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Tristetraprolin is involved in the glucocorticoid-mediated interleukin 8 repression

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ABSTRACT

Glucocorticoids have been widely used in various inflammatory disorders, and the transcriptional repression of inflammatory mediators has been considered to be the main mechanism of action. However, a previous study showed that dexamethasone inhibited interleukin 8 (IL-8) expression by promoting IL-8 mRNA decay, which implies a posttranscriptional regulation. Nevertheless, by which mechanism dexamethasone destabilized IL-8 mRNA was unclear. Another study indicated that an RNA-binding protein, tristetraprolin (TTP), could be induced by dexamethasone. TTP can bind to AU-rich elements (ARE) in the 3′-untranslated region of target mRNAs and promotes mRNA degradation. So, we speculated that dexamethasone destabilized IL-8 mRNA by upregulating TTP expression. Here, we report that dexamethasone reduced IL-8 expression through destabilizing IL-8 mRNA in human pulmonary microvascular endothelial cells (HPMECs). Dexamethasone stimulation increased TTP mRNA and protein levels. TTP silencing led to mRNA stabilization and protein upregulation of IL-8. These results provide the evidence that the glucocorticoid, in HPMECs, inhibits IL-8 expression through TTP at the posttranscriptional level.

1. Introduction

Glucocorticoids (GCs) are a kind of the most commonly used anti-inflammatory and immunomodulatory drugs. They have been used in a variety of inflammatory and autoimmune diseases [1,2]. GCs exert their anti-inflammatory and immunomodulatory effects mainly by inhibiting the expression of inflammatory mediators [3]. Most effects of GCs are mediated by the intracellular, glucocorticoid receptor (GR) [4]. The GR can regulate the expression of inflammatory mediators either by binding to the GC response elements (GREs) in the promoters of target genes or by interacting with nuclear factor-B (NF-κB) or activator protein-1 (AP-1), and then influencing the gene transcription [5,6]. Dexamethasone has been reported to inhibit expression of miRNA-146a/b [7], which negatively modulates interleukin-8 (IL-8) expression by suppressing IRAK-1 expression [8], this implied dexamethasone-mediated IL-8 repression was not via miRNA-146a/b. Another study demonstrated, in human glioblastoma cell lines, that dexamethasone inhibited IL-1-induced IL-8 gene expression by impairing NF-κB activation at the transcriptional level [9].

However, a previous study showed that dexamethasone downregulates IL-8 expression by promoting IL-8 mRNA decay, which demonstrates a posttranscriptional regulation mechanism [10]. IL-8 plays a critical role in the procedure of inflammation such as acute respiratory distress syndrome [11], nevertheless, the exact mechanism involved in dexamethasone-induced repression of IL-8 remains unclear.

The regulation of mRNA stability represents a major component in posttranscriptional regulation, which involves lots of RNA-binding proteins. Tristetraprolin (TTP) is a tandem zinc finger protein that can bind to the AU-rich elements (AREs) at the 3′-untranslated region (3′-UTR) of target mRNAs and promote target mRNA deadenylation and degradation [12]. According to the nucleotide database, the IL-8 mRNA also has AREs at 3′-UTR. Intriguingly, a previous study has shown that dexamethasone induces the TTP expression in A549 lung epithelial cells [13]. Based on the evidences above, we postulated that dexamethasone reduced IL-8 mRNA stability and then inhibited IL-8 release by upregulating TTP expression in human pulmonary microvascular endothelial cells (HPMECs). Here, we show that dexamethasone reduced IL-8 expression through destabilizing IL-8 mRNA in HPMECs. Dexamethasone stimulation increased TTP mRNA and protein levels. TTP silencing led to mRNA stabilization and protein upregulation of IL-8. These results provide the evidence that the glucocorticoid, in HPMECs, inhibits IL-8 expression through TTP at the posttranscriptional level.

