HIV protease inhibitors increase TNF-α and IL-6 expression in macrophages: Involvement of the RNA-binding protein HuR

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Abstract

HIV protease inhibitors (PIs) have been associated with the serious Metabolic Syndrome, which is the major risk factor of atherosclerotic cardiovascular disease. Atherosclerosis is widely considered to be a chronic inflammatory disease. Macrophages are the most prominent cell type present in atherosclerotic lesions and play essential roles in both early lesion development and late lesion complications. We previously reported that HIV PIs induced accumulation of intracellular free cholesterol and lipids, decreased endoplasmic reticulum (ER) calcium stores, activated the unfolded protein response (UPR), significantly increased apoptosis, and promoted foam cell formation in macrophages. HIV PI-induced ER stress and subsequent activation of the UPR, represents an important cell signaling mechanism of HIV PI-induced metabolic syndromes. Here we show that all HIV PIs, except amprenavir, increased expression of the major mediators of inflammatory response, TNF-α and IL-6, to varying degrees. Furthermore, we show that the RNA-binding protein, HuR, plays an important role in HIV PI-induced expression of TNF-α and IL-6. Atazanavir increased the cytoplasmic levels of HuR and enhanced the binding of HuR to 3′-UTR of TNF-α and IL-6. Down regulation of HuR expression by siRNA prevented atazanavir-induced increase of TNF-α and IL-6. These results suggest that HuR might have an impact on pathophysiological processes of HIV PI-induced atherosclerosis.

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Keywords: HIV protease inhibitor; ER stress; Inflammation; Atherosclerosis; HuR

1. Introduction

Incorporation of HIV protease inhibitors (PIs) in highly active anti-retroviral therapy (HAART) has effectively modified disease progression and significantly reduced the mortality and morbidity in HIV-infected patients [1]. However, the benefits of HIV PIs are compromised by a number of severe metabolic side effects, such as dyslipidemia, insulin resistance, and lipodystrophy, which are well-defined risk factors for premature atherosclerosis, myocardial infarction, and other forms of cardiovascular disease [2,3].

Atherosclerosis is a chronic inflammatory disease and inflammation appears to be involved in all stages of atherosclerotic development [4,5]. It also has been found that macrophages play a critical role in the initiation and progression of atherosclerotic lesions. In addition to the

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accumulation of free cholesterol in macrophages, another key characteristic of atherosclerotic lesions is the presence of abundant inflammatory cytokines [6]. Our recent studies have shown that ritonavir induces accumulation of intracellular free cholesterol and lipid, decreases ER calcium stores, activates the unfolded protein response (UPR), significantly increases apoptosis, and promotes foam cell formation in macrophages [7]. These results provide novel insights into the cellular mechanisms whereby HIV PIs may induce lipid deregulation and accelerate atherosclerosis. However, whether HIV PIs are able to modulate the expression of pro-inflammatory cytokines, such as TNF-α and IL-6, in macrophages is unknown.

TNF-α and IL-6 are major mediators of the inflammatory response and have been implicated in the pathogenesis of a wide variety of inflammatory diseases including atherosclerosis [8]. It is increasingly recognized that the post-transcriptional regulation is a critical control point for gene expression of inflammatory cytokines. Stabilization of mRNAs contributes to the strong and rapid induction of genes in the inflammatory response [8]. The turnover of short-lived mRNAs, such as those encoding cytokines, growth factors, and proto-oncogenes, is controlled through the association of RNA-binding proteins that recognize specific RNA sequences and either increase or decrease transcript half-life [9,10]. Best characterized among such RNA sequences are AU-rich elements (AREs), usually found in the 3′ untranslated regions (3′-UTR) of these short-lived mRNAs. HuR is ubiquitously expressed and is one of the most extensively studied RNA-binding proteins [11]. It has been shown to regulate the mRNA stability of TNF-α and IL-6 by binding to the AREs in the 3′-UTR [12,13]. Although the precise mechanisms regulating HuR function are not clear, it is becoming increasingly apparent that HuR’s subcellular localization is intimately linked to its function. It has been proposed that the mRNA-stabilizing function of HuR requires its translocation from the nucleus to the cytoplasm [11].

