Chemical inhibitors destabilize HuR binding to the AU-rich element of TNF-α mRNA

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Abstract

**Hu protein R (HuR) binds to the AU-rich element (ARE)
in the 3'UTR to stabilize TNF-α mRNA. Here, we identi-
fied chemical inhibitors of the interaction between HuR
and the ARE of TNF-α mRNA using RNA electro-
phoretic mobility gel shift assay (EMSA) and filter bind-
ing assay. Of 179 chemicals screened, we identified
three with a half-maximal inhibitory concentration
(IC_{50}) below 10 μM. The IC_{50} of quercetin, b-40, and b-41
were 1.4, 0.38, and 6.21 μM, respectively, for binding of
HuR protein to TNF-α mRNA. Quercetin and b-40 did
not inhibit binding of tristetraprolin to the ARE of TNF-α
mRNA. When LPS-treated RAW264.7 cells were treated
with quercetin and b-40, we observed decreased stabil-
ity of TNF-α mRNA and decreased levels of secreted
TNF-α. From these results, we could find inhibitors for
the TNF-α mRNA stability, which might be used ad-
vantageously for both the study for post-transcrip-
tional regulation and the discovery of new anti-in-
flammation drugs.**

Introduction

In eukaryotic cells, mRNAs undergo several types
of post-transcriptional regulation including splicing,
nuclear export, stabilization, and localization. The
stability of mRNA varies, as mRNA half-lives range
from approximately 15 min to more than 24 h
(Hollams *et al.*, 2002). In particular, the synthesis
of inflammatory cytokines such as TNF-α, IL-2,
IL-6, and IFN-γ are tightly controlled at the level of
mRNA stability. Both *cis*-elements (e.g., the AU-rich
element [ARE]) of the mRNA and trans-acting
RNA-binding proteins (e.g., Hu proteins) take part
in the regulation of mRNA half-life. In a steady
state, cytokine mRNAs are unstable due to the
presence of the ARE in the 3'-untranslated region
(UTR) with variable copies of the typical AUUUA
pentamer motif (Seko *et al.*, 2006). ARE-mediated
decay is differentially and rapidly regulated in
response to changes in the tissue environment
such as inflammation (Fan and Steitz, 1998; Fan *et
al.*, 2005).

TNF-α is an important inflammatory cytokine
that organizes the inflammatory response (Beutler
and Cerami, 1989). The TNF-α mRNA contains
AU-rich elements in the 3'-UTR that are usually
occupied by AU-binding proteins. Under normal
conditions, this leads to a blockade of translation or
rapid turnover of transcripts. T-cell intracellular
antigen-1 (TIA-1), one of the RNA recognition motif
(RRM) family proteins, is a translational silencer for
TNF-α mRNA. Tristetraprolin normally acts as a
destabilizer of TNF-α mRNA (Lai *et al.*, 1999;
Zhang *et al.*, 2002), while Hu proteins increase the
half-life of target mRNA (Seko *et al.*, 2006).

Hu proteins including HuR (HuA), HuB (Hel-N1),
HuC, and HuD, are a family of RNA-binding pro-
teins with homology to the *Drosophila* embryonic
lethal abnormal vision (ELAV) protein. Unlike the
other members of the ELAV family (HuB, HuC, and
HuD), that are exclusively found in neuronal tissue,
HuR is ubiquitously expressed (Ma *et al.*, 1996).
HuR consists of two N-terminal RNA recognition
motifs (RRM) with high affinity for an AU-rich
sequence, a nucleo-cytoplasmic shuttling sequence,

and a C-terminal RRM recognizing the poly(A) tail. HuR is mostly located in the nucleus, but certain events can trigger its translocation to the cytoplasm (Fan and Steitz, 1998). Stabilization of specific mRNAs by HuR is associated with extracellular stimuli. For example, HuR induces the stabilization of TNF-α mRNA in response to LPS challenge or chronic ethanol exposure in macrophages (Dean *et al.*, 2001; McMullen *et al.*, 2003). Moreover, HuR regulates the stabilization of p21 mRNA by UV light, and vascular endothelial growth factor (VEGF) mRNA by hypoxia (Levy *et al.*, 1998;

A

GST-HuR

GST | 1 | RRM | RRM | RRM | 327 (a.a.)

