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Differential regulation of ARE-mediated TNFα and IL-1β mRNA stability by lipopolysaccharide in RAW264.7 cells

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Abstract

Messenger RNA degradation is a mechanism by which eukaryotic cells regulate gene expression and influence cell growth and differentiation. Many protooncogene, cytokine, and growth factor RNAs contain AU-rich element (AREs) in the 3′untranslated regions which enable them to be targeted for rapid degradation. To investigate the mechanism of ARE-mediated RNA stability, we demonstrate the expression and regulation of TNFα and IL-1β mRNAs in LPS-stimulated macrophages. TNFα mRNA was rapidly induced by LPS and showed short half-life at 2-h induction, whereas IL-1β mRNA was induced slowly and had longer half-life. Electrophoretic mobility shift assays showed that the LPS-induced destabilization factor tristetraprolin (TTP) could bind to TNFα ARE with higher affinity than to IL-1β ARE. HuR was identified to interact with TNFα ARE to exert RNA stabilization activity. The expression and phosphorylation of TTP could be activated by p38 MAPK pathway during LPS stimulation. Moreover, ectopic expression with TTP and kinases in p38 pathway followed by biochemical assays showed that the activation of p38 pathway resulted in the phosphorylation of TTP and a decrease in its RNA-binding activity. The ARE-containing reporter assay presented that the p38 signal could reverse the inhibitory activity of TTP on IL-1β ARE but not on TNFα ARE. The present results indicate that the heterogeneity of AREs from TNFα and IL-1β could reflect distinct ARE-binding proteins to modulate their RNA expression.
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The mRNAs of many regulatory proteins of the inflammatory response are potentially unstable. The stability of mRNA is determined in many cases by interactions between specific RNA-binding proteins and *cis*-acting sequences located in the 3′untranslated region (3′-UTR) [1]. One of the best characterized *cis*-acting sequences is the adenylate/uridylate-rich elements (AREs) [2]. AREs can range in size and generally contain one or more copies of the pentameric sequence AUUUA and separate into class I, II, and III [3]. The number of the overlapping pentamer AUUUA may contribute to the mRNA half-life. The mRNA half-life analysis of endotoxin-stimulated monocytes showed that the half-lives in the class II category were significantly shorter than those of class I [4]. Studies using mRNAs with defined ARE sequence have demonstrated sequence-specific functional heterogeneity [5].

At least 14 apparently distinct proteins have been identified to interact with ARE in cell extracts by UV-crosslinking and gel-shift assays [2,6]. To date, three ARE-binding proteins have been shown to be involved in regulating rapid mRNA decay in vivo: the ARE- and poly(U)-binding and degradation factor AUF1/hnRNP D [7], tristetraprolin (TTP) [8] and HuR [9]. HuR is a ubiquitous member of the embryonic lethal abnormal vision (ELAV) family of RNA-binding proteins [10]. It is predominantly nuclear

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and shuttles between nucleus and cytoplasm by means of a sequence HNS [11]. Overexpression of HuR in transiently transfected mammalian cells can stabilize short-lived ARE-containing mRNAs [12]. HuR can respond to certain extracellular stimuli to mediate specific mRNAs stabilization [13–15]. For example, HuR regulates the stabilization of TNFα mRNA upon stimulation with LPS [16]. In contrast, tristetraprolin (TTP) is important for the destabilization of tumor necrosis factor and GM-CSF mRNAs, as shown in knockout mice [8,17] and in tissue culture by ectopic-overexpression studies [18]. TTP binds AREs of target mRNAs and induces deadenylation [19,20] or directs them to the exosome [21,22] or associates with RISC–microRNA complexes [23] for rapid degradation of target mRNAs. TTP was observed as an immediate-early gene that was induced in response to several kinds of stimuli, such as insulin and other growth factors and stimulators of innate immunity like LPS [24,25]. Phosphorylation of TTP by components of the p38 MAPK pathway may alter its ARE-binding activity [26–29] and, or subcellular distribution [28–31] to alter its mediated activity on degradation of ARE-containing transcripts.

TNFα and IL-1β are both important primary inflammatory mediators produced in macrophages. Their expression can be induced by LPS transcriptionally and post-transcriptionally [32,33]. HuR and TTP could bind to TNFα ARE to exert opposite effects on its RNA stability [16,18,27,34]. There was little investigation on the ARE-mediated IL-1β gene expression so far. Both TNFα and IL-1β are taken as targets to study the regulation between ARE binding proteins and different AREs in LPS-stimulated macrophages. Our results showed that TNFα and IL-1β mRNAs could be induced by LPS, however, their expression showed a differential kinetics and regulation.

