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Original Article

Proatherogenic stimuli induce HuR in atherosclerosis through MAPK/ErK pathway

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Abstract: Atherosclerosis is a chronic inflammatory disease inflicting the arterial wall, and endothelial activation and dysfunction play an important role in its pathogenesis. The RNA-binding protein HuR has been associated with events of inflammation and activation in endothelial cells, however, its connection with atherosclerosis remains unclear. Here, we show that the expression and RNA-binding activity of HuR are upregulated in human and mouse atherosclerotic lesions. In addition, proatherogenic stimuli, such as inflammatory lipids (Ox-PAPC) and cytokines (TNF-α and IL-1β), induce HuR in human aortic endothelial cells (HAECs) in vitro. Moreover, HuR is also induced in mouse aorta ECs fed a high-fat diet, and the inducible degree is correlated with proatherogenic hyperlipidemia. We further show that the MAPK/ErK pathway in ECs is activated by proatherogenic stimuli in vitro and by high-fat diet in vivo. Finally, we demonstrate that the MAPK/ErK pathway is required for HuR induction by proatherogenic stimuli. Altogether, our study uncovers the inducible effect of proatherogenic stimuli on HuR in ECs, and connects this effect to the activated MAPK/ErK pathway.

Keywords: Endothelium, HuR, atherosclerosis, proatherogenic stimuli, MAPK/ErK pathway

Introduction

Atherosclerosis, a chronic pathological process of artery wall, represents the main breading ground for cardiovascular diseases, and is a leading cause of morbidity and mortality worldwide. The maintenance of homeostasis in arterial endothelium plays an important role in preventing vascular pathology, and the endothelial dysfunction is closely linked to vascular diseases, including atherosclerosis [1]. Endothelial activation, a cellular status prelude to its dysfunction, is generally stimulated by proatherogenic factors like oxidized lipids, TNF-α and IL-1β, leading to induction of proinflammatory signaling responses and upregulated expression of surface adhesion molecules [2]. These pathological events synergistically stimulate an increase in lipid permeability and leucocyte recruitment to the arterial wall, which would cause intimal lipid accumulation and local inflammation that eventually promote the initiation and progression of atherosclerosis [3].

Human antigen R (HuR), also known as ELAV1, belongs to the ELAV (embryonic lethal abnormal vision) RNA-binding protein (RBP) family, which recognizes and binds 3' UTRs of mRNAs containing AU-rich element (ARE)- and U-rich element (URE) to regulate their stability and translation [4]. For fulfilling post-transcriptional control of target mRNAs, HuR is required to translocate from nucleus into the cytoplasm upon cellular perturbation [5]. HuR is ubiquitously expressed and its deficiency is embryonic lethal [6]. Presumably, due to its wide-spectrum targets, HuR is able to exhibit pleotropic effects, such as those implicated in cell cycle, apoptosis, tumorigenesis and angiogenesis [7]. Recently, HuR has been shown to mediate endothelial activation by suppressing endothelial nitric oxide synthase (eNOS) [8]. Another study has demonstrated that HuR controls the mRNA stability and expression of *CTSS*, which encodes a cysteine protease linked to atherosclerosis [9]. These observations hint that HuR might associate with atherosclerosis. However, to date, the role of HuR in atherosclerosis has not been determined. In the current study, we report the MAPK/ErK pathway-mediated regulation of HuR in aortic endothelial cells by pro-

atherogenic stimuli in vitro and in vivo, which might point to a potential connection between HuR and atherogenesis.

Materials and methods

Reagents and antibodies

The antibodies and reagents were purchased from the following sources: HuR (3A2) (Santa Cruz, sc-5261), SMCα-actin (Sigma, A2547), VEGFR2 (Cell Signaling, #2472), β-actin (AC-15) (Santa Cruz, sc-69879), SM22 (Santa Cruz, sc-373928), calponin (EP798Y) (abcam, ab46794), γ-Tubulin (GUT-88) (abcam, ab11-316), goat anti-rabbit IgG-HRP (Santa Cruz, sc-2004), goat anti-mouse IgG-HRP (Santa Cruz, sc-2302), goat anti-rat IgG-HRP (Millipore, AP136P); collagenase I (Sigma, C9891), collagenase II (Sigma, C6885) collagenase XI (Sigma, C7657), DNase I (Sigma, D5307), hyaluronidase (Sigma, H3506), recombinant IL1β (R&D System, NP-000567), recombinant TNF-α (R&D System, P01375), Ox-PAPC (InvivoGen, tlrl-oxp1); SB230580 (Sigma, S8307), PD980-59 (Sigma, P215), SP600125 (Sigma, S5567), stattic (Sigma, S7947).

Human coronary artery sampling

Coronary arteries were obtained from freshly removed hearts during transplant surgery at The Second Affiliated Hospital of Harbin Medical University. The tissue sampling and following cell isolation procedures and the informed consent were all in accordance with the terms of the Medical Ethical Committee of The Second Affiliated Hospital of Harbin Medical University. Samples of atherosclerotic coronary arteries were obtained from coronary artery disease (CAD) patients, while non-atherosclerotic samples were obtained from patients with dilated myocardiopathy that required heart transplantation surgery. Immediately after surgical resection, arteries were dissected, cut as rings approximately with 0.5 cm length, immersed in PBS, and deprived of connective and fat tissues under a low-magnification stereomicroscope. Samples were classified as atherosclerotic or nonatherosclerotic according to the presence or absence of fibrofatty tissue or visible plaques. After labeling, samples were immediately frozen in liquid nitrogen and stored at -80°C for further extracting total protein and RNA or used for enzymatic experiments.

