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HuR post-transcriptionally regulates TNF-α-induced IL-6 expression in human pulmonary microvascular endothelial cells mainly via tristetraprolin

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ABSTRACT

HuR and tristetraprolin (TTP) are both RNA-binding proteins, which are characterized as binding to the AU-rich elements (AREs) in the 3′-untranslated regions (3′-UTRs) of target mRNAs. Studies have shown that some ARE-containing mRNAs are stabilized by HuR, whereas are destabilized by TTP. Our previous study showed that HuR upregulated tumor necrosis factor-α (TNF-α)-induced interleukin-6 (IL-6) expression by stabilizing its mRNA in human pulmonary microvascular endothelial cells (HPMECs). Considering IL-6 mRNA has AREs, we decided to examine whether TTP was also involved in the regulation of TNF-α-induced IL-6 expression in HPMECs and whether HuR and TTP influenced each other at protein and mRNA level. Here, we report that TTP silencing increased IL-6 levels. HuR silencing increased TTP expression. TTP had no effect on HuR expression and subcellular localization. Compared to TTP silencing alone, double knockdown of HuR and TTP did not significantly reduce IL-6 release. The RNA-binding protein immunoprecipitation (RIP) results further showed that TTP but not HuR bound to intracellular IL-6 mRNA in HPMECs. We demonstrate for the first time that HuR post-transcriptionally regulates IL-6 expression mainly via TTP.

1. Introduction

HuR, which belongs to the embryonic lethal abnormal visual family of RNA binding protein, contains RNA-recognition motifs which recognize and bind to the AU-rich elements (AREs) in the 3′-untranslated regions (UTRs) of target mRNAs (Ma et al., 1996; Fan and Steitz, 1998). It has been reported that HuR increases the stability of tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), cyclooxygenase 2 (COX-2) and toll-like receptor 4 (TLR4) mRNAs (Dean et al., 2001; Lejbkowicz et al., 2005; Young et al., 2009; Lin et al., 2006). Tristetraprolin (TTP) is a tandem zinc-finger protein and contains two zinc-fingers which are necessary for TTP to bind to the AREs in the 3′-UTRs of target mRNAs (Lai et al., 2000). In contrast to HuR, TTP has been shown to destabilize TNF-α, COX-2 and VEGF mRNAs (Lai et al., 2000; Young et al., 2009; Lee et al., 2010). It seems that HuR and TTP are counter-regulators for the ARE-containing mRNAs, however, whether HuR and TTP interact with each other remains unknown.

Our unpublished data showed that HuR stabilized IL-6 mRNA (Xu et al., Unpublished results), which has six copies of the AUUUA motif at the 3′-UTR. A previous study has also reported the similar results that HuR increased IL-6 mRNA stability in macrophages (Zhou et al., 2007). Van Tubergen et al. (2011) have shown that TTP was involved in the regulation of IL-6 expression in head and neck squamous cell carcinoma cells, whereas whether TTP can regulate TNF-α-induced IL-6 release in human pulmonary microvascular endothelial cells (HPMECs) is not clear. Here we decided to examine whether TTP is involved in the regulation of TNF-α-induced IL-6 expression and whether HuR and TTP interact with each other at mRNA and protein level.

The present study showed that TTP negatively regulated IL-6 expression at the mRNA decay level. HuR silencing significantly increased TTP levels, whereas knockdown of TTP did not alter HuR expression and cytoplasmic localization. Double knockdown of HuR and TTP did not significantly reduce IL-6 expression compared with TTP silencing alone. The RNA-binding protein immunoprecipitation experiment was performed to detect whether HuR or TTP interacted with IL-6 mRNA and the results demonstrated that TTP but not HuR bound to intracellular IL-6 mRNA in HPMECs. It indicates for the first time that HuR post-transcriptionally regulates IL-6 expression in HPMECs mainly through TTP.