In the present studies, we directly examined the effects of HIV PIs including amprenavir, atazanavir, indinavir, lopinavir, nelfinavir and ritonavir on expression of pro-inflammatory cytokines, TNF-α and IL-6, in macrophages. The results indicate that different HIV PIs vary greatly in their ability to induce TNF-α and IL-6 release. Furthermore, we demonstrate that the RNA-binding protein, HuR, plays an important role in HIV PI-induced expression of TNF-α and IL-6. HIV PI-induced nucleus to cytoplasmic translocation of HuR contributes to the increased TNF-α and IL-6 expression in macrophages.

2. Methods

2.1. Cell culture and HIV PI treatment

Mouse J774A.1 macrophages were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO2. Cells from passages six to nine were used in these studies. Human THP-1 monotypic cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO2. THP-1 monocytes were treated with PMA (100 ng/ml) for 5 days to facilitate differentiation into macrophages. Amprenavir, atazanavir, lopinavir, nelfinavir and ritonavir were dissolved in dimethyl sulfoxide (DMSO). Indinavir was dissolved in H2O. HIV PIs were added directly to culture medium (final concentrations 5–50 μM) and incubated for 0.5–24 h. For each result, a minimum of three independent experiments were performed.

2.2. Enzyme-linked immunosorbent assays (ELISA) of cytokines

Mouse J774A.1 macrophages were treated with 15 μM of HIV PIs or vehicle control for various times, or various concentrations of HIV PIs (0–50 μM) for 24 h. At the end of the treatment, the culture media were collected and centrifuged at 14,000 rpm for 5 min. The supernatants were stored in aliquots at −70 °C. TNF-α and IL-6 levels in the media were determined by ELISA using mouse TNF-α and mouse IL-6 ELISA Max™ Set Deluxe Kits (BioLegend). The total protein concentrations of the viable cell pellets were determined using Bio-Rad Protein Assay reagent. Total amounts of the TNF-α and IL-6 in media were normalized to the total protein amounts of the viable cell pellets.

2.3. Western blot analysis

Total cell lysates, cytoplasmic and nuclear proteins were prepared as previously described [14]. The protein concentration was determined using the Bio-Rad Protein Assay reagent. The nuclear extracts (15 μg protein), cytoplasmic proteins (10 μg) or total cellular proteins (10 μg) were resolved on 10% Bis–Tris gels and transferred to Nitrocellulose membranes. Immunoblots were blocked overnight at 4 °C with 5% non-fat milk in TBS buffer and then incubated with antibodies to HuR, lamin B, or actin. Immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibody and the Western Lightning Chemiluminescence Reagent Plus. The density of the immunoblot bands was analyzed using Image J computer software (NIH) [7].

2.4. Immunofluorescent staining

Mouse J774A.1 macrophages were treated with vehicle control (DMSO), amprenavir (15 μM) or atazanavir (15 μM) for 24 h. Cells were fixed using 3.7% formaldehyde. The expression of HuR was detected by using mouse monoclonal antibody against HuR and Donkey anti-mouse antibody conjugated with Alexa Fluor-594. The nuclei were stained using DAPI. HuR expression was visualized using a confocal flu-
orescence microscope with a 63× oil immersion objective using a dual filter set for DAPI and rhodamine.

2.5. RNA isolation and real-time quantitative PCR

Total cellular RNA was isolated from mouse J774A.1 macrophages or human THP-1 cell-derived macrophages after treatment with HIV PIs (15 µM), or vehicle control (DMSO) for 24 h, using Ambion RNAqueous kit. Total RNA (10 µg) was used for first-strand cDNA synthesis using High-Capacity cDNA Archive Kit. The mRNA levels of TNF-α and IL-6 were quantified using the specific gene expression assay kits for mouse TNF-α and IL-6 on a DNA Engine Opticon2 Sequence Detection System (MJ Research, Inc.). The mRNA levels of CHOP in THP-1-derived macrophages were quantified using the following TaqMan primers and probe: forward primer 5′-CTGAATTCGACCAAAGC ATGA-3′, reverse primer 5′-AAGGTGGGTAGTGTTGGCCC-3′, Taq-Man probe 6-FAM-CATGGGTGACATGCTCCGC ACT-BHQ-1 [15]. The mRNA of HuR in mouse J774A.1 cells was quantified using the following primers: forward primer 5′-TGTGCCCAGGAATGCT-3′, reverse primer 5′-TCACGAAATCTTTTACATCGTG-3′. iQTM SYBR Green Supermix (Bio-Rad Laboratories) was used as a fluorescent dye to detect the presence of double-stranded DNA. The mRNA values for each gene were normalized to internal control β-actin mRNA. The ratio of normalized mean value for each treatment group to vehicle control group (DMSO) was calculated.