Animals and diet

All mice were maintained under a specific pathogen-free condition at the facilities of The Second Affiliated Hospital of Harbin Medical University, and all procedures of animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees of The Second Affiliated Hospital of Harbin Medical University for animal welfare. The sacrifice of mice was euthanized by CO_2 inhalation. The transgenic mouse strain ApoE$^{-/-}$ (*B6.129P2-Apoe^{tm1Unc}/J*) was obtained from the Jackson Laboratory. Eight-week-old wild-type and ApoE$^{-/-}$ mice were fed a HFD diet (containing 10.8% total fat and 0.75% cholesterol) (S8492-E010, Ssniff, Germany) or chow diet for 16 weeks according to different experimental purposes.

Serum lipid detection

Serum samples were obtained from the whole blood collected with an intra-cardiac needle from the fasted mice. The concentration of the total cholesterol, HDL and LDL cholesterol was determined by a colorimetric enzymatic assay using Biochemistry Analyzer (ADVIA 1650, Bayer, Tarrytown NY, USA) according to the manufacturer's instructions.

Isolation of human and mouse aortic endothelial cells

Human aortic ECs (HAECs) were isolated from aortic explants of donors receiving heart transplant surgery as mentioned above. Mouse aortic ECs (MAECs) were isolated from C57BL/6 wild-type mice. The isolation of high purity of HAECs was conduced with the selective enzymatic digestion as previously described [10, 11]. Briefly, the aorta lumen was filled with 1 mg/mL collagenase II (Sigma) using a 24-gauge cannula, and incubated for 1 hour at 37°C. ECs were removed from the aorta by flushing with DMEM containing 20% FBS. After culture for 1 week in complete medium (DMEM 20% FBS, penicillin-streptomycin, glutamine, NEAA, sodium pyruvate, HEPES, heparin and ECGS), ECs were purified with mouse-anti-human CD102 (BD Pharmingen) and sheep-anti-mouse coated magnetic beads (Life Technologies) or rat-anti-mouse-CD102 (BD Pharmingen) and sheep-anti-rat coated magnetic beads (Life Technologies). The purity of ECs was confirmed by FACS analysis.

Culture and treatment of HAECs

HAECs were maintained in gelatin-coated plates in complete endothelial medium (DMEM containing 20% FBS, penicillin-streptomycin, glutamine, NEAA, sodium pyruvate, HEPES, heparin and ECGS). Confluent HAECs cultured in complete Medium 199 (Corning) supplemented with 1% heat-inactivated fetal bovine serum and 1% antibiotics were treated with Ox-PAPC as previously described [12], 5 ng/ml or 10 ng/ml recombinant human TNF, 5 ng/ml or 10 ng/ml IL-1β, 25 μg/ml or 50 μg/ml dose of Ox-PAPC were utilized based on our pilot experiments and other report [13]. Within this dosage range, Ox-PAPC consistently shows atherogenic effect. For pharmacological inhibitor screening, confluent HAECs were treated with vehicle control or recombinant IL1β, TNF-α, Ox-PAPC in the presence or absence of 10 μM pharmacological inhibitors like SB230580, PD98059, SP600125 or Stattic for different periods according to experimental purposes.

Transfections and siRNA-mediated knockdown

In vitro cultured HAECs were transfected with small interfering RNA using 40 nM siRNA and reagent Lipofectamine RNAimax (Invitrogen) twice at day 1 and day 2 and further cultured for total 3 days according to the manufacturer's instructions. siRNA targeting luciferase with same concentration was used as a negative control.

Western blotting analysis

For tissue samples, proteins were initially extracted with ultrasonication process in RIPA buffer with protease inhibitors (Sigma) on ice. For isolated or cultured cells, proteins were directly secreted in lysed cells using RIPA buffer with protease inhibitors (Sigma) on ice for 10 min. Protein samples in supernatants were denatured in 1 × loading buffer. Equal amount of proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membrane (Millipore), blocked by 5% skimmed milk (BD Difco) in TBS supplemented with 0.1% Tween (TBST) before probed with primary antibodies at 4°C overnight. Membranes were washed with TBST for three times and incubated with secondary antibodies at room temperature for 1 hour. After the repeated wash in TBST, membranes were incubated with the enhanced chemiluminescence (GE Healthcare, RPN2209) for protein detection visualized by GE ImageQuant LAS 4000 instrument. Western bands were quantified using ImageJ software.

RNA purification and real-time reverse transcriptase quantitative PCR

Homogenization of tissue or cell and the following total RNA isolation with Trizol-based method (Thermo Fisher Scientific, #15596026) were conducted based on the manufacturer's instructions. mRNA levels were determined by SYBR green real-time PCR kit (TakaRa, #RR-420A) with the 7500 Real-Time PCR System (Applied Biosystems, USA). Data were analyzed using the comparative Ct method with *Actin* as an endogenous control throughout. Sequences of primers probing human or mouse genes are available upon request.