2. Materials and methods

2.1. Materials

Human pulmonary microvascular endothelial cells (HPMECs) and Endothelial Cell Medium were purchased from ScienCell

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(Carlsbad, USA). Lipofectamine 2000 (Cat. NO. 11668-027) and Opti-MEM I reduced serum medium were obtained from Invitrogen (Carlsbad, USA). HuR small interfering RNA (siRNA), TTP siRNA and negative control siRNA were purchased from GenePharma (Shanghai, China). TNF-α (300-01A) was purchased from PeproTech (Rocky Hill, USA). Protease Inhibitor Cocktail (#R1321) was purchased from Fermentas UAB (Vilnius, Lithuania). Protein assay kit and an enhanced chemiluminescent (ECL) kit were purchased from Pierce (Rockford, USA). The Nuclear and Cytoplasmic Protein Extraction Kit (Cat. NO. P0027) and the RIPA Lysis Buffer (Cat. NO. P0013C) were purchased from Beyotime Institute of Biotechnology (Nantong, China). The following antibodies were used: mouse anti-HuR antibody (ab14371) was purchased from Abcam (Cambridge, USA), mouse anti-HuR antibody (sc-5261) and goat anti-Tristetraprolin antibody (sc-8458) were purchased from Santa Cruz Biotechnologies (Santa Cruz, USA), rabbit anti-β-actin antibody (#4967) and rabbit anti-Histone H3 (3H1) antibody (#9717) were purchased from Cell Signaling (Danvers, USA). An IL-6 ELISA Kit (Cat. NO. 430508) was purchased from Biolegend (San Diego, USA). Actinomycin D (ActD) was purchased from Sigma–Aldrich (St. Louis, USA). RevertAid First Strand cDNA Synthesis Kit (#K1622) was purchased from Fermentas UAB (Vilnius, Lithuania), SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, USA).

2.2. Cell culture, treatment, and transfection

HPMECs were cultured and treated as previously described (Su et al., 2008). HuR siRNA and TTP siRNA were used to silence HuR and TTP respectively. HPMECs were seeded in 6-well plates and transfections were performed according to the manufacturer's recommendations using Lipofectamine 2000 when cells were 30–50% confluent. The final concentration of HuR siRNA and TTP siRNA was 40 nM and 80 nM, respectively. Cells were incubated in a CO2 incubator for 24 h, transfection medium was changed, and recovered for another 24 h before the addition of TNF-α (10 ng/ml). Control cells were transfected with negative control siRNA and Lipofectamine 2000. For double knockdown of HuR and TTP, both siRNAs were simultaneously transfected. The sequences of siRNAs are as follows: HuR siRNA: sense (5′-GGA UGA GUU ACG AAG CCU GTT-3′) and antisense (5′-CAG GCU UCG UAA CUC AUC CTG-3′); 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′).

2.3. Western immunoblot experiments

The procedure of western immunoblot was as previously described (Su et al., 2008). The primary antibodies (HuR antibody (Abcam), TTP antibody, β-actin antibody and Histone H3 (3H1) antibody) were diluted 1:1000 with PBST containing 5% BSA. The horseradish peroxidase (HRP)-conjugated secondary antibodies were diluted 1:5000 using PBST containing 5% BSA. After the immune complexes were visualized on FluorChem FC2 system (Cell Biosciences, Santa Clara, USA), densitometric analysis of bands was done by ImageJ software and the data of the target protein was normalized to β-actin or histone (http://rsb.info.nih.gov/ij/).

