400.

Western blot analysis

Proteins extracted from cells were immunoblotted with various antibodies following the published protocols [38]. The primary antibodies used were all diluted at 1:4000, whereas the secondary antibody was diluted 1:10,000.

Luciferase assay

The BIC promoter–luciferase plasmid along with the NF-κB, AP-1, and Ets mutated ones were kindly provided by Dr. Eric Flemington (Tulane University, New Orleans, LA, USA). RAW264.7 cells were transfected with the indicated plasmids and *Renilla* luciferase pRL-TK vector (Promega, Madison, WI, USA) using an Amaxa Cell Line