GST-TTP

GST | 1 | ZnF | ZnF | 327 (a.a.)

Mu TNF
AUAUUUAUAUUUGCACUUAUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUGCUUAUGAAUGUAUUUAUUUG

Mu COX2
UGACCAUAUUUAUUUAUUUAUGUGAAGAAUUUAAUUUAAUUAUUUAAUAUUUAUA

Hu IL6
GCGUUAGGACACUAUUUUAAUUAUUUUUAAUUUAUUAAUAUUUAAAUAUG

Mu cFOS
GGAUUCUCAGAUAUUUAUAUUUUUAUUUUAUUUUUUUCUACCCUGAGGUC

B

GST-mHuR (μM) 0 0.15 0.3 0.45 0.6 0.75 0.9 1.05 1.2 1.35

HuR-RNA complex

mTNF-α ARE → Free probe

GST-mTTP (μM) 0 0.5 1 1.5 2 2.5 3 3.5 4 4.5

TTP-RNA complex

mTNF-α ARE → Free probe

C

Bound probe (% of input) vs Protein (nM)
● HuR
▼ TTP
◆ GST

Figure 1. Binding affinity of HuR and tristetraprolin to the ARE of TNF-α mRNA. To make fusion proteins, we attached a GST tag to the N-terminal region of HuR (NP_034615) and tristetraprolin (NP_035886). For RNA ligands, we cloned the ARE sequence from TNF-α, COX-2, IL-6, and cFos mRNAs. RRM, RNA-recognition motif; ZnF, Zn^{2+}-finger binding motif. (A) HuR (upper) and tristetraprolin (TTP, lower) bound to the ARE of TNF-α mRNA in RNA EMSA analysis. (B) ^{32}P-labeled TNF-α ARE RNAs were incubated with the indicated concentration of HuR, tristetraprolin or GST protein. After incubation at 25°C for 20 min, the reaction mixture was resolved by gel electrophoresis in a 6% PAGE gel. (C) The affinity of HuR, tristetraprolin, and GST for TNF-α was determined by filter binding assay.

Wang *et al.*, 2000). HuR can function as an adaptor protein for the nuclear export of many ARE-containing mRNAs (Brennan and Steitz, 2001).

In a previous study, we demonstrated that flavonoids can inhibit the binding of HuC to the ARE of TNF-α mRNA (Kwak *et al.*, 2009). For the present study, we screened chemicals for their ability to interfere with the interaction of HuR protein with TNF-α mRNA. Use of chemical inhibitors that act on the stability of TNF-α mRNA may represent an improvement over the current therapeutic strategy of using anti-TNF-α antibody for the treatment of chronic inflammatory diseases.

Results

Binding affinity of HuR and tristetraprolin to ARE of TNF-α mRNA

We created recombinant GST fusion proteins with HuR and tristetraprolin, and used these fusion proteins and radiolabeled RNA containing the ARE sequence from TNF-α (Figure 1A) to perform RNA EMSA (Figure 1B). HuR:ARE complexes of two different molecular weights were formed in a concentration-dependent manner (Figure 1B). In RNA EMSA, we observed that HuR bound more strongly than tristetraprolin to ARE sequences. The binding efficiencies of the two proteins were also quantified by filter binding assays using the same recombinant proteins and RNA probe (Figure 1C). The binding affinity of HuR to TNF-α mRNA was higher than that of tristetraprolin. In addition, the total amount of protein:RNA complex was more abundant for HuR:ARE.

