Original Article

Glucocorticoids inhibit production of exosomes containing inflammatory microRNA-155 in lipopolysaccharide-induced macrophage inflammatory responses

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Abstract: Glucocorticoids are anti-inflammatory agents that are widely used in clinical practice. Increasing evidence has identified exosomes as important mediators in inflammation, but it is unknown whether glucocorticoids regulate exosome secretion and function. In the present study, we observed a reduction of exosome secretion in lipopolysaccharide (LPS)-induced RAW264.7 macrophages following treatment with dexamethasone. Importantly, exosomes isolated from LPS-induced RAW264.7 macrophages increased TNF-α and IL-6 production in RAW264.7 cells. However, this increase was less pronounced following treatment with exosomes isolated from dexamethasone-treated cells. Moreover, dexamethasone decreased expression of pro-inflammatory microRNA-155 in exosomes from LPS-induced RAW264.7 macrophages. We postulate that exosomes are novel targets in the anti-inflammatory effect of glucocorticoids in LPS-induced macrophage inflammatory responses. These findings will benefit the development of new approaches for anti-inflammatory therapeutics.

Keywords: Exosome, glucocorticoid, inflammation, microRNA-155

Introduction

Over the past few decades, glucocorticoids have been widely applied in the clinic to treat a variety of inflammatory and immune diseases such as asthma, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, sepsis, eczema, and psoriasis [1, 2]. Today, the most common clinical use of glucocorticoids is for therapy of inflammatory disorders, in which they have become established as first-line treatment for both adults and children [1, 3-6].

Given the importance of glucocorticoids in therapeutics, intense efforts by the pharmaceutical industry and academic research have been made to understand their mechanisms of action. At the cellular level, glucocorticoids reduce numbers of inflammatory cells, including T lymphocytes and mast cells, at sites of injury or activation [7-11]. The predominant effect of glucocorticoids is to switch off the expression of multiple inflammatory genes that are activated during acute and chronic inflammatory processes. Such genes encode inflammatory cytokines, adhesion molecules, chemokines, receptors, and enzymes, which are involved in the initiation or amplification of the immune system [9, 12]. At higher concentrations, glucocorticoids have additional effects on the synthesis of anti-inflammatory proteins. A classic working model is the “glucocorticoid receptors (GR)” hypothesis, in which glucocorticoids diffuse readily across cell membranes and bind to GRs in the cytoplasm. GRs are normally bound by molecular chaperones or transcription factors such as heat shock protein-90, FK-binding protein, nuclear factor (NF)-κB, and Ap-1, and their binding regulates target gene expression [4, 7]. Although the exact mechanisms of glucocorticoids are far from clear, the emergence of new inflammatory mediators is likely to offer further insights.
Exosomes are small particles with a diameter of 30 to 100 nm, which are released from both normal and diseased cells such as macrophages, T cells, and tissue cells [13-16]. They are composed of a lipid bilayer containing transmembrane proteins and components derived from donor cells. They have recently attracted interest as mediators of intercellular communication, and function as regulators in normal cell physiological activity, tumor pathogenesis, and most importantly in inflammation [13, 14, 17, 18]. However, it remains unknown whether glucocorticoids regulate exosome secretion and function. To address this, we investigated the effect of glucocorticoids on exosomes using the lipopolysaccharide (LPS)-induced macrophage cell model. This is the first report to postulate that exosomes are a novel target involved in the anti-inflammatory effect of glucocorticoids.

Materials and methods

Cell lines and cell culture

RAW264.7 cells were originally obtained from ATCC and maintained in our institution. They were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 2 μM glutamine, and 100 μg/ml streptomycin sulfate.

RNA extraction

RNA was extracted from cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s protocol, and then stored at -80°C.

mRNA expression analysis

A total of 1 μg RNA per sample was used to synthesize cDNA with the primerScript RT reagent Kit (TaKaRa, China) following the manufacturer’s protocol. Quantitative reverse transcription PCR (qRT-PCR) was performed using the SYBR Green Master kit (ABI, USA) according to the manufacturer’s instructions. Experiments were performed in triplicate. GAPDH was amplified for normalization as a housekeeping gene. Primers were designed using PrimerExpress software, and primer sequences were as follows: GAPDH: 5'-TGACCACCAA-CTGTTACC-3' (forward) and 5'-CATCCGTGTTGTCATG-3' (reverse); TNF-α: 5'-GGCAGGCTACTTTGAGGATCATTG-3' (forward) and 5'-ACATTCCACGGCTCCAAT-GTGACATG-3' (reverse); IL-6: 5'-ACAACCCAC-GGCCTTCC-CTACCT-3' (forward) and 5'-CACGATTTCCAGAGAATG-3' (reverse).