Materials and methods

Plasmid constructs. The cDNAs of HuR and TTP were PCR synthesized by using primers 5′-ATGTCTAATGGTTATGAAGAC-3′ and 5′-ATGAGCGAGTTATTTGTGGG-3′ for HuR and 5′-CTCAGAGACA GAGATACGATTG-3′ and 5′-ATGGATCTCGCCATCTAC-3′ for TTP and the 2 h LPS-treated RAW264.7 cDNA as template. The PCR fragments were ligated into pGEM-Teazy vector (Promega). After DNA sequence confirmation, the *Eco*RI fragment was further cloned into both bacterial expression vector pGEX (Amersham–Pharmacia) and mammalian cell expression vector pCMV-Tag2 (Stratagene). The 3′ AREs of TNFα and IL-1β were PCR cloned by using primers 5′-TGAGGTGCAATGCACAGC-3′ and 5′-CCGGCCTTCCAAATAAA TAC-3′ for TNFα as well as 5′-AGGGTCACAAGAAACCATGG-3′ and 5′-AGGCTATGACCAATTCATCC-3′ for IL-1β. The PCR fragments were cloned into pGEM-Teazy vector (Promega) to prepare riboprobe for EMSA. For heterologous 3′-UTR assay, these ARE fragments were inserted into 3′ end of CMV-driven luciferase gene (Stratagene). The pRSV-Flag-MKK3(Ala), pRSV-Flag-MKK3(Glu), pCMV5-Flag-p38, and pCMV-Flag-p38(AGF) were kindly provided by Prof. Roger J. Davis.

Cell culture. Mouse macrophage RAW264.7 and HEK293T cells were grown at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin.

Recombinant protein preparation and antibody generation. The recombinant GST, GST–HuR, and GST–TTP proteins were produced from *Escherichia coli* and purified by glutathione–Sepharose column (Amersham–Pharmcia). The GST–TTP protein was boosted into rabbits to generate polyclonal antibodies. The antibody used in EMSA supershift assay was purified by protein A column.

RNA isolation and RT-PCR. Total RNA was extracted from the cultures using Blue extract reagent (LTK, Inc., Taiwan) following the procedures recommended by the manufacturer. Five microgram of total RNA extracted from RAW264.7 treated with LPS for different time intervals was reverse-transcribed to produce cDNA using reverse transcriptase and oligo(dT) (Promega, Madison, WI) as a primer. The specific cDNA was amplified using 5% of the RT reaction in 20 μl containing 10 pmol of forward primer, 10 pmol of reverse primer, and lypholized *Taq* DNA polymerase, buffer, and dNTPs (LTK, Inc., Taiwan). The sequences of the primers used for IL-1β, TNFα, GAPDH, and actin are: 5′-TTGACG GACCCCAAAAGATG-3′ and 5′-AGAAGGTGCTCATGTCCTCA-3′ for IL-1β; 5′-ATGAGCACAGAAAGCATGATC-3′ and 5′-CAGAGCA ATGACTCCAAAG-3′ for TNFα; 5′-ACCCCCAATGTGTCCGTCGT-3′ and 5′-TACTCCTTGGAGGCCATGTA-3′ for GAPDH; 5′-TCCTTC CTGGGCATGGAGTC-3′ and 5′-ACTCATCATACTCCTGCTTG-3′ for actin. The expected size of the PCR product is 204 bp for IL-1β, 707 bp for TNFα, 299 bp for GAPDH, and 300 bp for actin. The PCR was performed in a Robocycler gradient 96 PCR thermal machine (Stratagene) using the following conditions: 94 °C (3 min) for one cycle, 94 °C (40 s), 55 °C (40 s), 72 °C (depending on the product length, 1 min/1 kb) for 20–25 cycles, and a final incubation at 72 °C for 3 min. The PCR products were separated in agarose gel and quantitated by UVP LabWork 4.5 software.

Real-time PCR. Real-time PCR was performed with the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) in a total volume of 25 μl. Expression of TNFα, IL-1β, and actin was analyzed using SYBR Green PCR Master Mix (Applied Biosystems) containing 50 ng cDNA and 160 nM of each primer: 5′-GACCC TCACACTCAGATCATCTTCT-3′ and 5′-CCTCCACTTGGTGGTTT GCT-3′ for TNFα; 5′-TCGTGCTGTCGGACCCATAT-3′ and 5′-GTCGTTGCTTGGTTCTCCTTGT-3′ for IL-1β; the primers for actin were identical as used in semi-quantitative RT-PCR. The real-time PCR amplification conditions were 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The real-time PCR data were analyzed using the $2^{-\Delta\Delta C_T}$ relative quantitation method, according to the manufacturer's directions.