Screening of inhibitors for HuR:ARE complex formation

We screened for chemical inhibitors for HuR:ARE binding based on RNA EMSA and filter binding assays. We obtained a subset of a publicly available chemical library from the Korea Research Institute of Chemical Technology (KRICT) and screened 179 different chemicals using an electrophoretic mobility gel shift assay with recombinant HuR. In primary screening, we applied chemicals at 100-μM concentrations to HuR:ARE complexes. Among the chemicals, nine candidates showed a strong inhibitory effect (cut-off ≥ 25% inhibition) on
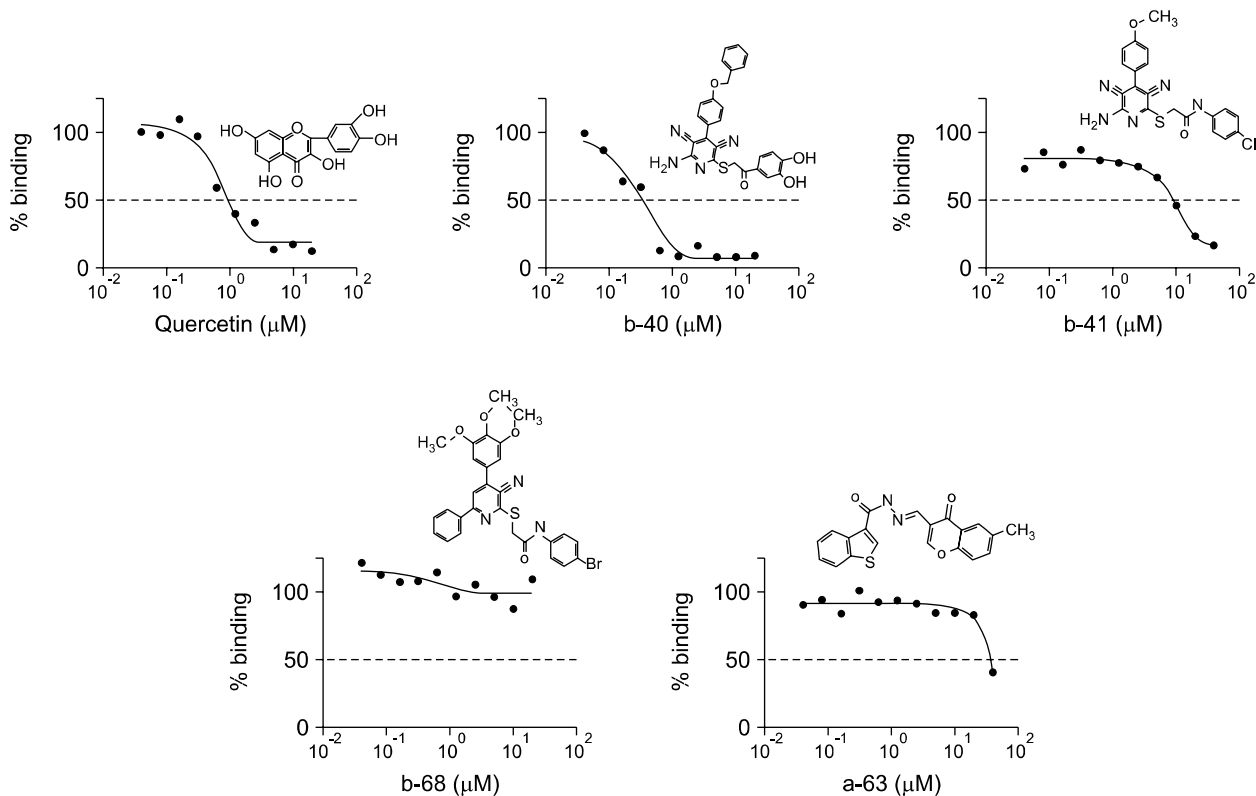
Figure 2. Inhibitory activity of six candidate chemicals for HuR binding to the ARE of TNF-α mRNA. We tested six candidate chemicals after primary screening and checked the IC$_{50}$ values of each chemical using filter binding assay. The upper figure of each line graph shows the chemical structure.

Table 1. List of candidate inhibitors of ARE and HuR interaction with IC_{50} values.

ID	Name	Formula	IC_{50} (μM)
Quercetin	Quercetin	$C_{15}H_{10}O_7$	1.40
b-40	2-amino-4-(4-(benzyloxy)phenyl)-6-(2-(3,4-dihydroxyphenyl)-2-oxoethylthio)pyridine-3,5-dicarbonitrile	$C_{28}H_{20}N_4O_4S$	0.38
b-41	2-(6-amino-3,5-dicyano-4-(4-methoxyphenyl)pyridin-2-ylthio)-N-(4-chlorophenyl)acetamide	$C_{22}H_{16}ClN_5O_2S$	6.21
b-68	N-(4-bromophenyl)-2-[3-cyano-6-phenyl-4-(3,4,5-trimethoxyphenyl)-2-pyridinyl]thio]acetamide	$C_{29}H_{24}BrN_3O_4S$	–
a-63	Benzo[b]thiophene-3-carboxylic acid,[(6-methyl-4-oxo-4H-1-benzopyran-3-yl)methylene]hydrazide(9CI)	$C_{20}H_{14}N_2O_3S$	–

the binding of HuR to ARE.