2.6. Construction of lentiviral siRNA for HuR

The sense sequence of the siRNA cassettes specifically targeting the nucleotides of HuR was designed through siRNA Target Finder (Ambion, Austin, TX). A two-step polymerase chain reaction (PCR) strategy was performed using two separate reverse primers to generate a siRNA expression cassette (SEC) consisting of human U6 promoter and a hairpin siRNA sequence. The antisense strand was amplified by PCR using the following primers: forward primer 5′-GCAGGACACA GCTTGG GCTAC-3′, reverse primer 5′-CTGAATCTGCACCAAGC ATGA-3′, Taq-Man probe 6-FAM-CATGGGTGACATGCTCCGC ACT-BHQ-1 [15]. The mRNA of HuR in mouse J774A.1 cells was quantified using the following primers: forward primer 5′-TGTGCCCAGGAATGCT-3′, reverse primer 5′-TCACGAAATCTTTTACATCGTG-3′. iQTM SYBR Green Supermix (Bio-Rad Laboratories) was used as a fluorescent dye to detect the presence of double-stranded DNA. The mRNA values for each gene were normalized to internal control β-actin mRNA. The ratio of normalized mean value for each treatment group to vehicle control group (DMSO) was calculated.

2.7. HuR lentivirus transduction in macrophages

Mouse J774A.1 macrophages were incubated with lentivirus at 100 ‘multiplicity of infection’ (MOI) for 48 h and cultured with DMEM plus 10% FBS. HuR RNA silencing effect of the lentiviral siRNA was confirmed by real-time RT-PCR and Western blot analysis.

2.8. Preparation of synthetic RNA transcripts

pSP-72 vector containing the entire coding region (CR) or 3′-UTR for mouse TNF-α (GI: 54844, CR: 157–864, 708 bp); 3′-UTR: 1110–1350, 241 bp) and IL-6 (GI: 10834983, CR: 31–666, 636 bp; 3′-UTR: 667–1062, 396 bp) was used as a template for PCR amplification of the CR and 3′-UTR of TNF-α and IL-6. T7 primer and gene specific 3′ primers for TNF-α and IL-6 were used. To prepare the CR and 3′-UTR of TNF-α, the following primers were used: 5′-CAGCAATTTCATGACATGAGAAGTCTTC-3′ and 5′-GCCGGATCCTAATACATTATCAGTCA AGC-3’. To prepare the CR and 3′-UTR for IL-6, the following primers were used: 5′-GACGAATTCATGGTGTGCGAGTAG-3′ and 5′-GCAGGATCCGAAGACAGTCTAAAC-3’. PCR-amplified products were used as templates to transcribe biotinylated RNAs by using T7 RNA polymerase in the presence of biotin-cytidine 5′-triphosphate (CTP) as described [17].