Preparation of cytoplasmic and nuclear extracts and Western blotting assay. To prepare cell extract, 5×10^6 cells were resuspended in 400 μl buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 100 μg/ml PMSF, and phosphatase inhibitors). The cell suspension was on ice for 15 min, and then 25 μl of 10% NP-40 was added followed by vortexing for 10 s. After centrifugation at 10,000*g* for 30 s, the supernatant was collected as cytoplasmic extract. The nuclear pellets were resuspended in 100 μl of buffer C (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 100 μg/ml PMSF, and phosphatase inhibitors) and rocked on ice for 20 min. After centrifugation at top speed for 10 min, the supernatant was collected as nuclear extract. Then the samples were aliquoted and stored at −80 °C for further assays. The proteins separated by SDS–PAGE were transferred to PVDF membranes (Millipore) and Western blotting was done using anti-HuR (Santa Cruz), anti-TTP and anti-α-tubulin antibody.

RNA electrophoretic mobility shift assay (EMSA). TNFα and IL-1β ARE probes were in vitro transcribed by T7 RNA polymerase in the absence or presence of [α-^{32}P]UTP. 2×10^5 cpm of probe was incubated with 10 μg of cytoplasmic extract or 100 ng of recombinant proteins at room temperature for 40 min in a final volume of 10 μl containing 15 mM Hepes (pH 7.9), 10 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 0.2 mM DTT, 30 μg heparin sulfate, and 5 μg of yeast total RNA. Unbound RNAs were digested by 20 U RNase T1 at 37 °C for 20 min. Gel mobility supershift analysis was performed by the addition of 1 μg of antibody and then incubated at 37 °C for another 20 min. In competition assay, the indicated molar ratio of cold TNFα or IL-1β ARE was added to the reaction mixture. Binding mixtures were then loaded onto native 5% polyacrylamide gel (acryl:bis = 40:1) containing 2.5% glycerol in 0.25×

Tris–borate–EDTA buffer. After electrophoresis at 15 V/cm for 80 min, the gel was dried and exposed to Kodak XAR film at −70 °C for appropriate time.

Transfection, luciferase, and β-galactosidase assay. The HEK293T cells (2×10^5) were seeded in each well of a 6-well plastic culture plate. Cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) with 1 μg of indicated luciferase constructs, 1 μg of SV40-β-galactosidase plasmid (Promega), and other expression vector. After 24 h, cells were harvested and the cell lysates were assayed for luciferase and β-galactosidase activity. Luciferase activity was determined in a luminometer (Packard) with Promega luciferin as substrate. β-Galactosidase activity was determined by a standard colorimetric assay using *o*-nitrophenyl βD-galactopyranoside as substrate. The luciferase assay results were normalized with β-galactosidase activity to correct for variations in transfection efficiency. Each treatment group contained duplicate cultures and each experiment was repeated three to four times. Relative luciferase activity defined as luciferase light units/β-galactosidase activity is presented as means ± SE.

Results

Differential IL-1β and TNFα mRNA stability in LPS-stimulated RAW264.7

To study the regulation of ARE-mediated mRNA expression, the LPS-stimulated expression profiles of TNFα and IL-1β mRNAs were monitored in mouse macrophage cell line RAW264.7. The steady state mRNA amounts of TNFα increased rapidly at 30-min induction and then decreased gradually, whereas that of IL-1β was accumulated at 2–4 h post-induction and nearly disappeared at 8-h stimulation (Fig. 1A). In the presence of actinomycin D to stop RNA synthesis, we found that the turnover rate of TNFα mRNA was slower at 30-min induction than that at 1- and 2-h induction (Fig. 1B). IL-1β mRNA was more stable than TNFα's at 2-h induction (Fig. 1C). The IL-1β mRNA was too rare to be well analyzed at 30 min- and 1-h induction. This assortment of observations document that, although both TNFα and IL-1β mRNA contain ARE elements, they presented differential responses to LPS treatment. The TNFα transcript was rapidly induced following LPS stimulation and exhibited short half-life, while IL-1β mRNA transcript was increased slowly and showed higher stability.