Enzyme-linked immunosorbent (ELISA) assay

Protein levels of TNF-α and IL-6 were measured by the ELISA kit (eBioscience, USA) according to the manufacturer’s instructions.

Exosome isolation

RAW264.7 macrophage cells were cultured in FBS-free DMEM medium, then treated with Dexamethasone (DEX, 10⁻⁷ M) or DMSO for 1 h prior to incubation with 1 μg/ml LPS. After 24 h, exosomes were isolated from culture supernatants and purified using ExoQuick (System BioSciences, USA). Exosome production was quantified by the BCA Protein Assay Kit (Pierce, USA).

Transmission electron microscopy (TEM)

Exosomes were re-suspended in 150 mM ammonium bicarbonate buffer. Experimental samples were placed on electron microscopy grids for 5 min, and the excess was wiped off using clean fibuff paper. The grids were dried at room temperature, then stained with 5 ml 2% filtered uranyl acetate solution. The grids were loaded onto a TITAN 80-300 Scanning Transmission Electron Microscope, and images were taken with Tecnai software (FEI).

MicroRNA (miRNA) expression analysis

Approximately 20 ng of RNA was converted into cDNA using the High-Capacity cDNA archive kit (ABI, USA) with a miR-155-specific primer (ABI, USA). The U6 gene (ABI, USA) was used as a normalization control.

Statistical analysis

Data are expressed as mean ± SEM of three independent repeats. Statistical tests were performed using Prism 5.0 software (GraphPad, USA). P values less than 0.05 were identified as statistically significant.

Results

Glucocorticoids inhibit the LPS-induced macrophage inflammatory response

DEX is a representative member of the glucocorticoid family [19-21], and was used as an experimental drug in the present study.
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RAW264.7 cells were pretreated with DEX or DMSO for 1 h, then stimulated with LPS for 24 h. Real-time PCR analysis showed that DEX significantly decreased expression of $\text{TNF-}\alpha$ and $\text{IL-6}$ mRNA compared with DMSO controls (Figure 1A). Accordingly, TNF-\(\alpha\) and IL-6 protein expression were also significantly decreased following treatment with DEX (Figure 1B).

Glucocorticoids reduce exosome secretion in LPS-induced RAW264.7 macrophage cells

To further investigate the effect of glucocorticoids on exosome secretion, we isolated exosomes from DEX- or DMSO control-treated RAW264.7 cells with LPS stimulation. Purified exosomes were confirmed and evaluated using TEM. As shown in Figure 2A, the purified vesicles were of the expected size of exosomes (30-100 nm). Quantification using the BCA assay showed that LPS increased the level of exosome secretion to almost twice that of the control. However, after DEX treatment, exosome secretion was significantly decreased (Figure 2B). These results suggest that glucocorticoids reduce exosome secretion in the LPS-induced macrophage inflammatory response.

Glucocorticoids impair the inflammatory effect of exosome in LPS-induced RAW264.7 macrophage cells

To further investigate the effect of glucocorticoids on exosome function, RAW264.7 cells were treated with exosomes isolated from LPS-induced RAW264.7 cells with or without DEX. These exosomes were found to augment TNF-\(\alpha\) and IL-6 production in RAW264.7 cells, but the increase was lower following treatment with exosomes isolated from DEX-treated cells (Figure 3). These results suggest that glucocorticoids regulate exosome function and impair the inflammatory effect of exosomes in LPS-induced RAW264.7 cells.
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Figure 2. Glucocorticoids reduce exosome secretion in LPS-induced RAW264.7 macrophage cells. RAW264.7 cells cultured in serum-free medium were pre-treated with DEX ($10^{-7}$ M) or DMSO for 1 h, then treated with LPS (1 μg/ml) stimulation for 24 h. A: Exosomes were evaluated using electron microscopy and images were taken with Tecnai software. B: Exosome production was quantified by a BCA Protein Assay Kit. Data are representative of three independent experiments (**P<0.01).