RNA immunoprecipitation

RNA immunoprecipitation (RIP) studies were carried out as described previously [14], and a MagnaRIP Kit (Millipore) was utilized. Briefly, cellular inclusion of arterial tissues was extracted with ultrasonication process using RIP lysis buffer (10 mM HEPES, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 0.5% NP-40, 1 mM DTT) for 40 min on ice; For cultured cell samples, cells were directly lysed with RIP lysis buffer. The obtained lysates were incubated at 4°C overnight with magnetic protein A-protein G beads (Sigma) coupled with 5 μg normal mouse IgG (Millipore) or HuR monoclonal antibody (Santa Cruz, sc-5261) to gain RNA-protein immunocomplexes. Beads were then washed for three times and incubated with proteinase K buffer (Millipore) for 30 min at 55°C, followed by RNA isolation from the immunoprecipitates according to the manufacturer's protocol (Millipore). cDNA was prepared in each sample by First-strand cDNA Synthesis System (Thermo scientific). qRT-PCR was performed by amplifying the 300-bp region in the 3' UTR of each transcript. 3' UTR of GAPDH was amplified as a negative control throughout. Sequences of primers are available upon request.

Statistical analysis

All statistical analyses were performed with GraphPad Prism 6. Data are representative of at least 3 independent experiments and

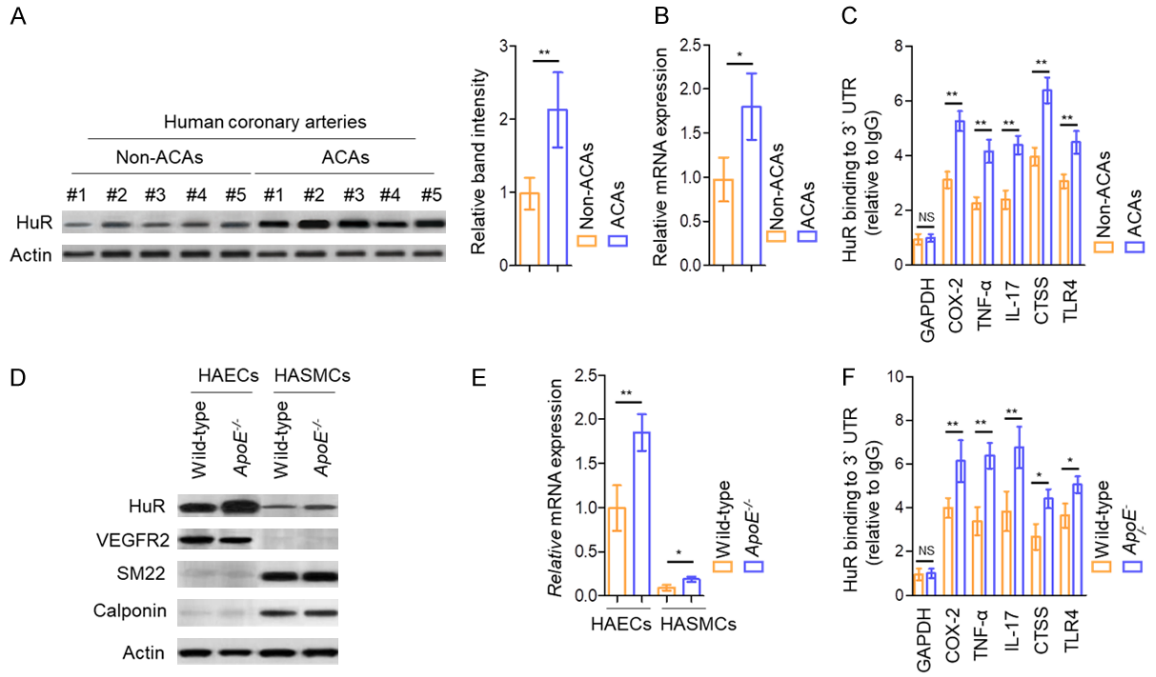

Figure 1. HuR expression and its RNA-binding activity are upregulated in human and mouse atherosclerosis. (A) Western blot analysis of protein level of HuR in arterial extracts from non-atherosclerotic or atherosclerotic human coronary arteries (n = 5). Human Actin was used as a loading control. The representative images (left) and statistical analysis of band intensity (right) are shown. (B) qRT-PCR analysis of mRNA level of *HuR* in arterial extracts from non-atherosclerotic (n = 9) or atherosclerotic (n = 12) human coronary arteries. The results represent the mean value of 3 replicates and are normalized to human Actin. (C) Arterial extracts from non-atherosclerotic or atherosclerotic human coronary arteries were immunoprecipitated by HuR antibody or isotype IgG control antibody (n = 5). Total RNAs were purified from immunoprecipitates and reversely transcribed to cDNA and analyzed by qRT-PCR to measure the level of transcripts known to be associated with HuR, including COX-2, TNF-sure the level of transcripts known to be associated with than internal negative control. The results represent the mean value of 3 replicates. The enrichment value relative to IgG group for each transcript is normalized to human GAPDH. (D) Western blot analysis of protein level of HuR in HAECs and HASMCs isolated from arteries of 24-week-old C57BL/6 wild-type mice (n = 5) or ApoE$^{-/-}$ mice (n = 5) fed a chow diet. VEGFR2, SM22 and Calponin were used as biomarkers. Representative images are shown. (E) qRT-PCR analysis of mRNA level of *HuR* in arterial extracts from 24-week-old C57BL/6 wild-type mice (n = 5) or ApoE$^{-/-}$ mice (n = 5) fed a chow diet. The results represent the mean value of 3 replicates and are normalized to mouse Actin. (F) As described in (C), arterial extracts from 24-week-old C57BL/6 wild-type mice or ApoE$^{-/-}$ mice fed a chow diet were subjected to in vitro RNA immunoprecipitation assay (n = 5). The enrichment value relative to IgG group for each transcript is further normalized to mouse GAPDH. The statistical analysis was performed using unpaired Student's *t*-test. Data are mean ± s.e.m. **, P < 0.01; *, P < 0.05; NS, not significant.

expressed as mean ± s.e.m. Statistical analysis was assessed by unpaired Student's *t*-test, unless indicated otherwise. Values with P < 0.01 or P < 0.05 indicate a statistically significant difference, NS represents nonsignificant difference.