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2. Materials and methods

2.1. Materials

HPMEC and Endothelial Cell Medium were purchased from ScienCell (Carlsbad, USA). Dexamethasone (Cat. NO. 02194561) was purchased from MP Biomedicals (California, USA) and dissolved in sterile water. An IL-8 ELISA Kit (Cat. NO. 431507) was purchased from Biolegend (San Diego, USA). Lipofectamine 2000 (Cat. NO. 11668–027), Opti-MEM I reduced serum medium and TRIzol reagent were purchased from Invitrogen (Carlsbad, USA). RevertAid First Strand cDNA Synthesis Kit (#K1622) was purchased from Fermentas UAB (Vilnius, Lithuania). Actinomycin D (ActD) (Cat. NO. A1410) was purchased from Sigma-Aldrich (St. Louis, USA). Taq DNA Polymerase PCR Kit (Cat. NO. GK8006) was purchased from Generay Biotech (Shanghai, China). TNF-α (300-01A) was obtained from PeproTech (Rocky Hill, USA). RIPA Lysis Buffer (Cat. NO. P0013C) was obtained from Beyotime Institute of Biotechnology (Nantong, China). Protease Inhibitor Cocktail (#R1321) was purchased from Fermentas UAB (Vilnius, Lithuania). Protein assay reagent (Cat. NO. KGPBCA) was purchased from Keygen Biotec (Nanjing, China). Goat anti-Tristetraprolin antibody (sc-8458) was purchased from Santa Cruz Biotechnologies (Santa Cruz, USA). Mouse anti β-actin antibody (#BM0627) was purchased from Boster (Wuhan, China). TTP siRNA and negative control siRNA were purchased from GenePharma (Shanghai, China). Lipofectamine 2000 (Cat. NO. 11668–027) and Opti-MEM I reduced serum medium were purchased from Invitrogen (Carlsbad, USA).

2.2. Cell culture, treatment, and transfection

HPMECs were cultured in Endothelial Cell Medium at 37 °C in an incubator supplied with 5% CO_2. Cells were subcultured to passages 5 to 8 for all experiments. Cells were seeded in six-well plate at ~5×10^4 cells per well in 2 ml of Endothelial Cell Medium and stimulated with dexamethasone (2 μM), TNF-α (10 ng/ml), or transfected with siRNAs in certain experiments. Dexamethasone was added when cells were ~70% confluent. siRNA was used to silence TTP. The procedure of TTP siRNA transfection was as previously described [14], and the final concentration of TTP siRNA was 80 nM. The target sequences of siRNAs are as follows: TTP siRNA 1: sense (5′-ACG ACU UUA UUU AUU CUA AUA TT-3′) and antisense (5′-UAU UAG AAU AAA UAA AGU CGU TT-3′), TTP siRNA 2: sense (5′-UAG CAU AUU UAA GGG AGG CAA TT-3′) and antisense (5′-UUG CCU CCC UUA AAU AUG CUA TT-3′), TTP siRNA 3: sense (5′-UAG AAU CUU AUG UGC UGU GAA TT-3′) and antisense (5′-UUC ACA GCA CAU AAG AUU CUA TT-3′), negative control siRNA: sense (5′-AAU UCA CAU GAU UAA UAG UAA TT-3′) and antisense (5′-UUA CUA UUA AUC AUG UGA AUU TT-3′).

2.3. Enzyme-linked immunosorbent assay (ELISA)

The cell culture supernatant was collected after HPMECs were treated with dexamethasone (2 μM) for 24 h or transfected with siRNAs for 48 h. IL-8 in cell culture supernatant was measured using IL-8 ELISA kit. Human IL-8 Standard was used to construct standard curves. The experiments were performed according to the manufacturer's instructions. Absorbances were read at 450 nm and 570 nm using a microplate reader, and the concentrations of IL-8 were calculated according to the standard curves.