2.9. RNA–protein binding assays

For biotin pull-down assays, 25 nmolar of biotinylated transcripts of CR or 3′-UTR for TNF-α or IL-6 were incubated with 120 µg of cytoplasmic lysate for 30 min at room temperature. Complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads and analyzed by Western blotting. To assess the association of endogenous HuR with endogenous TNF-α and IL-6 mRNA complexes, performed as described [11]. Briefly, after treatment with HIV PIs or vehicle control (DMSO), cells were harvested, counted, and the same number of cells per condition (3 × 10⁵) were pelleted and resuspended in approximately two cell pellet volumes of polysome lysis buffer containing 100 mM KC1, 5 mM MgCl2, 10 mM HEPES (pH 7.0), 0.5% Nonidet P-40 with 1 mM DTT, 100 U/ml RNaseOUT, 0.2% vanadyl-ribonucleoside complex, 0.2 µM PMSF, 1 µg/ml pepstatin A, 5 µg/ml bestatin, and 20 µg/ml leupeptin. Cell lysates were centrifuged at 16,000 × g for 10 min at 4 °C. For IP, protein A-Sepharose beads were swollen 1:1 (v/v) in NT2 buffer (50 mM Tris (pH 7.4), 150 mM NaCl, and 1 mM
MgCl₂/0.05% Nonidet P40] supplemented with 5% BSA. A 100-μl aliquot of the preswollen protein A bead slurry was used for each IP reaction and incubated for 4 h at room temperature with excess immunoprecipitating Ab (30 μg), using either a mouse mAb specific for HuR, or an IgG1 isotype control Ab. RNA in IP materials was reverse transcribed and used to detect the presence of TNF-α and IL-6 mRNAs by real-time PCR [9].

2.10. Statistical methods

Student’s t-test was employed to analyze the differences between sets of data. Statistics were performed using GraphPad Pro (GraphPad, San Diego, CA).

3. Results

3.1. Effects of HIV PIs on TNF-α and IL-6 expression in macrophages

Accumulation of inflammatory cytokines is another key characteristic of the advanced atherosclerotic lesions. It has been demonstrated that lipid-laden macrophages secrete significant amounts of proinflammatory cytokines, such as TNF-α and IL-6. To examine whether HIV PIs also increase the synthesis of the inflammatory cytokines in macrophages, we measured TNF-α and IL-6 in the culture medium of HIV PI-treated mouse macrophages using ELISA. The results demonstrated that the abilities of individual HIV PIs to induce TNF-α and IL-6 release were different. As shown in Fig. 1A, atazanavir, indinavir, lopinavir, nelfinavir and ritonavir significantly increased TNF-α and IL-6 synthesis in a dose-dependent manner after 24 h treatment, but amprenavir did not have any effect on either TNF-α or IL-6 synthesis even at 50 μM concentration (Fig. 1Aa). Twenty-four-hour treatment with 15 μM of atazanavir increased the synthesis of TNF-α and IL-6 by 73% and 61%, respectively; indinavir increased the synthesis of TNF-α and IL-6 by 37% and 29%, respectively; lopinavir increased the synthesis of TNF-α and IL-6 by 60% and 53%, respectively; nelfinavir increased the synthesis of TNF-α and IL-6 by 36% and 25%, respectively; ritonavir increased the synthesis of both TNF-α and IL-6 by 29%.

Expression of inflammatory cytokines is a highly regulated process. It can be regulated at the levels of transcription, translation and secretion [18]. Modulation of mRNA stability is an important mechanism in the regulation of TNF-α and IL-6 biosynthesis. To identify the potential cellular mechanisms of HIV PI-induced expression of TNF-α and IL-6, we compared the effect of atazanavir and amprenavir on TNF-α and IL-6 mRNA stability in macrophages. The results indicated that atazanavir significantly increased the half-life of the mRNAs of TNF-α (from 0.8 h to 1.5 h) and IL-6 (from 0.8 h to 2 h), but amprenavir had no significant effect (Fig. 1B).

3.2. Atazanavir increased cytoplasmic HuR levels

Previous studies have shown that HuR binds to the 3′-UTR regions of TNF-α and IL-6 in response to inflammatory stimuli in macrophages [9]. HuR primarily resides in the nucleus in resting cells, but translocates to the cytoplasm in response to a number of stimuli, such as LPS and ethanol [8]. We initially investigated whether the different effects of amprenavir and atazanavir on TNF-α and IL-6 release were correlated with the different effects on the intracellular localization of HuR. As shown in Fig. 2A, atazanavir increased cytoplasmic HuR levels and correspondingly decreased nuclear HuR levels; total cellular HuR levels were unchanged. In contrast, amprenavir had no significant effect on HuR translocation. Atazanavir-induced cytoplasmic translocation of HuR was further confirmed by immunofluorescent staining (Fig. 2B).