Results

Increased expression and RNA-binding activity of HuR in human and mouse atherosclerosis

To explore the role of HuR associated with atherosclerosis, we first examined the clinical relevance by comparing HuR expression levels between human non-atherosclerotic coronary arteries (non-ACAs) and atherosclerotic coronary arteries (ACAs). Western blot analysis showed a significant increase in HuR protein level in ACAs obtained from coronary artery disease (CAD) patients compared with non-ACAs collected from patients with dilated myocardiopathy (2.16-fold; *P* < 0.01; **Figure 1A**). In concert, its mRNA level was also upregulated in ACAs, as compared with non-ACAs (1.85-fold; *P* < 0.05; **Figure 1B**). In addition, to test whether the RNA-binding activity is also affected, we measured the level of commonly known mRNAs bound and stabilized by HuR, such as COX-2 [15], TNFα [16], IL-17 [17], CTSS [9] and TLR4

[18], through performing RNA immunoprecipitation assay with lysates of non-ACAs and ACAs. As shown in **Figure 1C**, the levels of COX-2, TNFα, IL-17, CTSS and TLR4 associated with HuR were all increased in ACAs compared with non-ACAs, and these target mRNAs were not present in IgG immunoprecipitate but manifested striking enrichment compared with the mRNA of non-target GAPDH, indicating that the RNA-binding activity of HuR was indeed enhanced in ACAs. Together, these lines of evidence imply a clinical relevance of HuR upregulation to human atherosclerosis.

Next, we investigated HuR in mouse atherosclerotic model. Since atherosclerosis-prone apolipoprotein E-deficient (ApoE$^{-/-}$) mice spontaneously develop advanced atherosclerosis after 15 weeks of age [19], we therefore measured the HuR expression in aortic root cryosections from 24-week-old C57BL/6 wild-type mice and ApoE$^{-/-}$ mice fed a chow diet. To better relate HuR protein in aortic roots to atherosclerotic lesions, we checked its expression in isolated endothelial cells (ECs) and smooth muscle cells (SMCs), respectively. The results showed *en masse* increased HuR expression in ApoE$^{-/-}$ mice compared with wild-type mice (**Figure 1D**). Noticeably, although HuR was present in both ECs and SMCs, probably indicating its fundamental role in vascular biology [6], a large portion of HuR appeared in ECs (**Figure 1D**). This may imply a heterogeneous functional assignment for HuR in different regions of artery, especially in endothelium at least in our experimental condition. Consistent with these results, the transcript of HuR was preferably expressed in ECs and upregulated in arteries of ApoE$^{-/-}$ mice compared with that of wild-type mice (**Figure 1E**). Moreover, similar to the results obtained from human samples (**Figure 1C**), RNA immunoprecipitation analysis showed that the RNA-binding activity of HuR was also enhanced in arteries of atherosclerotic ApoE$^{-/-}$ mice compared with non-atherosclerotic wild-type mice (**Figure 1F**). Collectively, these data reveal the increased expression and RNA-binding activity of HuR in both human and mouse atherosclerosis, and also suggest a possible role that HuR may play during atherogenesis.

Proatherogenic stimuli induce HuR expression and activity in vitro

To understand how HuR expression is regulated in human aortic cells, we utilized human aortic endothelial cells (HAECs) and human aorta smooth muscle cells (HASMCs) cultured in vitro. Western blot analysis showed that HuR was more abundantly expressed in HAECs than HASMCs (**Figure 2A**), coinciding with the results observed in mouse aortic roots (**Figure 1D**). Since the dysfunction of endothelium plays a central role in atherosclerosis development and progression in human and mice [20], we therefore focused on investigating HuR in HAECs. Proatherogenic stimuli such as hyperlipidemia and inflammatory cytokines are hallmarks of atherosclerosis [21], and events that launch atherosclerosis require endothelial cell activation generally driven by these molecules such as lipid oxidation products [22], TNF-α [23] and IL-1β [24]. We hypothesized that a causal link may exist between these proatherogenic factors and HuR upregulation. Thus, in order to test it, we treated HAECs with inflammatory cytokines (TNF-α and IL-1β) or inflammatory lipids (oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine, Ox-PAPC), which strongly activate proatherogenic pathways in HAECs [25]. The results showed that both the mRNA (**Figure 2B**) and protein (**Figure 2C**) levels of HuR were induced upon the treatment of TNF-α and IL-1β in a dose-dependent manner. Similar to these results, Ox-PAPC treatment also induced the mRNA (**Figure 2D**) and protein (**Figure 2E**) expression of HuR. We also detected the effects of these proatherogenic factors on the RNA-binding activity of HuR. Intriguingly, the RNA-binding activity of HuR in HAECs was also enhanced by IL-1β or Ox-PAPC treatment (**Figure 2F**). These results indicate that proatherogenic stimuli induce HuR expression and activity in HAECs in vitro.