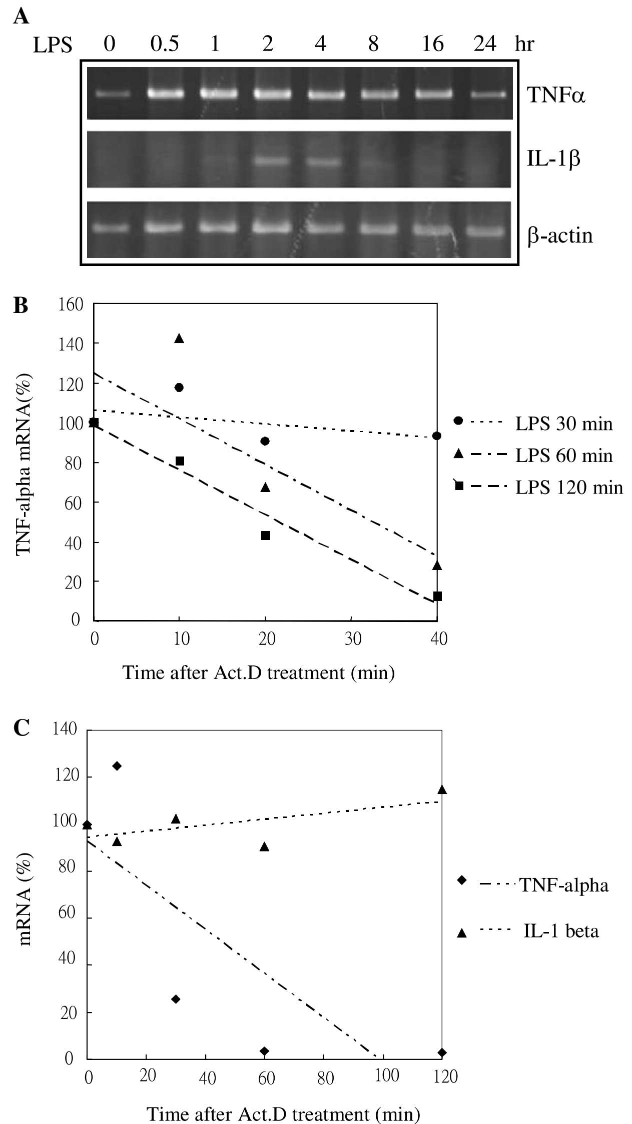

Fig. 1. The mRNA expression profiling of TNFα and IL-1β by LPS in RAW264.7 cells. (A) RAW264.7 macrophages were treated with 100 ng/ml LPS for the indicated time and total RNAs were isolated. Semi-quantitative RT-PCR was performed using specific primers for TNFα, IL-1β, and actin, respectively. (B,C) RAW264.7 cells were treated for LPS for 30 min, 1 h or 2 h. Transcription was then stopped by adding 10 μg/ml of actinomycin D (Act. D) for 5, 10, 20, and 40 min. All samples were analyzed by real-time PCR with specific primers for TNFα, IL-1β, and actin. Levels of TNFα and IL-1β RNA were normalized to those of β-actin in each sample.

ARE-binding of HuR and TTP

To understand the biochemical basis of the distinct responses to LPS on TNFα and IL-1β mRNA expression, the AREs of TNFα and IL-1β were synthesized and their RNA-binding proteins were analyzed. The ARE sequence of TNFα is a typical class II ARE that contain multiple overlapping copies of AUUUA motif, but the ARE of IL-1β only contains two overlapping copies of AUUUA motif and other two scattered motifs (Fig. 2A). Gel shift assay showed that three RNA–protein complexes formed on TNFα ARE, labeled with A, B, and C (Fig. 2B). The complex C could be recognized by anti-HuR antibody, and the complex A and LPS-induced complex B could be supershifted by anti-TTP antibody. On the other hand, only a weak LPS-induced ARE-protein complex formed on IL-1β ARE and it also could be supershifted by anti-TTP antibody (Fig. 2C). The anti-HuR antibody could not recognize the complex on IL-1β ARE (data not shown). The recombinant GST-TTP could interact with TNFα and IL-1β AREs (Fig. 3A). When increasing amount of GST–TTP was incubated with ARE probes, the RNA–protein complexes became larger gradually (Fig. 3A). Unlabeled TNFα ARE could compete TTP binding more efficiently than unlabeled IL-1β ARE

A **TNFα ARE**
5’UGAGGUGCAAUGCACAGCCUUCCUCACAGAGCCAGCCCCCCUCUAUUUAUAUUUG
CACUUAUUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUGCUUAUGAAUGAUUUAU
UUGGAAGGCCGG 3’

IL-1β ARE
5’AGGGUCACAAGAAACCAUGGCACAUUCUGUUCAAAGAGAGCCUGUGUUUCCUCCUU
GCCUCUGAUGGGCAACCACUUACCUAUUUAUUUAUGUAUUUAUUGAUUGGUUGAUCU
AUUUAAGUUGAUUCAAGGGGACAUUAGGCAGCACUCUCUAGAACAGAACCUAGCUGU
CAACGUGUGGGGGAUGAAUUGGUCAUAGCCU 3’

Fig. 2. RNA gel-shift analysis of complexes bound to TNFα and IL-1β ARE. (A) The sequences of mouse TNFα and IL-1β AREs used in this study. Radiolabeled RNA containing the ARE of TNFα (B) or IL-1β (C) was incubated with cytoplasmic lysates from RAW264.7 macrophages stimulated with 100 ng/ml LPS for the indicated time intervals. Prior to separation by non-denaturing PAGE, the antibody against TTP or HuR was added to the reactions as indicated. The ARE-complexes and antibody supershifted bands are indicated by arrows.