IC_{50} of candidate chemicals

To determine the IC_{50} of each chemical on the stability of HuR:ARE complexes, chemicals were applied in various doses to measure the formation of the RNA-protein complex by filter binding assay. The half-maximal inhibitory concentration (IC_{50}) was determined using GraphPad Prism software (Figure 2). As summarized in Table 1, IC_{50} values were 1.4 μM for quercetin, 0.38 μM for b-40, and 6.21 μM for b-41.

## Specificity of chemical inhibitors of the HuR:ARE complex

Although we screened chemicals using HuR and the ARE of TNF-α mRNA, HuR can bind to other ARE sequences, and the ARE of TNF-α can be occupied by tristetraprolin. To evaluate the specificity of the inhibitory activity of b-40 and quercetin for RNA-binding proteins, we substituted tristetraprolin for HuR and analyzed the inhibitory effect of these chemicals on complex formation with the ARE of TNF-α (ARE[TNF-α]) (Figure 3). As shown in Figure 1B, tristetraprolin:ARE binding (Kd = 2.194×10^{-7} M) was lower than that of HuR:ARE (Kd = 0.677×10^{-7} M), and tristetraprolin:ARE binding was not inhibited by either quercetin or b-40.

We also tested the specificity of the inhibitory activity for target ARE sequences from COX2, IL-6 and cFos (Figure 3). Quercetin treatment at 0.5 μM did not effectively inhibit any RNA-protein interactions except HuR:ARE (TNF-α) (Figure 3A). Treatment of b-40 at 0.25 μM inhibited formation of the complexes HuR:ARE (TNF-α) and HuR:ARE (cFos), but the binding of HuR to the ARE of COX2 and IL-6 mRNAs was not altered by b-40 treatment.
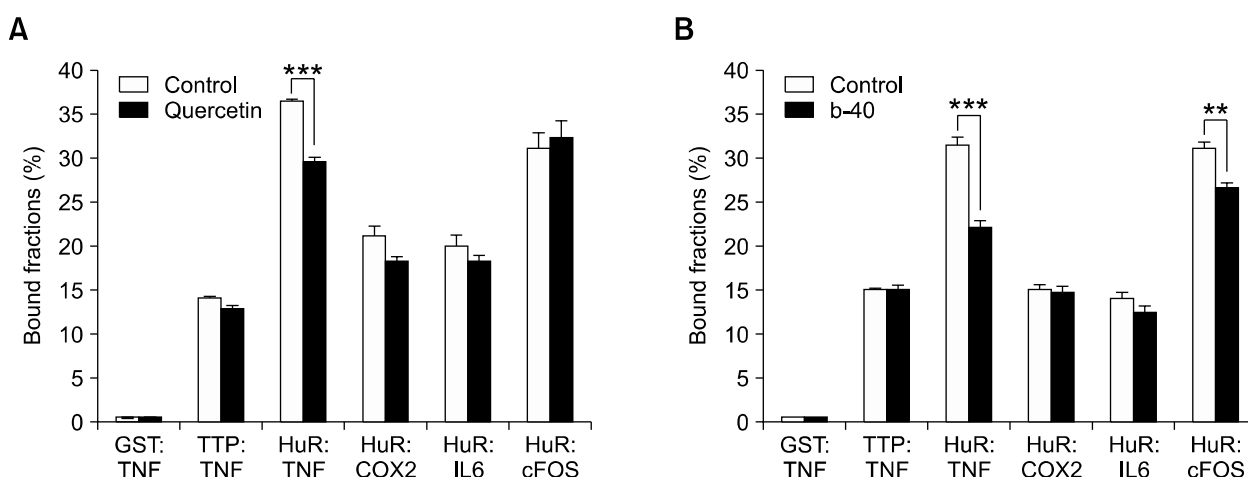