Proatherogenic hyperlipidemia induces HuR expression and activity in vivo

The observations of HuR upregulation in human and mouse atherosclerotic lesions do not clarify their underlying causal relationship. As far as we know, at the initial stage of atherosclerosis development, the disruption of endothelial function is often preceded by a status of 'activation', in which hyperlipidemia plays a fundamental role [26]. Considering a possibility that HuR upregulation may occur synchronously with atherogenesis, we thus asked whether hyperlipidemia is a stimulating factor of endothelial HuR expression in arteries in vivo. We found that compared with mice fed a standard

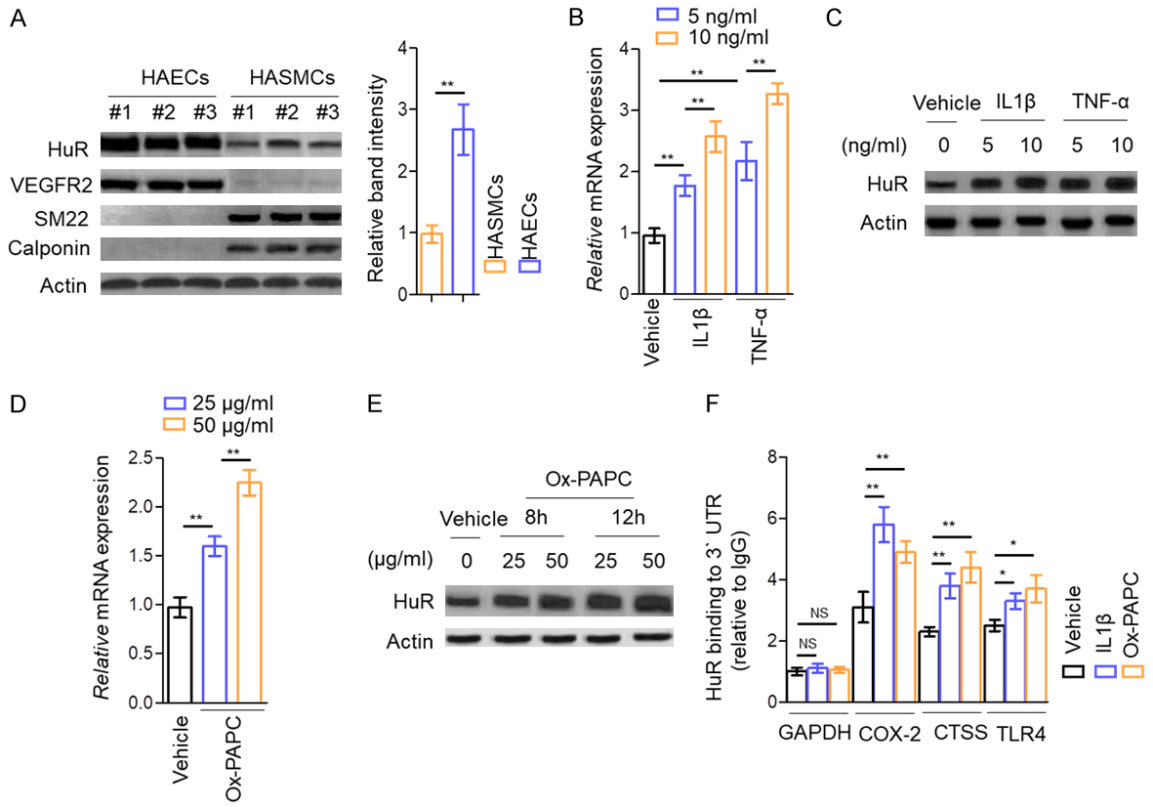

Figure 2. Proatherogenic stimuli induce HuR expression and activity in vitro. (A) Western blot analysis of protein level of indicated proteins in cultured HAECs and HASMCs (n = 3). VEGFR2, SM22 and calponin were used as marker proteins. Human Actin was used as a loading control. The representative images (left) and statistical analysis of HuR band intensity (right) are shown. (B, C) Cultured HAECs were treated with vehicle or different concentrations of recombinant IL1β or TNF-α for 8 hours. (B) The mRNA level of HuR was measured by qRT-PCR analysis. The results represent the mean value of 3 replicates and are normalized to human Actin. (C) The protein level of HuR was measured by Western blot. Human Actin was used as a loading control and the representative images are shown. (D, E) Cultured HAECs were treated with vehicle control or different concentrations of Ox-PAPC for 8 or 12 hours. (D) The mRNA level and (E) protein level of HuR were determined as in (B, C). (F) HAECs were treated with vehicle control, 10 ng/ml recombinant IL1β or 50 μg/ml Ox-PAPC and cultured for 8 hours. Cell extracts were subjected to in vitro RNA immunoprecipitation assay as depicted in (**Figure 1C** and **1F**). The enrichment value relative to IgG group for each transcript is normalized to GAPDH. The statistical analysis was performed using unpaired Student's *t*-test. Data are mean ± s.e.m. **, P < 0.01; *, P < 0.05; NS, not significant.

diet (chow), mice had higher circulating cholesterol level in plasma when fed a HFD for 4, 7 or 10 days (**Figure 3A**). Meanwhile, qTR-PCR analysis of isolated aortic ECs revealed that a HFD diet significantly increased HuR expression (**Figure 3B**). When analyzing the pooling data of cholesterol level and HuR expression, the mRNA level of endothelial HuR was found positively correlated well with the circulating cholesterol level (R^2 = 0.1506; P = 0.0133; **Figure 3C**). Moreover, the protein expression (**Figure 3D**) and RNA-binding activity (**Figure 3E**) of HuR were both upregulated in isolated aortic ECs from mice fed a HFD diet for different days compared with those fed a chow diet. In sum, these data not only suggest that hyperlipidemia may positively modulate HuR expression in aortic ECs in vivo, but also suggest an early regulation of HuR in response to proatherogenic hyperlipidemia.