2.4. RNA isolation, cDNA synthesis, and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated and synthesized as previously described [14], and then analyzed with reverse transcription-PCR (RT-PCR) using the Taq DNA Polymerase PCR Kit according to the manufacturer's instructions. GAPDH mRNA was used as the control. The RT-PCR conditions were as follows: 1 cycle of 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and 1 cycle of 72 °C for 10 min. The PCR products were analyzed on a 2% agarose gel containing ethidium bromide and visualized under UV light. The visualized band was analyzed by ImageJ software (http://rsb.info.nih.gov/ij/). The primers used for the RT-PCR are listed as follows: GAPDH forward, AGA AGG CTG GGG CTC ATT TG; GAPDH reverse, AGG GGC CAT CCA CAG TCT TC; IL-8 forward, ATG ACT TCC AAG CTG GCC GTG GCT; IL-8 reverse, TCT CAG CCC TCT TCA AAA ACT TCT C; TTP forward, TTC GCC CAC TGC AAC CTC; and TTP reverse, CGC CCA CTC TCT GAG AAG GTC.

2.5. Western immunoblot experiments

The procedure of western immunoblot was as previously described [14]. Total proteins were extracted after HPMECs were treated with dexamethasone (2 μM). The primary antibodies (TTP antibody and β-actin antibody) were diluted 1:1000 with PBST containing 5% BSA. The alkaline phosphatase (AP)-conjugated secondary antibodies were diluted 1:5000 using PBST containing 5% BSA. The membrane was incubated with the BCIP/NBT Alkaline Phosphatase Color Development Kit (#C3206, Beyotime Institute of Biotechnology) until color development was achieved. The band images were acquired using the BenQ Scanner (5560C) and analyzed by the ImageJ software (http://rsb.info.nih.gov/ij/).

2.6. Analysis of mRNA stability by reverse transcription polymerase chain reaction (RT-PCR)

HPMECs were treated with dexamethasone (2 μM) for 24 h or transfected with siRNAs for 48 h, and then stimulated with TNF-α (10 ng/ml) for 2 h, and ActD (5 μg/ml) was added at 0, 60, 120 and 240 min to stop transcription. RNA was isolated, synthesized and analyzed with reverse transcription-PCR as described above. IL-8 mRNA levels were normalized to GAPDH mRNA. The normalized value of TNF-α 0 h was set as 100%. The half-life of IL-8 mRNA was calculated using GraphPad Prism software version 5.01 on a one phase exponential decay model. The semi-logarithmic curves were also plotted using the GraphPad Prism software.

2.7. Cytotoxicity assay

The cytotoxicities of dexamethasone, the siRNA-lipofectamine mix (control siRNA-lipofectamine mix and TTP siRNA-lipofectamine mix), or ActD were measured by testing the amounts of mitochondrial dehydrogenase (MDH) released from the HPMEC. ~5×10^3 HPMECs were seeded in 96-well plate and treated using dexamethasone, siRNA-lipofectamine mix, or ActD as described above. The cytotoxicity was determined by MTT Cell Proliferation and Cytotoxicity Assay Kit (C0009, Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions. For dexamethasone and the siRNA-lipofectamine mix cytotoxicity test, the cells were tested at 0, 12, 24, 48 h of treatment. For ActD cytotoxicity test, cells were treated with dexamethasone for 16 h or the siRNA-lipofectamine mix for 48 h, stimulated with TNF-α (10 ng/ml) for 2 h, and the cells were tested at 0, 1, 2, and 4 h of addition of ActD. Absorbances were measured by a microplate reader. The cytotoxicity was calculated as: [(OD of controls − OD of treatment) / OD of controls] × 100%.

2.8. Statistical analysis

Statistical analysis was performed using SPSS 19.0 (Chicago, USA). ANOVA test and a *t*-test were used to analyze the differences between the different time intervals within the same group and between the different groups, respectively. Two-tailed probability values of <0.05 were considered statistically significant. Error bars on images represent SEM.