Figure 3. Specificity of the inhibitory activity of quercetin and b-40. TNF-α, COX2, IL-6 or cFos ARE RNA was incubated with GST (128 nM), tristetraprolin (128 nM) and HuR (32 nM). After the reaction mixture was treated with quercetin at 0.5 μM (A) or b-40 at 0.25 μM (B) the percentage of RNA complexed with protein was quantified using filter-binding assay. The results are means $\pm$ standard deviation (SD) of duplicates from two independent experiments. Statistical analysis was performed by *t*-test using Graph Pad Prism5 software. A *P* value of less than 0.05 was considered statistically significant (**$P < 0.01$, ***$P < 0.001$).
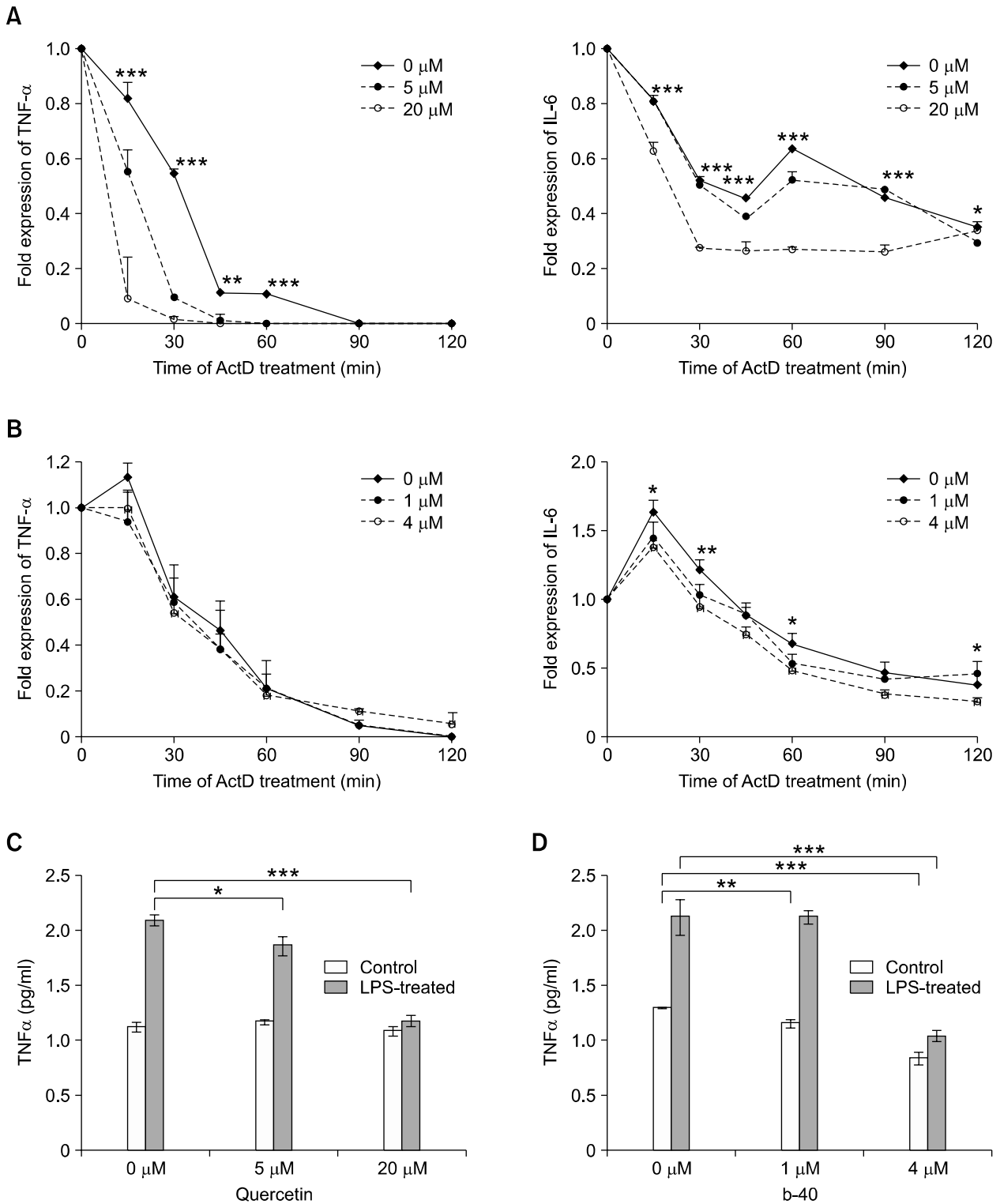
Figure 4. Effect of quercetin and b-40 on TNF-α and IL-6 expression in LPS-treated RAW264.7 cells. RAW264.7 cells were pre-treated with 1 μg/ml LPS for 1 h and transcription was blocked using Actinomycin D (5 mg/ml) and quercetin (A) or b-40 (B). Total RNA was isolated at the time points indicated for quantification of TNF-α and IL-6 mRNA using semi-quantitative RT-PCR. Error bars present ± standard deviation (SD) of three independent experiments. Statistical analysis was performed by one way ANOVA (Tukey post-test) using Graph Pad Prism5 software. A P value of less than 0.05 was considered statistically significant ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Levels of secreted TNF-α and IL-6 proteins were measured using an ELISA kit after 24 h treatment with quercetin (C) or b-40 (D) in LPS-activated RAW264.7 cells. Data of (C) and (D) are shown as a mean ± SD (n = 3). Statistical analysis was performed by t-test using Graph Pad Prism5 software.