MAPK/ErK pathway is activated by proatherogenic stimuli

To discover the possible intracellular signaling pathway that mediates HuR upregulation induced by different proatherogenic stimuli, we particularly paid attention to the p44/p42 mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ErK) pathway, since many RNA-binding proteins were reported to be regulated by this pathway when their

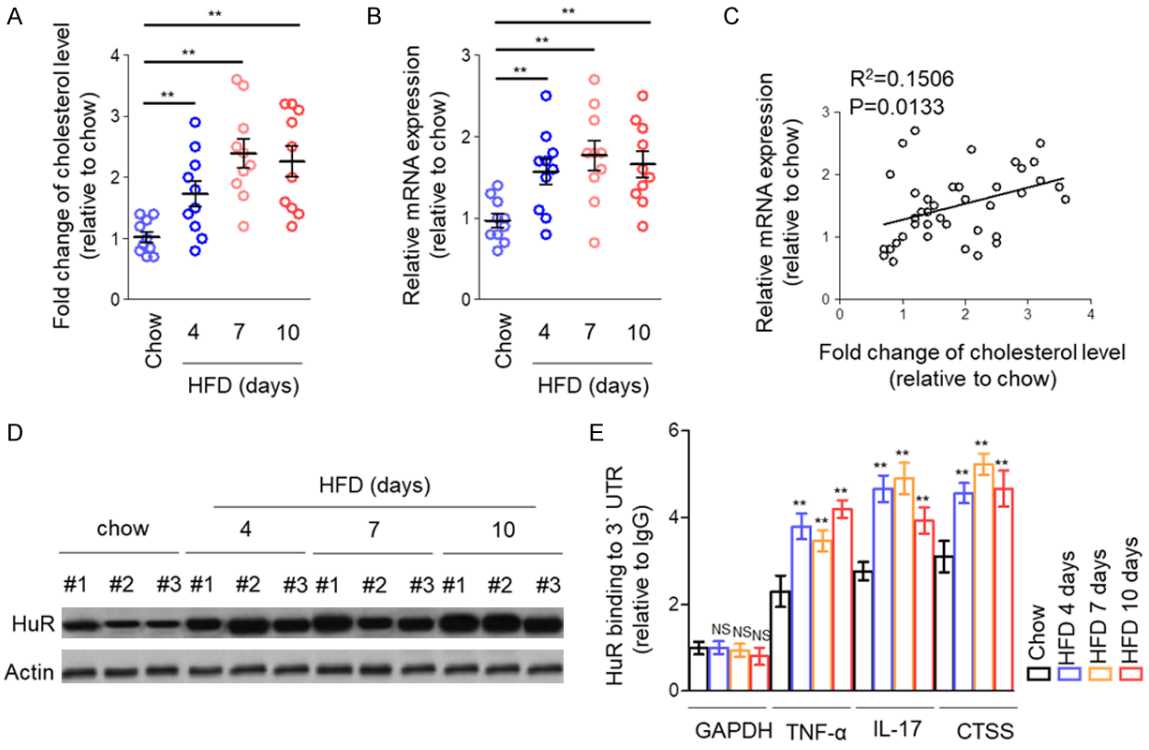

Figure 3. Proatherogenic stimuli induce HuR expression and activity in vivo. (A) The circulating cholesterol level of C57BL/6J wild-type mice fed a chow diet or HFD diet for 4, 7 or 10 days followed by an overnight fast treatment. (B) Mice were treated as in (A), qRT-PCR was performed to determine the mRNA level of HuR in ECs isolated from the descending aorta. In (A and B), each group contains 10 mice and each symbol represents the mean value of 3 replicates for every sample. The data are represented as fold change to the mean value of chow group. (C) The circulating cholesterol level from (A) and mRNA level of HuR from (B) in each mouse were pooled together and their correlation was analyzed (n = 40). R^2 and P value are shown. (D) Mice were treated as in (A), the protein level of HuR in ECs isolated from descending aortas was analyzed by Western blot (n = 3). Mouse Actin was used as a loading control. The representative images are shown. (E) Mice were treated as in (A), ECs extracts from each group were subjected to in vitro RNA immunoprecipitation assay (n = 5). The enrichment value relative to IgG group for each transcript is normalized to mouse GAPDH. The statistical analysis was performed using unpaired Student's *t*-test. Data are mean ± s.e.m. **, P < 0.01; NS, not significant.

activity is involved in inflammation [18, 27]. To test this possibility, we first examined whether this signaling pathway is activated by proatherogenic stimuli. Indeed, the results showed that the MAPK/ErK pathway was activated by TNF-α and IL-1β (**Figure 4A**) in cultured HAECs. Similarly, Ox-PAPC treatment also activated the MAPK/ErK pathway (**Figure 4B**). In vivo, the MAPK/ErK pathway was activated in isolated aortic ECs from mice fed a HFD diet for different days compared with those fed a chow diet (**Figure 4C**). Therefore, these data indicate that the MAPK/ErK pathway can be activated by proatherogenic stimuli in vitro and in vivo.