Fig. 3. Differential binding affinity of TTP on TNFα and IL-1β AREs. (A) Increasing amounts of recombinant GST-TTP (1, 5, 20, and 100 ng) were incubated with radiolabeled AREs of TNFα or IL-1β. (B) Twenty nanogram of GST-TTP was incubated with radiolabeled ARE of TNFα in the presence of increasing amounts of cold IL-1β or TNFα ARE.

(Fig. 3B). It implies that TTP has higher binding affinity to TNFα ARE. The variety of ARE sequences might reflect the differential protein binding properties.

Expression of HuR and TTP in LPS-stimulated RAW264.7

To explore how the HuR and TTP regulate the mRNA expression of cytokines, their protein expression level and subcellular localization were determined in RAW264.7 during LPS stimulation. The cytoplasmic and nuclear extracts from control and LPS-stimulated cells were isolated for Western blotting assay. During LPS treatment, the expression levels of HuR were almost consistent in the cytoplasmic fraction. The expression of TTP was significantly induced by LPS in cytoplasmic extract and produced smear multiple forms (Fig. 4). After alkaline phosphatase treatment, the higher bands could be returned to lower position indicating that the multiple bands were due to protein phosphorylation (data not shown). Another ARE-binding protein, AUF1, predominantly located in nuclear extract of RAW264.7 cells (data not shown). When RAW264.7 cells were treated with SB203580 to block p38 MAPK, both phosphorylation and expression of TTP proteins were inhibited, and the expression of HuR was not affected by this p38 inhibitor (Fig. 4). Our results showed that HuR is a constitutive factor, whereas TTP is a inducible and p38 signal-sensitive proteins in LPS-stimulated RAW264.7 cells.

Functional characterization of HuR and TTP on gene expression

To dissect the functional role of HuR and TTP on ARE-mediated gene expression, cotransfection and reporter assays were performed. In Fig. 5A, 293T cells were cotransfected with increasing amounts of HuR or TTP expression plasmids and a reporter gene encoding luciferase fused to TNFα or IL-1β AREs. The result showed that HuR could enhance the TNFα ARE-containing luciferase activity but the effect was not prominent on IL-1β ARE. This correlates with HuR binding assay. On the contrary, TTP diminished the ARE-containing reporter activity in a dose-dependent manner. However, the dosage effect of TTP on TNFα and IL-1β ARE was different. Higher dose of TTP could gradually restore the TNFα ARE-containing luciferase activity, but it caused greater reduction of IL-1β ARE-containing luciferase activity. In the presence of 1 or 2 μg HuR, we observed that the very little amounts of TTP (0.1 μg) could highly suppress the HuR activated TNFα ARE-containing luciferase activity (Fig. 5B and C). The TTP-mediated suppression of IL-1β ARE-containing luciferase activity seemed not to be affected by the presence of HuR (Fig. 5B and C). The results suggest that on TNFα or IL-1β AREs, HuR or TTP displays their functions in different ways.

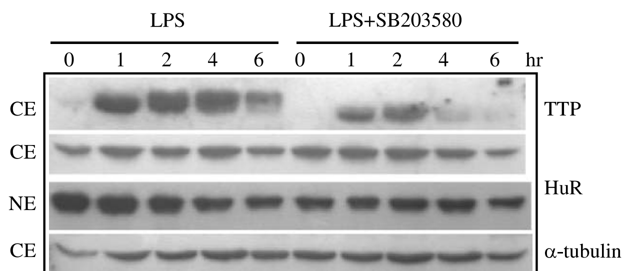

Fig. 4. The protein expression profiling of TTP and HuR in LPS-stimulated macrophages. RAW264.7 cells were treated with LPS alone or pre-treated with 10 μM SB203580 for 30 min followed by LPS treatment for 0, 1, 2, 4, and 6 h. The cytosolic (CE) and nuclear extracts (NE) were harvested for Western blotting assay by using anti-TTP, anti-HuR, and control anti-α-tubulin antibodies separately.

p38 signaling pathway and HuR- and TTP-regulated gene expression

p38 MAPK signaling pathway has been reported to be involved in regulation of TTP activity [28,29]. We further checked whether p38 signaling pathway could affect TNFα and IL-1β mRNA stability through TTP or HuR. Activation of p38 was via the activation of upstream MAP kinase kinase (MKK) 3 and MKK6 [35,36]. The dominant negative mutants of MKK3(Ala) or the constitutively activated MKK3(Glu) were used to inhibit or activate p38 activity, respectively. They were cotransfected with TTP and pLuc-TNFα (ARE) or pLuc-IL-1β (ARE) into 293T cells. Western blotting assay demonstrated that the presence of MKK3(Glu) resulted in the production of higher molecular weight of TTP (Fig. 6A) compared to the presence of MKK3(Ala). It indicates that TTP could be phosphorylated by the p38 pathway. Gel shift assay showed that p38 pathway-phosphorylated TTP has lower ARE-binding activity than unphosphorylated TTP, and the presence of HuR did not affect the TTP-binding (Fig. 6B). Interestingly, the luciferase assays presented the fact that the activation of p38 MAPK could restore the TTP-mediated suppression of IL-1β ARE-containing gene expression to the original level, but showed a very weak effect on the TNFα ARE-containing luciferase activity (Fig. 6C). The HuR activity was not affected by the p38 signal (Fig. 6D).