To further determine whether atazanavir-induced cytoplasmic translocation of HuR was associated with increased TNF-α and IL-6 mRNA binding activity, we carried out two experiments. First, we used biotinylated 3′-UTRs of TNF-α and IL-6 to pull down endogenous cytoplasmic HuR. Cytoplasmic HuR bound to biotinylated 3′-UTRs of TNF-α and IL-6 was detected by Western blotting. As shown in Fig. 3, atazanavir significantly increased binding of HuR to 3′-UTRs of TNF-α and IL-6, while amprenavir had no effect and such assays failed to pull down HuR when using biotinylated coding regions (CRs) of TNF-α and IL-6. Second, we examined the effect of atazanavir and amprenavir on in vivo association of endogenous TNF-α and IL-6 mRNAs with HuR by IP using cytoplasmic lysates from HIV PI-treated cells or vehicle control (DMSO) treated cells. The mRNAs associated with HuR were measured by real-time RT-PCR. As shown in Fig. 4, the mRNAs of TNF-α and IL-6 were more abundant in the IP material obtained from atazanavir-treated cells after HuR IP, compared with their abundance in control IgG1 IP. But amprenavir had no significant effect.

3.3. HuR silencing reduced atazanavir-induced expression of TNF-α and IL-6

In order to directly test the involvement of HuR in atazanavir-induced expression of TNF-α and IL-6, we constructed lentiviral siRNA directly targeting HuR to knock down HuR expression in macrophages. As shown in Fig. 5A, this lentiviral siRNA effectively down-regulated HuR expression after 48 h transduction (~90%). This intervention prevented atazanavir-induced increase of mRNA and protein expression of TNF-α and IL-6 (Fig. 5B and C). Consistently, atazanavir-induced stabilization of TNF-α and IL-6 mRNAs was also abolished by silencing HuR (Fig. 5D and E). These results suggest that HIV PI-induced cytoplasmic translocation of HuR represents a key cellular mechanism of HIV PI-induced inflammatory cytokine release in macrophages.
Fig. 1. Effect of HIV PIs on TNF-α and IL-6 expression and mRNA stability in mouse J774A.1 macrophages. (A) Effect of HIV PIs on TNF-α and IL-6 expression. Mouse J774A.1 macrophages were incubated with different concentrations of HIV PIs (0–50 μM): (a) amprenavir; (b) atazanavir; (c) indinavir; (d) lopinavir; (e) nelfinavir; (f) ritonavir for 24 h. The vehicle control group was treated with the same volume of DMSO. At the end of incubation, the culture media and cells were collected separately. The amounts of TNF-α and IL-6 released to the media were analyzed by ELISA as described under Section 2 and expressed as percent of vehicle control. The TNF-α amount of vehicle control was 0.45 ng ± 0.064 ng/mg protein; the IL-6 amount of vehicle control was 11.45 ± 0.96 ng/mg protein. Each bar represents mean ± S.E. of three independent experiments. Statistical significance relative to vehicle control, *p < 0.05. (B) Effect of HIV PIs on mRNA stability of TNF-α and IL-6. Mouse J774A.1 macrophages were treated with vehicle control (DMSO), AMPV (15 μM) or ATZV (15 μM) in the presence of actinomycin D (2.5 μg/ml). Total cellular RNA was isolated at 0, 1, 2, and 4 h, and the remaining mRNA levels of TNF-α and IL-6 were determined by real-time quantitative RT-PCR as described in Section 2. Values are the means ± S.E. from three independent experiments: (a) TNF-α mRNA and (b) IL-6 mRNA.

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Fig. 2. Effects of HIV PIs on HuR cellular distribution in mouse J774A.1 macrophages. Mouse J774A.1 macrophages were treated with 15 μM of HIV PIs for 24 h. Total cell lysates, cytoplasmic and nuclear proteins were prepared for Western blot analysis as described in Section 2. (A) Representative Western blots: (a) cytoplasmic proteins; (b) nuclear proteins; (c) total cell lysates. β-Actin and lamin B were used as loading controls for total and cytoplasmic proteins, and nuclear proteins. (B) Fluorescent confocal images of cellular distribution of HuR. Cells were treated with (a) Vehicle control (DMSO); (b) amprenavir (AMPV, 15 μM); (c) atazanavir (ATZV, 15 μM) for 24 h; (d) negative control of the secondary antibody staining. Expression of HuR was detected with mouse monoclonal antibody against HuR and donkey anti-mouse antibody conjugated with Alex Fluor-594. Nuclei were stained with DAPI. HuR expression was visualized under a confocal fluorescence microscope with a 63× oil immersion objective using a dual filter set for DAPI and rhodamine. Three experiments were performed that showed similar results.