MAPK/ErK pathway is required for proatherogenic stimuli-induced HuR expression and activity

To test whether the activated MAPK/ErK pathway is responsible for HuR induction by proatherogenic stimuli, we evaluated the reversal effect of pharmacological inhibitors targeting MAPK/ErK pathway or other pathways, which were used as controls. Among the tested inhibitors, a p38 inhibitor SB203580 and an ERK inhibitor PD98059 were found able to completely abolish the stimulating effect of TNF-α and IL-1β on HuR expression in HAECs at both mRNA (**Figure 5A**) and protein levels (**Figure 5B**). However, a JNK inhibitor SP600125 or STAT3 inhibitor Stattic did not show obvious effect (**Figure 5A**, **5B**). Similar results were obtained when HAECs were treated with Ox-PAPC (**Figure 5C**, **5D**). Of note, as shown in these results, SB203580 and PD98059 exhibited similar degree of reversal effect, which might suggest a common intracellular signaling pathway shared by different proatherogenic stimuli to specifically activate HuR expression in HAECs. Furthermore, immunofluores-

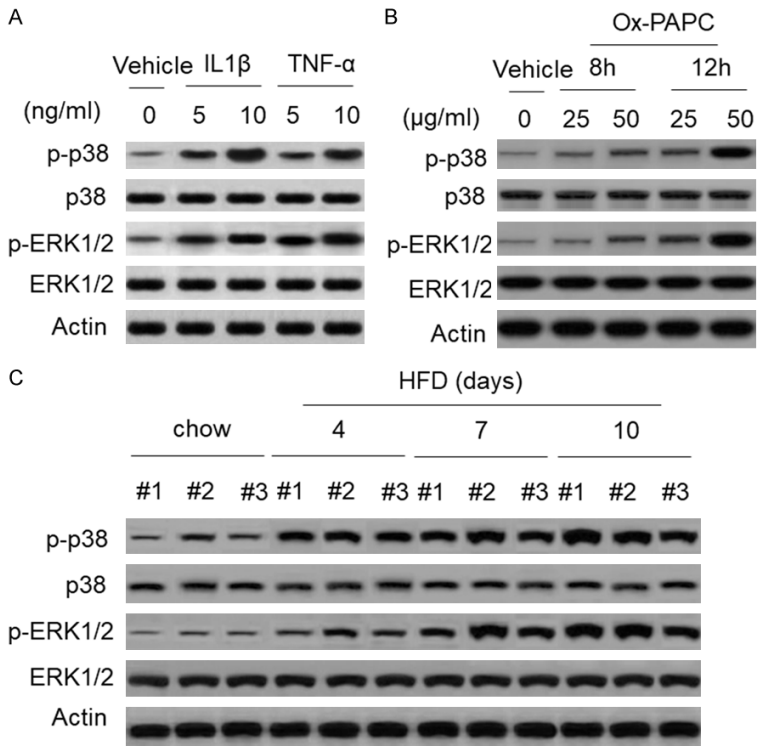

Figure 4. MAPK/ErK pathway is activated by proatherogenic stimuli. (A) Cultured HAECs were treated with vehicle or different concentrations of recombinant IL1β or TNF-α for 8 hours. Protein level was measured by Western blot analysis. Human Actin was used as a loading control and the representative images are shown. (B) Cultured HAECs were treated with vehicle or different concentrations of Ox-PAPC for 8 or 12 hours. Protein levels were detected as described in (A). (C) Mice were treated as in **Figure 3D**, protein level in ECs isolated from 3 representative descending aortas was analyzed by Western blot. Mouse Actin was used as a loading control and the representative images are shown.

cent data indicated that the proatherogenic stimuli-induced HuR expression was abrogated when MAPK/ErK pathway was inhibited by SB203580 (**Figure 5E**), confirming the conclusion that the activation of MAPK/ErK pathway is required for proatherogenic stimuli-induced HuR expression in HAECs. Intriguingly, even though SB203580 or PD98059 completely abolished the induced expression of HuR, they only partially diminished its enhanced RNA-binding activity (**Figure 5F**), implying that its activity might not be wholly dependent on its expression level and other unknown factors participate in determining its association with target mRNAs in these conditions. Considering that the MAPK/ErK pathway is activated in response to proatherogenic stimuli in vitro and in vivo, our evidence suggests that the MAPK/ErK pathway may be indispensable for the regulation of HuR by proatherogenic stimuli.

Discussion

In this study, we found the upregulated expression and enhanced activity of HuR in human and mouse atherosclerotic lesions, and that proatherogenic molecules, such as Ox-PAPC, TNF-α and IL-1β, induce HuR expression and enhance its activity in cultured HAECs, which imitates the phenotypes observed in atherosclerotic lesions. In addition, compared with a chow diet, a HFD diet induces endothelial HuR expression and activity in arteries of mice, and the degree of induction is correlated well with the level of circulating cholesterol in plasma. These results indicate a stimulatory role of proatherogenic molecules in regulating HuR in endothelial cells. Meanwhile, the MAPK/ErK pathway is also activated by proatherogenic stimuli, and inhibiting this pathway diminishes HuR induction. Taken together, our study discovers the link between proatherogenic molecules and HuR regulation in ECs in vitro and in vivo, and shows that the MAPK/ErK pathway is important for this effect.