Discussion

In this study, we provide evidence to present the differential regulation in ARE-containing transcripts during LPS treatment. LPS could induce TNFα mRNA expression rapidly and change its mRNA stability in different time intervals, while the expression of IL-1β mRNA was induced slowly by LPS and its mRNA had longer half-life than TNFα's. Distinct combination and regulation of ARE-binding proteins on TNFα and IL-1β mRNAs were observed to modulate their mRNA expression.

The RNA–protein interaction assays showed the involvement of TTP in the binding of TNFα and IL-1β AREs. TTP seems to have higher binding affinity to TNFα ARE. In a previous report, immobilized TTP protein was

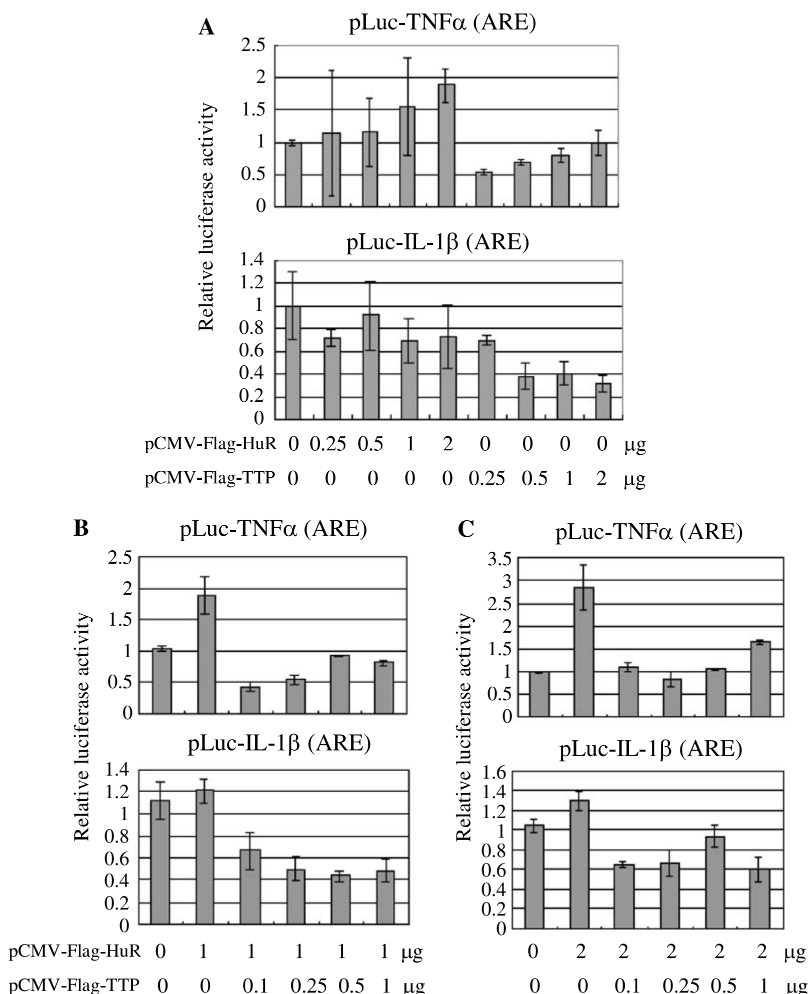

Fig. 5. Functional characterization of HuR and TTP mediated ARE-containing mRNA expression. (A) 293T cells were cotransfected with 0, 0.25, 0.5, 1, and 2 μg pCMV-Flag-HuR or pCMV-Flag-TTP together with 1 μg of reporter pLuc-TNFα (ARE) or pLuc-IL-1β (ARE). 293T cells were cotransfected with 1 μg pCMV-Flag-HuR (B) or 2 μg pCMV-Flag-HuR (C) and increasing amounts of pCMV-Flag-TTP as indicated together with 1 μg of reporter pLuc or pLuc-TNFα (ARE) or pLuc-IL-1β (ARE).