Control of mRNA stability by quercetin and b-40

Because quercetin and b-40 can inhibit HuR:ARE (TNF-α) complex formation, these two chemicals may act by affecting the stability of TNF-α mRNA. We examined TNF-α and IL-6 mRNA in LPS-treated RAW264.7 mouse macrophage cells after the addition of actinomycin D. In the absence of quercetin, TNF-α and IL-6 mRNA levels were decreased by the addition of actinomycin D. When we added quercetin to LPS-treated RAW264.7 cells, the degradation of TNF-α and IL-6 mRNA was faster than that of untreated controls (Figure 4A). The effect of b-40 on mRNA stability was minimal for TNF-α, but b-40 induced more rapid degradation of IL-6 mRNA (Figure 4B).

To confirm the effect of quercetin and b-40, we measured levels of TNF-α protein secreted into the medium of LPS-treated RAW264.7 cells using an ELISA kit. As shown in Figure 4C, the amount of TNF-α protein in quercetin-treated cells decreased in a dose-dependent manner. In addition, treatment with b-40 effectively reduced the synthesis of TNF-α at a dose as low as 4 μM (Figure 4D).

Discussion

The half-life of mRNA can be determined by its stability in response to a variety of stimuli. The stability of mRNA plays an important role in the homeostasis, differentiation, development, and growth of specialized cells. HuR protein increases expression of several mRNAs, including vascular endothelial growth factor (VEGF) and TNF-α. The HuR-mediated stabilization of mRNA has an important role in tumorigenesis and inflammation (Hollams *et al.*, 2002). Chemicals such as SB-242235 and RWJ-67657 act as effectors of anti-inflammatory activity by blocking the production of TNF-α, IL-1, and IL-6 (Kumar *et al.*, 2003). However, the mechanism of action of these inhibitors in post-transcriptional regulation of inflammatory mediators has not been investigated. In a previous report, we described the interference of flavonoids with the interaction of the stabilizing factor HuC and the key inflammatory cytokine TNF-α (Kwak *et al.*, 2009). In this study, we have verified the inhibitory effect of candidate chemicals for RNA-protein binding using EMSA and filter binding assay.

Details of the mechanism of action of the selected chemicals' interference with the HuR binding to the ARE of TNF-α mRNA should be investigated at the molecular level in further studies. Because quercetin and b-40 showed specificity for HuR, we speculate that these chemicals might recognize the structure of HuR, but not tristetraprolin. The chemicals might

destabilize the structure of the protein or sterically hinder its interaction with RNA. Meisner and coworkers have identified three low-molecular-weight inhibitors for HuR, and demonstrated the action of these chemicals in interfering with the formation of HuR dimers before RNA binding (Meisner *et al.*, 2007). In Figure 3, we used 0.5 μM quercetin and 0.25 μM b-40 to check the inhibitory activity on the binding of HuR to various ARE sequences. Although higher concentration of both chemicals could inhibit the HuR:ARE binding, we could find that HuR:TNF binding was inhibited at the lowest concentration. In addition, we used higher concentration of two chemicals in Figure 4 to check the inhibitory activity in LPS-treated RAW264.7 cells. As expected, the inhibitory concentration was higher in *in vivo* cell culture assay.