Figure 3. Glucocorticoids impaired the inflammatory effect of exosomes in LPS-induced RAW264.7 macrophage cells. RAW264.7 cells cultured in serum-free medium were pre-treated with DEX ($10^{-7}$ M) or DMSO for 1 h, then treated with LPS (1 μg/ml) stimulation for 24 h. RAW264.7 macrophage cells were then treated with exosomes at 40 μg/ml for 24 h. Protein levels of TNF-α and IL-6 in the supernatant were examined by ELISA. Data are representative of three independent experiments (**P<0.01).

Discussion

Great progress has been made in the study of exosomes during recent years and they have been shown not only to be potential biomarkers but also mechanisms for the development of many physiological and pathological conditions [13, 14]. Exosomes have immunostimulatory or immunosuppressive activities, and are therefore involved in regulation of the immune system [17, 28].

Exosomes derived from dendritic cells carry MHC family proteins and co-stimulatory molecules such as CD80 and CD86, which are effective at stimulating T cell responses both in vitro and in vivo [29, 30]. When purified exosomes from the body fluids of carcinoma patients were exposed to the human monocytic leukemia cell line THP-1, they induced expression of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and IL-23 at both the mRNA and protein level [31]. Moreover, exosomes derived from red blood cells also showed a pro-inflammatory effect by binding to monocytes and inducing...
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the release of pro-inflammatory cytokines, which boosted in vitro T cell-mediated responses [28].

BALF exosomes from asthmatic patients have been reported to trigger secretion of pro-inflammatory leukotrienes and IL-8 in a human bronchial epithelial cell line [32]. Moreover, exosomes isolated from Mycobacterium avium-infected macrophages stimulated a pro-inflammatory response in resting macrophages [33, 34]. Similarly, our study found that exosomes isolated from LPS-activated macrophages triggered the production of pro-inflammatory cytokines TNF-α and IL-6, indicating a pro-inflammatory role for exosomes in the macrophage inflammatory response. We also show that pretreatment with glucocorticoids significantly reduced exosome secretion in the cell supernatant, which would contribute to the anti-inflammatory effect of glucocorticoids.

Exosomes are involved in cell-cell communication at the level of synapses or by delivering proteins and RNA, as well as modifying recipient cells within the microenvironment and at a distance through the systemic circulation. Recent reports also have shown that exosomes harbor genetic information in the form of miRNAs [13, 14]. These are a group of non-coding small RNAs around 20-25 nucleotides in length. They regulate target genes through partial sequence complementarity to the 3′ untranslated region, causing mRNA degradation and/or translational repression [35, 36]. They are also emerging as key regulators in the pathogenesis of inflammation. Indeed, several miRNAs are induced in response to inflammatory signals, and functionally promote or inhibit inflammation [18, 22, 26, 37, 38].

miR-146a and miR-155 are two functional members of the TLR4 pathway. miR-146a was reported to target TRAF6, IRAK1, and IRAK2, and to inhibit NF-κB activation, thus limiting the inflammatory response [22, 23]. Conversely, miR-155 transgenic mice exhibited increased pro-inflammatory cytokine production in response to LPS, suggesting a pro-inflammatory role for miR-155 [37, 38].

Despite advances in our understanding of miRNA biology, little is known about regulation of miRNAs by glucocorticoids in the inflammatory process. In the present study, we found that miR-146a and miR-155 were both present within exosomes. The expression of miR-146a and miR-155 in exosomes was significantly upregulated following LPS stimulation, which is in line with previous observations [23, 26, 37, 39]. It is possible that increased miR-146a and miR-155 synthesis in macrophages would result in their increased expression within exosomes. Interestingly, treatment with DEX as a representative glucocorticoid decreased miR-155 expression in exosomes. Given the powerful pro-inflammatory effect of miR-155 in the inflammatory process, we propose that its downregulation by glucocorticoids helps limit inflammation, which may explain the loss of the inflammatory effect by exosomes from glucocorticoid-treated cells.

In summary, this study further extends the biological role of exosomes in inflammation and...
for the first time identifies exosomes as a possible novel target in the anti-inflammatory effect of glucocorticoids. These findings provide a basic rationale for the use of exosomes in drug development and the treatment of inflammation.

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Disclosure of conflict of interest

None.

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