used to select its optimal binding site by RNA SELEX and revealed a strong preference for the extended sequence UUAUUUAUU, rather than UAUUUAU and a simple AUUUA motif [37]. Comparing the AREs of TNFα and IL-1β, we find that TNFα ARE contains three overlapping UUAUUUAUU motifs, while IL-1β ARE has only three UAUUUAU and one AUUUA sequences. Moreover, the LPS-induced phosphorylation and expression of TTP could be blocked by the p38 inhibitor in RAW264.7 cells (Fig. 4 and [27]). Using ectopic expression experiment in the culture cells, it has been confirmed that p38 signal could phosphorylate TTP and cause a decrease in its RNA-binding activity [26]. The functional analysis by using ARE-containing reporter gene showed that suppression activity of TTP could be reversed by p38 signal especially on IL-1β ARE-containing reporter. However, this reversal was unobvious to TNFα ARE containing reporter. A recent report showed the similar result that p38 kinase phosphorylated TTP did not alter its function on TNFα ARE [38]. This result was correlated with Fig. 1C's observation that TTP may be almost phosphorylated under 2 h LPS-stimulation and lost its suppression effect on IL-1β mRNA stability but not on TNFα's. Our explanation is that TTP has differential binding affinity on TNFα and IL-1β AREs, and therefore less amount of TTP could bind to TNFα ARE to trigger the RNA destabilization. Consequently, TNFα ARE has little response to TTP phosphorylation. Our data imply that the negative RNA stability regulator TTP is able to respond to p38 signal to control its target ARE-containing mRNA expression differentially.

EMSA showed that cytoplasmic HuR could bind to TNFα ARE to promote ARE-mediated gene expression. Its activity was not affected by MKK3 pathway. This was consistent with the previous study that activation of p38 by the expression of MKK6 active mutant with HuR did not result in any alteration in HuR activity [16]. The interaction between IL-1β ARE and HuR was only observed upon using the recombinant HuR (data not shown). It may be too low for the affinity of HuR for IL-1β ARE to be detected in cytoplasmic extracts. However, TNFα ARE was the target of both TTP and HuR that were proteins with different functions. It might possibly explain the