Tristetraprolin and TIA-1 mediate the degradation of TNF-α mRNA, but HuR can stabilize the transcripts upon the inflammatory stimulation like LPS treatment (Fan and Steitz, 1998; Fan *et al.*, 2005; Seko *et al.*, 2006). Because we wanted to find the chemicals inhibiting the production of TNF-α at the post-transcription level, we focused on the inhibition of mRNA stabilization by HuR. We did not test the inhibitory activity of these two chemicals against TIA-1, but the chemical inhibitors could suppress the stability of TNF-α mRNA in ELISA (Figure 4).

TNF-α has become a major target of numerous pharmaceutical projects. Etanercept (Enbrel[TM]), infliximab (Remicade[TM]), and adalimumab (Humira[TM]) act as neutralizing antibodies of TNF-α. Grattendick and coworkers have published the effects of three anti-TNF-α drugs on levels of secreted TNF-α and cell-associated TNF-α *in vitro* (Grattendick *et al.*, 2008). They have also described many adverse effects of protein-based anti-TNF-α drugs, which induce partial neutralization of TNF-α bioactivity. The development of drugs regulating stability of TNF-α mRNA may provide a better therapeutic strategy for the treatment of chronic inflammatory diseases.

In response to genotoxic stress, HuR can associate with the 3'-UTR of p53 mRNA to stabilize transcripts (Mazan-Mamczarz *et al.*, 2003). Exposure to UV light (UVC) induced an increase of p53 protein levels, and overexpression of HuR in RKO cells resulted in elevation of observed levels of p53 using western blot analysis. However, cells with a reduced level of HuR showed decreased levels of p53. HuR has been demonstrated to bind to p53 mRNA to enhance its translation. The results of this study indicate that control of mRNA stability may be the mechanism for the radioprotective effect of quercetin .

Although quercetin and b-40 might inhibit the transcription of TNF-α, we could check the production of TNF-α mRNA in steady state levels, and there was no significant change between groups with increasing concentration of chemicals (Figure 4A and 4B). In addition, the production of TNF-α protein was not decreased upon the treatment of quercetin and b-40 (Figure 4C and 4D). These data might suggest that the possible effect of the chemicals on the transcription of TNF-α might be quite minimal.

In our studies, we identified chemical inhibitors of the interaction of TNF-α mRNA with HuR protein. The chemicals quercetin, b-40, and b-41 strongly inhibited binding of HuR to TNF-α mRNA *in vitro*. These chemicals induced rapid decay of cytokine mRNA in LPS-stimulated RAW264.7 cells. These observations may be useful for study of both the post-transcriptional regulation of cytokines and as a possible alternative to the treatment of inflammatory disease with TNF-α antibody.

Methods

Chemicals

We used representative chemicals provided by the Korea Chemical Bank of the Korea Research Institute of Chemical Technology (Yuseong-gu, Daejeon, Korea).

DNA constructions

Full-length recombinant mouse HuR and tristetraprolin genes were cloned into pGEX4T1 vectors. Vectors incorporating clones were transformed into *Escherichia coli* (*E. coli*) strain BL21 for protein expression as described previously (Kang *et al.*, 2002). For *in vitro* synthesis of TNFα-ARE transcripts, sense oligonucleotides were used as a template for PCR reactions. All oligonucleotides contained the 5' flanking sequence 5'-GGGAGATAC-GGTCCACTACC-3' and the 3' flanking sequence 5'-CGA-AGTCTGAGTGATGCATG-3'. The PCR templates were amplified using forward primer 5'-GCTAATACGACTCAC-TATAGGGAGATACGGTCCACTACC-3' and reverse primer 3'-GCTTCAGACTCACTACGTAC-5'.