2.4. Subcellular fractionation

HPMECs were grown in 6-well plates, cytoplasmic and nuclear fractions were prepared as manufacturer's recommendations. Cells were rinsed with ice-cold PBS, 200 μl of cytoplasmic protein extraction reagent A containing 1 mM phenylmethylsulfonyl fluoride was added, vortexed for 5 s, incubated on ice for 15 min, 10 μl of cytoplasmic protein extraction reagent B was added, vortexed for 5 s, incubated on ice for 1 min, and vortexed again for 5 s, the supernatant was collected after high-speed centrifugation (16,000 × *g* for 5 min at 4 °C). For collecting nuclear fractions, nuclear pellets were incubated in 50 μl of nuclear protein extraction reagent with 1 mM phenylmethylsulfonyl fluoride, vortexed for 30 s every 2 min, centrifuged (10 min, 16,000 × *g*, 4 °C) after 30 min, and supernatants were saved.

2.5. Enzyme-linked immunosorbent assay (ELISA)

IL-6 ELISA kit was used to measure the IL-6 levels in cell culture supernatant. The experiment was performed according to the manufacturer's directions. Absorbances were read at 450 nm and 570 nm using a microplate reader, the absorbance at 570 nm was subtracted from the absorbance at 450 nm.

2.6. RNA isolation, cDNA synthesis, and real-time polymerase chain reaction (PCR)

Total RNA was isolated and synthesized as previously described (Su et al., 2008). Real-time PCR analysis was performed using an ABI 7300 Real Time PCR System (Applied Biosystem, Foster City, USA). GAPDH mRNA was used as the control. Amplification conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s, melting curve analysis was used to confirm the specificity of primers. The primers used for the amplifications are as follows: GAPDH forward, AGA AGG CTG GGG CTC ATT TG; GAPDH reverse, AGG GGC CAT CCA CAG TCT TC; IL-6 forward, GGT ACA TCC TCG ACG GCA TCT; IL-6 reverse, GTG CCT CTT TGC TGC TTT CAC; HuR forward, CCG TCA CCA ATG TGA AAG TG; HuR reverse, TCG CGG CTT CTT CAT AGT TT; TTP forward, TTC GCC CAC TGC AAC CTC; TTP reverse, CGC CCA CTC TCT GAG AAG GTC. $2^{-\Delta\Delta Ct}$ was used in mRNA quantitation.

2.7. Analysis of mRNA stability by real-time PCR method

HPMECs were transfected, and then stimulated with TNF-α (10 ng/ml) for 8 h, ActD (5 μg/ml) was added to stop transcription. RNA was isolated, synthesized and amplified as previously described (Su et al., 2008). IL-6 mRNA levels were normalized to GAPDH mRNA. The normalized value at TNF-α 0 h was set as 100%. The half-life of IL-6 mRNA was calculated with GraphPad Prism software version 5.01 on a one phase exponential decay model, and the semi-logarithmic curves were also plotted using the GraphPad Prism software.

2.8. RNA-binding protein immunoprecipitation

RNA-binding protein immunoprecipitation (RIP) was performed to examine the interaction of intracellular IL-6 mRNA with HuR or TTP as described previously (Tenenbaum et al., 2002). In brief, 100 μl of preswelled Protein A/G Agarose Beads (A10001, Abmart, China) were washed with 1 ml of NT2 buffer (50 mM Tris (pH 7.4), 1 mM $MgCl_2$, 150 mM NaCl, 0.05% Nonidet P-40) and mixed with 10 μg of mouse anti-HuR antibody (sc-5261, Santa Cruz, USA), 10 μg of goat anti-TTP antibody (sc-8458, Santa Cruz, USA), or 10 μg of mouse IgG (A7028, Beyotime Institute of Biotechnology, China) or goat IgG (A7007, Beyotime Institute of Biotechnology, China). HPMECs were cultured as described above and treated with TNF-α (10 ng/ml) for 4 h, then ∼2×10^7 cells were lysed in 120 μl of polysome lysis buffer (5 mM $MgCl_2$, 100 mM KCl, 10 mM HEPES (pH 7.0), 0.5% Nonidet P-40, 1 mM DTT, 100 U/ml RNase OUT (Invitrogen, USA)), 0.2% vanadyl-ribonucleoside complex (Invitrogen, USA),