Although we provide evidence to show the clinical relevance of HuR in atherosclerosis, given the limited sample size we enrolled, more clinical investigations with larger samples are needed to strengthen these observations. On the other hand, in view of the early emerging response of endothelial HuR to distinct proatherogenic stimuli, it seems that the aberrant upregulated expression of HuR may be useful to serve as a novel molecular signature for predicting atherosclerosis [28]. In addition, the enhanced RNA-binding activity of HuR indicates the intercellular mislocation of HuR, since the nucleocytoplasmic translocation of HuR is necessary for its activity [5], therefore, this characteristic may also be considered as an useful indicator. In fact, the dysregulation of HuR is

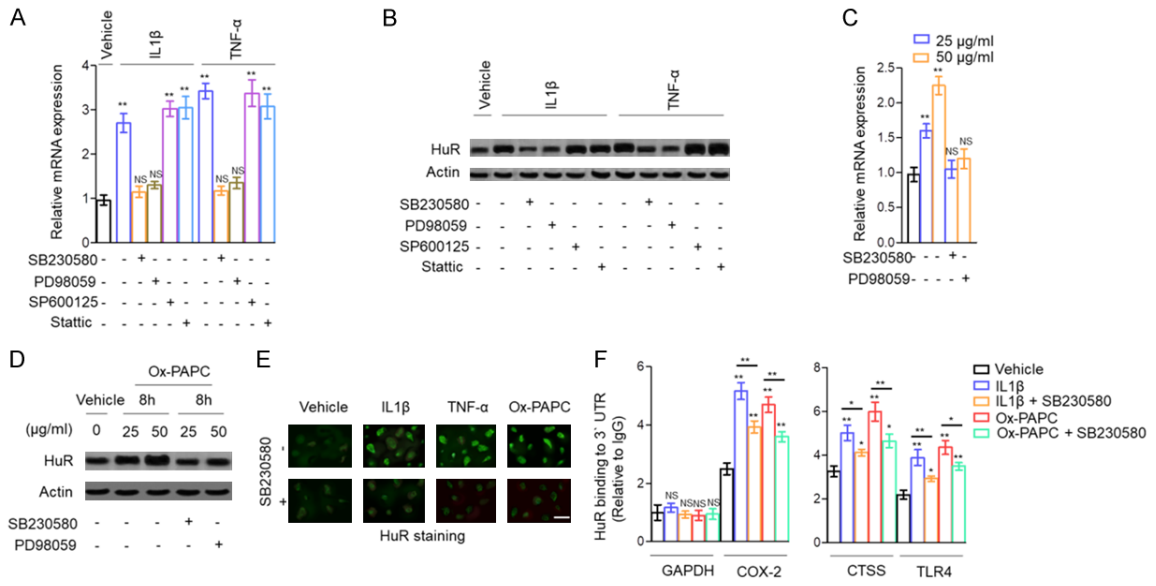

Figure 5. MAPK/ErK pathway is required for proatherogenic stimuli-induced HuR expression and activity. (A, B) HAECs were treated with vehicle or 10 ng/ml recombinant IL1β or TNF-α in the presence or absence of 10 μM pharmacological inhibitors like SB230580, PD98059, SP600125 or Stattic as indicated and cultured for 8 hours. (A) The mRNA level of HuR was measured by qRT-PCR analysis. The results represent the mean value of 3 replicates and are normalized to human Actin. (B) The protein level of HuR was measured by Western blot. Human Actin was used as a loading control and the representative images are shown. (C, D) HAECs were treated with vehicle or different concentrations of Ox-PAPC in the presence or absence of 10 μM SB230580 or PD98059 and cultured for 8 or 12 hours. (C) The mRNA level and (D) protein level of HuR were detected as described in (A, B). (E) HAECs were treated with vehicle or 10 ng/ml recombinant IL1β, TNF-α or 50 μg/ml Ox-PAPC in the presence or absence of 10 μM SB230580 for 8 hours. The expression of HuR was detected by immunofluorescent staining (green). Scale bar, 25 μM. (F) HAECs were treated with vehicle, 10 ng/ml recombinant IL1β or 50 μg/ml Ox-PAPC in the presence or absence of 10 μM SB230580 and cultured for 8 hours. Cell extracts were subjected to in vitro RNA immunoprecipitation assay as depicted in (**Figure 1C** and **1F**). The enrichment value relative to IgG group for each transcript is normalized to GAPDH. The statistical analysis was performed using unpaired Student's *t*-test. Data are mean ± s.e.m. **, $P < 0.01$; *, $P < 0.05$; NS, not significant.

not limited to atherosclerosis, but has also been associated with progression of some types of cancer, where HuR mainly acts as a regulator for stabilizing mRNAs and controlling gene expressions [29]. Except for the regulation of endothelial HuR in atherosclerosis revealed in our study, other correlations can not be excluded, such as treatment responses or prognosis, and it is worthwhile to demonstrate these possibilities in future investigations.

The mechanisms by which the HuR expression is transcriptionally regulated are often overlooked. We show here that the expression of HuR is induced by inflammatory cytokines through MAPK/ErK pathway. In a previous study, the MAPK pathway was implicated in mediating LPS-induced HuR expression [18]. Hence, it appears that the MAPK/ErK kinases are important mediators transducing extracellular inflammatory stimulations to activate HuR expression, at least in these scenario, but we still do not know which downstream transcriptional factor(s) is responsible for this activity. We also noticed the enhanced RNA-binding activity of HuR independent of its steady protein expression when the MAPK/ErK pathway is inactivated by pharmacological inhibitors. It has been demonstrated that HuR can be phosphorylated by MAPK and translocates from nucleus to cytosol to exhibit higher activity [30]. Therefore, except for MAPK kinase, other participators may be involved in determining HuR activity upon inflammatory stimulations. This is very possible, because the posttranslational modification of HuR can also be accomplished by other factors and results in altered activity, such as Cdk1 [31] and protein kinase c [32]. The mechanisms of modulating HuR activity merit further studies.

In summary, our data provide a direct link between proatherogenic molecules and HuR regulation, and also uncover the mechanism responsible for their connection. Based on our evidence, we propose that the endothelial HuR may be a potential participator involved in atherosclerosis pathogenesis. In vivo lines of evidence obtained from animal models may be useful to demonstrate this notion.

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Disclosure of conflict of interest

None.

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