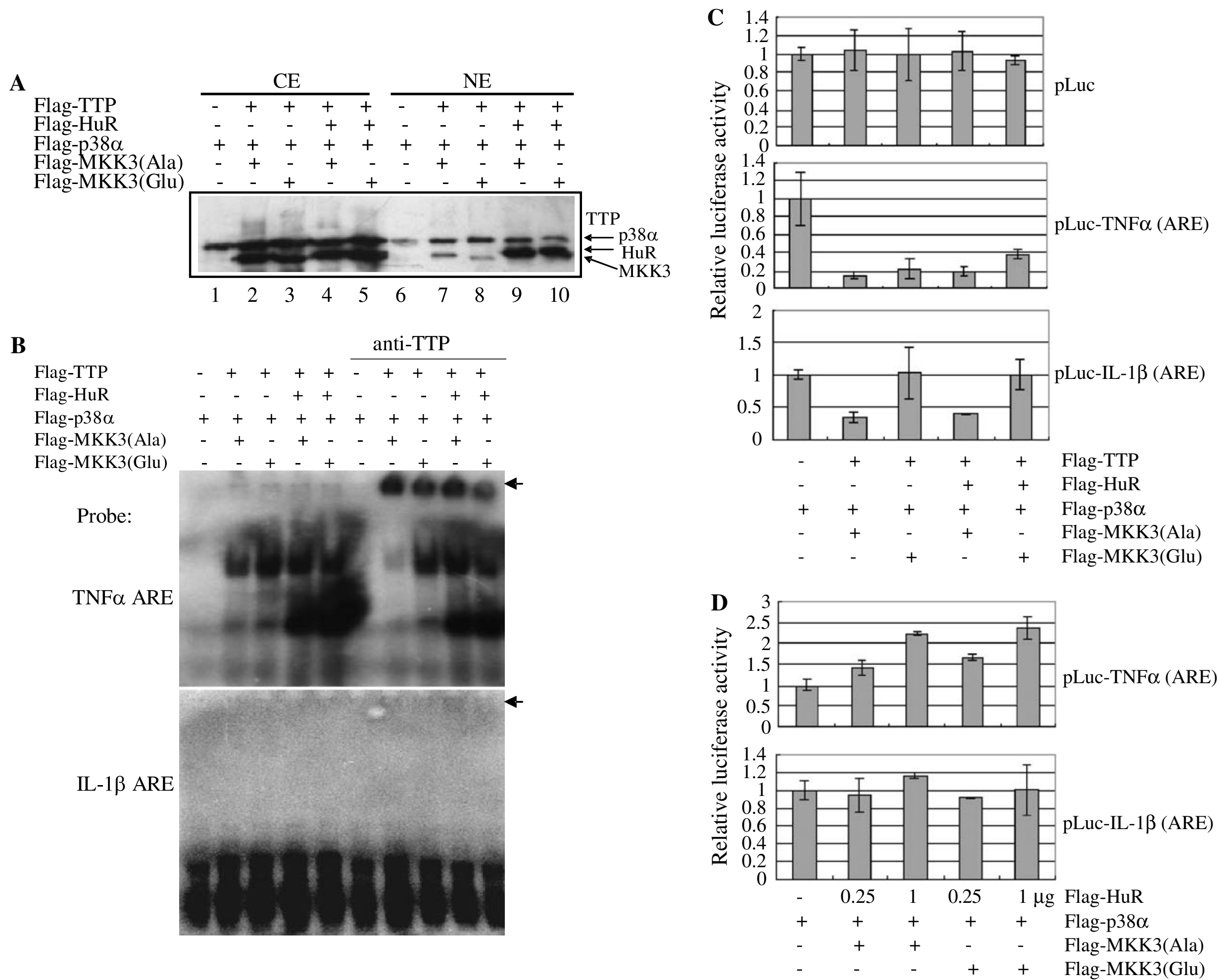

Fig. 6. p38 signaling in HuR and TTP mediated ARE-containing mRNA expression. 293T cells were cotransfected with 0.5 μg of either pRSV-Flag-MKK3(Ala) or pRSV-Flag-MKK3(Glu) and 0.5 μg pCMV-Flag-TTP, 1 μg pCMV-Flag-HuR and 0.5 μg pCMV5-p38α as indicated. Cytosolic and nuclear extracts were isolated for Western blotting with anti-Flag antibody (A). Moreover, the cytoplasmic extracts were incubated with radiolabeled TNFα and IL-1β ARE for gel shift assay and anti-TTP antibody was added in the supershift assay (B). The above experiments combined cotransfection with control pLuc, pLuc-TNFα (ARE) or pLuc-IL-1β (ARE) for luciferase assay (C). (D) pLuc-TNFα (ARE) or pLuc-IL-1β (ARE) was cotransfected with 0.25 or 1 μg pCMV-Flag-HuR together with 0.5 μg pRSV-Flag-MKK3(Ala) or pRSV-Flag-Mkk3(Glu).

difference of expression profiling of both TNFα and IL-1β during LPS stimulation: when the TNFα transcripts were induced, HuR could stabilize them and cause rapid mRNAs accumulation, however, IL-1β mRNAs had less protection from HuR. The previous report presented that distinct ARE domains of GM-CSF mRNA could respond to HuR and p38/MAPKAPK-2 individually [39]. HuR and the other mRNA destabilization factor AUF1 could bind to distinct sites of the p21 and cyclin D1 mRNAs to regulate the mRNA fate by protein abundance, stress condition, and subcellular localization [40]. The results indicated that HuR and other ARE-binding proteins could concurrently bind to common target mRNAs. The functional competition between HuR and TTP was observed in the experiment on IL-3 ARE [41]. Our cotransfection assay also showed that TTP could almost overcome the HuR effect in TNFα ARE even when the amount of TTP was lower than HuR. The detailed functional interaction between HuR and TTP on TNFα ARE will be further investigated.

We also observed that the low dose of TTP had higher suppression activity than high dose on TNFα ARE as reported in a previous study [18]. Our protein binding assay provides an explanation that the high dose of TTP could form large protein complex with TNFα ARE, which may block the TTP interaction of other mRNA decay enzymes. The other possibility is that the high amount of TTP could override the mRNA decay enzymes. A recent study suggests that the TTP protein family functions as a molecular link between ARE-containing mRNAs and the mRNA decay machinery by the recruitment of mRNA decay enzymes including deadenylation, decapping, and exonucleolytic decay [42]. Moreover, as a negative factor for cytokines production, the expression of TTP seemed to be controlled delicately. The autoregulation of feedback inhibition was observed [43,44].

The ARE-dependent RNA stability is the target of several different signaling mechanisms, and p38 mitogen-activated protein kinase pathway is one of them [4,43–48]. Several ARE-binding proteins including TTP, hnRNP A1, and hnRNP A0 have been reported that could respond to p38 signal to modulate the target mRNA stability or translation [28,29,49,50]. Thus, it is a very complicated signaling pathway to control the ARE-mediated gene expression. Our result showed that TNFα mRNA has longer half-life after exposure to LPS for 30 min (Fig. 1B). We also found p38 MAPK inhibitor SB203580 could decrease TNFα mRNA half-life at this time interval (data not shown). It seemed that LPS could stabilize TNFα mRNA and there is a p38-sensitive protein involved in this regulation. The detailed molecular linkage of p38 pathway and ARE-mediated cytokines expression is to be investigated.

Acknowledgments

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