4. Discussion

There are currently nine different HIV PIs approved for the treatment of HIV infection. Most HIV PIs are classified as peptidomimetic inhibitors in structure [19]. Accumulating clinical evidence indicates that HAART has changed the clinical profile of HIV infection from a sub-acute lethal disease to a chronic ambulatory disease. HIV PI-induced atherosclerotic cardiovascular disease has become an important cause of morbidity and mortality in HIV-infected patients [20,21]. Although the mechanism underlying HIV PI-induced atherosclerosis remains to be fully identified, abundant evidence from cell culture experiments and animal studies, along with clinical studies has clearly indicated...
that individual HIV PIs differ in their ability to cause dyslipidemia, and multiple mechanisms may be involved in HIV PI-associated metabolic syndrome [22].

Our previous studies have shown that HIV PIs activated the UPR, induced accumulation of intracellular free cholesterol and lipids, and promoted foam cell formation, both in normal cholesterol-loaded macrophages and Ac-LDL-loaded macrophages. These observations represent a potential mechanism by which HIV PIs may promote atherosclerosis and cardiovascular diseases [7,23]. Recently, we also demonstrated that HIV PIs activate the UPR and disrupt lipid metabolism in hepatocytes [24].

Atherosclerosis is a chronic inflammatory disease. There is growing evidence that inflammation plays a pivotal role in the development of atherosclerosis. Both local inflammation in the arterial walls and the systemic inflammatory response are associated with atherosclerosis [5]. The major risk factors for atherogenesis, such as dyslipidemia, insulin resistance and lipodystrophy also contribute to inflammatory conditions by increasing secretion of pro-inflammatory cytokines, such as TNF-α and IL-6. In addition to augmenting the local inflammatory response in atherosclerotic plaques, TNF-α also systemically affects a number of mediators of the atherosclerotic process and perturbs lipid homeostasis [4,5].

It has been demonstrated that most of the inflammatory cytokines present in the advanced atherosclerotic lesions are secreted by macrophages [4]. Recent studies done by Feng et al. and Li et al. have shown that free cholesterol loading activates the UPR and induces the synthesis and secretion of two important inflammatory cytokines, TNF-α and IL-6, through NF-κB, JNK and MAP kinase pathways in macrophages [6,25]. The results presented in Fig. 1 show clearly that individual HIV PIs also vary in their ability to increase TNF-α and IL-6 expression in macrophages, which is correlated to their ability to activate the UPR. Atazanavir has the most significant effect, while amprenavir has no effect at all.

Production of inflammatory cytokines is a tightly regulated process. Post-transcriptional regulation is emerging as a critical control point for labile cytokine biosynthesis [18]. Among the post-transcriptional regulatory events, modulation of mRNA stability plays an important role in the control of cytokine gene expression. A significant amount of literature has been devoted to understanding the mechanisms that regulate cytokine mRNA stability in various cell types, and the role of RNA-binding proteins in this process.