Preparation of recombinant HuR and tristetraprolin proteins

Colonies containing recombinant mouse HuR and tristetraprolin genes were grown in LB culture medium (10 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) at 37°C with shaking until the A600 reached 0.4. Then IPTG was added to a final concentration of 0.5 mM, and the culture was incubated for an additional 4 h. After resuspension in 5 ml of ice-cold lysis buffer (50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 5 mM DTT), the cells were disrupted by sonication (30 W, 90 s). The lysate was incubated for 30 min at 4°C and then centrifuged at 12,000 $\times$ g for 30 min at 4°C. The

supernatant was mixed with Glutathione Sepharose 4B (GE Healthcare, Sweden) and incubated for 90 min at room temperature with gentle agitation. The sepharose medium was washed three times with TBS (50 mM Tris-HCl (pH 8.0), 120 mM NaCl), and the fusion protein was eluted into elution buffer (50 mM Tris-HCl pH 8.0, 20 mM reduced glutathione).

In vitro transcription of RNA probe

The RNA probe was generated from a PCR template containing a T7 RNA polymerase promoter site. The template was transcribed using [α-^{32}P] UTP (GE Healthcare, Sweden) and the T7 MEGAscript kit (Ambion, TX) according to the manufacturer's instructions. Transcribed probe was treated with DNase I for 15 min at 37°C, extracted with phenol:chloroform, and precipitated using isopropanol.

RNA electrophoretic mobility gel shift assay (RNA EMSA)

Following mixing of the labeled RNA probe and the GST-HuR protein in binding buffer (50 mM Tris-HCl (pH 8.0), 120 mM NaCl), each chemical was added in a DMSO solution. The mixtures were incubated for 20 min at room temperature. The protein-RNA complexes were separated from free RNA on a 6% polyacrylamide gel prepared and pre-electrophoresed in 0.5$\times$ TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA). Electrophoresis was performed in 0.5$\times$ TBE buffer at 8 mA for 1 h (Jung *et al.*, 2006). Bands were visualized using a BAS2500 automated detector system (Fujifilm, Tokyo, Japan), and their intensities were measured by image analysis software (Multi-Gauge V3.0, Fujifilm, Tokyo, Japan).

Protein-RNA filter binding assay

Binding reactions contained ^{32}P-labeled RNA and GST-HuR or GST-tristetraprolin protein in binding buffer in a total volume of 100 μl. After incubation for 20 min at room temperature, reaction solutions were filtered through 0.45 μm nitrocellulose disks (Millipore, MA), which had been previously boiled for 30 min in binding buffer. The filters were washed twice with binding buffer and analyzed in a Tri-Carb 2900TR liquid scintillation counter (Perkin Elmer, CT).

Cell culture

The RAW264.7 mouse macrophage cell line was cultured in Dulbecco's modified Eagle's medium (Welgene, Daegu, South Korea), supplemented with 10% fetal bovine serum (Hyclone Laboratories, UT) and 1% penicillin/streptomycin (Invitrogen, CA) at 37°C in a 5% CO_2 atmosphere.

mRNA stability studies

For RNA stability assays, actinomycin D (5 μg/ml) was added to RAW264.7 cells to inhibit transcription. Total RNA extracted from cells was treated with Actinomycin D and/or

quercetin (0, 5, and 20 μM) or b-40 (0, 1, and 4 μM) for different time intervals. Total RNA (200 ng) prepared using TRIZOL reagent was reverse-transcribed to produce cDNA using reverse transcriptase and oligo-dT primer. PCR was performed using 1 or 2 μl of cDNA with specific primers.

RT-PCR

Reverse transcriptase with an oligo-dT primer (Invitrogen, CA) was used to prepare cDNA from 0.1 μg of total RNA. PCR with specific primers was performed using 1 μl of cDNA. The primer sets were as follows: TNF-α: forward 5'-CCAGGCAGTCAGATCATCTTC-3' and reverse 5'-TT-GATGGCAGAGAGGAGGTT-3'; IL-6: forward 5'-AACGA-TGATGCACTTGCAGA-3' and reverse 5'-GGAAATTGGG-GTAGGAAGGA-3'; and GAPDH: forward 5'-TTCACCAC-CATGGAGAAGGC-3' and reverse 5'-GGCATGGACTGT-GGTCATGA-3'. PCR conditions were as follows: 30 cycles of denaturation at 95°C for 30 s followed by annealing at 55°C for 30 s and extension at 72°C for 30 s; and a final step at 72°C for 10 min. PCR products were separated by electrophoresis on 1.5% agarose gels containing 0.1 μg/ml ethidium bromide and were visualized under UV light with a bioimaging system (Syngene, Cambridge, UK).

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