Fig. 3. Binding of cytoplasmic HuR to TNF-α and IL-6 transcripts in mouse J774A.1 macrophages. Mouse J774A.1 macrophages were treated with vehicle control (DMSO), amprenavir (AMPV, 15 μM) or atazanavir (ATZV, 15 μM) for 24 h. Cytoplasmic lysates were prepared as described under Section 2. One hundred and twenty micrograms proteins of each lysate were used to incubate with 25 nmol of biotinylated TNF-α or IL-6 3′-UTR or CR (coding region) for 30 min at 25 °C, and the resulting RNP complexes were pulled down by using streptavidin-coated beads. Representative HuR immunoblots of the pull down materials from (A) 3′-UTR and CR of TNF-α; (B) 3′-UTR and CR of IL-6. Three experiments were performed that showed similar results.
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erature has indicated the involvement of the RNA binding protein HuR in modulation of TNF-α and IL-6 mRNA stability [8,18]. HuR, a ubiquitously expressed member of the Elav family of RNA-binding proteins, is a potential trans-acting factor in RNA stability. HuR has been shown to bind with high affinity and specificity to the 3′-UTR U- and A-rich sequences (AREs) in a variety of mRNAs [26]. Although the precise mechanisms regulating HuR expression are still unclear, its cytoplasmic localization appears to be crucial for its function. Under nonstressed conditions, HuR is predominantly present in the nucleus; in response to various stress signals, HuR can be exported to the cytoplasm. It is currently believed that after binding to its target mRNAs in the nucleus, the HuR–mRNA complex is transported to the cytoplasm where HuR prevents the degradation of bound mRNA [9,27].

In the present study, we provide strong evidence that stabilization of mRNAs contributes to the HIV PI-induced TNF-α and IL-6 expression in macrophages and HuR is a key regulator of this process. HIV PI, atazanavir, significantly increased TNF-α and IL-6 mRNA stability (Fig. 1B) and induced intracellular translocation of HuR from nucleus to cytoplasm (Fig. 2). Consistent with previous reports, our data indicate that HuR binds to the 3′-UTRs of TNF-α and IL-6 mRNAs and this binding is significantly increased by HIV PI, atazanavir (Fig. 3). IP assays demonstrated the in vivo association of TNF-α and IL-6 mRNAs with HuR, which is also enhanced by atazanavir (Fig. 4). Furthermore, the RNA interference data presented in Fig. 5 showed that knock down of HuR expression by siRNA abrogated atazanavir-induced increase of TNF-α and IL-6 expression and mRNA stabilization. These observations provide strong support for the hypothesis that HuR is involved in the HIV PI-induced inflammatory cytokine expression in macrophages. However, how HIV PIs regulate HuR function remains to be further investigated.

It has been demonstrated that AMP-activated protein kinase (AMPK) and mitogen-activated protein kinases (MAPKs), p38 and ERK, are involved in the regulation of TNF-α mRNA stability and HuR function [11,26,28]. Activation of ERK and p38 MAPKs enhanced the cytoplasmic presence of HuR, while activation of AMPK reduced the cytoplasmic abundance of HuR [26,28]. It has also been shown that ER stress signaling pathways are involved in ERK activation and regulation of TNF-α and IL-6 expression in macrophages [6]. Our preliminary results indicate that HIV PIs activate the ERK and JNK, but not p38 MAPK. HIV PI-induced TNF-α and IL-6 protein increase can be blocked by ERK specific inhibitor (unpublished data). We also have preliminary evidence suggesting the involvement of ERK activation in HIV PI-induced HuR translocation. As shown in online supplemental data Fig. 1, atazanavir-induced ERK activation. Atazanavir-induced increase of cytoplasmic HuR was inhibited by ERK specific inhibitor, PD98059 (online supplemental data Fig. 2). The precise mechanisms by which HIV PIs modulate ERK activation and HuR function have not been identified and are the focus of our ongoing studies.

Taken together, the current findings and our previous studies [7] demonstrate that HIV PIs vary greatly in their ability to activate the UPR, increase inflammatory cytokine release and promote foam cell formation in macrophages. Activation of the UPR further increases the synthesis of pro-inflammatory cytokines. Post-transcriptional modulation of TNF-α and IL-6 represents an important cellular mechanism by which HIV PIs promote inflammatory response. These results provide important information for dissecting the mechanisms of the HIV PI-associated metabolic abnormalities in patients taking these drugs. Activation of the UPR, accumulation of intracellular free cholesterol and lipids, and the increase of inflammatory cytokine synthesis may represent the key cellular events by which HIV PIs alter lipid homeostasis and promote atherogenesis in HIV patients undergoing HAART. A better understanding of the cellular and molecular mechanisms of the HIV PI-induced metabolic abnormality may provide useful information for the development of new drugs and therapeutic strategies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2007.04.008.

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