Regulation of AT1R expression through HuR by insulin

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ABSTRACT

Angiotensin II type 1 receptor (AT1R) has a pathophysiological role in hypertension, atherosclerosis and heart failure. Type 2 diabetes is hyperinsulinemic state and a major risk factor for atherosclerosis and hypertension. It is known that hyperinsulinemia upregulates AT1R expression post-transcriptionally by increasing the half-life of AT1R mRNA, but little is known about the mechanism of this effect. In the present study, we first identified AT1R 3'-UTR as a mediator of insulin effect. Using 3'-UTR as a bait, we identified through analysis of insulin-stimulated cell lysates by affinity purification and mass spectrometry HuR as an insulin-regulated AT1R mRNA binding protein. By ribonucleoprotein immunoprecipitation, we found HuR binding to AT1R to be increased by insulin. Overexpression of HuR leads to increased AT1R expression in a 3'-UTR-dependent manner. Both insulin and HuR overexpression stabilize AT1R 3'-UTR and their responsive element within 3'-UTR are located within the same region. Cell fractionation demonstrated that insulin induced HuR translocation from nucleus to cytoplasm increased HuR binding to cytoplasmic AT1R 3'-UTR. Consistent with HuR translocation playing a mechanistic role in HuR effect, a reduction in the cytoplasmic levels of HuR either by silencing of HuR expression or by inhibition of HuR translocation into cytoplasm attenuated insulin response. These results show that HuR translocation to cytoplasm is enhanced by insulin leading to AT1R upregulation through HuR-mediated stabilization of AT1R mRNA.

INTRODUCTION

Biological actions of angiotensin II, a peptide hormone central in regulating cardiovascular structure and function, are mediated by its interaction with specific cell surface receptors expressed on the cell membranes of cardiovascular and renal cells (1). Angiotensin II has two receptors that confer its effects: the widely expressed type 1 receptor (AT1R) is a G-protein-coupled seven times membrane spanning receptor that mediates most of the deleterious remodeling effects of angiotensin II, while the angiotensin II type 2 receptor is much more restricted in its expression and counteracts most of the cellular effects of AT1R receptor (2). Acute stimulation with angiotensin II regulates electrolyte homeostasis and vasoconstriction, increasing blood pressure, while chronic stimulation promotes adverse remodeling in the myocardium and vasculature. AT1R expression is upregulated in the healing phase of myocardial infarction, in failing myocardium and in atherosclerotic arteries. Consistent with the central pathophysiological role of AT1R, pharmacologic therapy that reduces the activity of AT1R has been shown in numerous clinical trials to be beneficial in attenuating the progression of atherosclerosis, heart failure as well as chronic renal disease.

Type 2 diabetes mellitus (T2DM) is an important risk factor for atherosclerosis and the majority of diabetic patients die of cardiovascular disease (3). The renin–angiotensin system (RAS) has a central role in the development of vascular disease in type 2 diabetes (4). Attenuation of RAS activity has been shown to be beneficial in the setting of both primary and secondary prevention of new ischemic events

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and mortality (4). It is possible that T2DM is linked to RAS via hyperinsulinemia as it is a typical feature T2DM. Hyperinsulinemia has been associated with the increased activity of AT1R. In atherosclerotic regions of arteries, the production of angiotensin II and the expression of AT1R are significantly increased (5). In carotid endarterectomy samples derived from diabetic or non-diabetic patients, AT1R expression is increased in comparison to the samples from non-diabetic patients. This effect is also seen in isolated vascular smooth muscle cells (VSMCs), in which the baseline expression of AT1R in cells retrieved from patients with T2DM is higher and insulin response stronger when compared to non-diabetic subjects (6). These results suggest clinically meaningful relationship between AT1R receptor expression and insulin levels.

Post-transcriptional regulation of AT1R is the predominant mechanism by which estrogen, progesterone, insulin, statins and angiotensin II exert their effect on AT1R (7–12). Prior data shows that insulin regulates AT1R by stabilizing its mRNA (11). However, the mechanisms by which insulin regulates AT1R mRNA stabilization remain obscure. We herein identified the 3′-UTR of AT1R as a mediator of this response. In our assays with insulin-stimulated lysates, we found HuR protein to bind 3′-UTR transcript. HuR (also known as ELAVL1), a member of the ELAV (embryonic lethal abnormal vision) RNA-binding protein (RBP) family, is an ubiquitously expressed protein that has been shown to have role in regulation of cancer (13), hypoxic (14) and genotoxic responses via mRNA stabilization and effects on translation (15). Insulin sensitivity of the HuR interaction with AT1R 3′-UTR was confirmed by ribonucleoprotein immunoprecipitation (RNP-IP), gel shift and affinity purification experiments. Our data suggest that HuR mediates the insulin effect on AT1R expression via mRNA stabilization.

MATERIALS AND METHODS

Cell culture, luciferase assay and protein extraction

HEK293 cells were grown in DMEM that was supplemented with 10% fetal bovine serum (FBS), with ampicillin/streptomycin, and glutamine. Cells were used for 6–10 passages before replacement with early passage stocks. Coronary artery VSMCs were purchased from Clonetics. Early passage VSMC were cultured on smooth muscle growth medium-2 with 5% FBS. Constructs were transiently transfected in HEK293 cells using a standard Fugene 6 protocol (Roche). Small interfering RNAs (siRNAs) (30 nM) for HuR, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Tudor and nuclease domains containing protein p100, AU-rich binding protein (AUF1) and negative control siRNAs (Ambion and Qiagen) were transfected using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions. HEK293 cells or VSMCs were serum starved for 24 h before stimulation by 75 nM of insulin for 12 h unless otherwise indicated. Cells were harvested 24–48 h after transfection and firefly luciferase activities were measured using the Luciferase Assay System (Promega). The luciferase activity was normalized to renilla activity or total protein content.

The luciferase results were calculated from an average of three independent experiments performed in triplicate for each construct. Leptomycin B (LMB) was purchased from Sigma. Cell fractionation was done with Cell Fractionation kit (Pierce) according to manufacturer's instructions. Protein concentrations were determined by Bradford assay (Bio-Rad).

Lentivirus preparation and transduction

cDNA encoding full-length of human HuR was cloned into the pLenti6-V5-DEST vector, and virus was generated in the 293FT viral packaging cell line. Equal titers of test or vector control virus were used in subsequent experiments. Five lentiviral vectors expressing a 21-nt HuR short hairpin RNA (shRNA) in pLenti6 vector were purchased from Sigma. VSMCs were infected with virus-containing supernatant in the presence of polybrene, and stably transduced cells were selected with Blasticidin. HuR expression levels were detected by western blot.

Constructs

All the constructs were based on pGL3 (Promega) vector have been described in an earlier study (16). All the constructs were confirmed by sequencing prior use.

RNA probe preparation, affinity purification and RNA electrophoretic mobility-shift assay

AT1R (including 3′-UTR) cDNA was used as a template for PCR reactions whereby T7 RNA polymerase promoter sequence was added to the 5′-end of all fragments. For producing RNA probes for affinity purification, a 30-base long polyA tail was included in the 3′-oligo. RNA probes used in RNA electrophoretic mobility-shift assay (REMSA) were synthesized without polyA tail and labeled with biotin. More detailed protocols and primer sequences were described (17). Gel shift was performed as described (29).

Western blotting

Affinity-purified proteins and cell lysates were separated in SDS–PAGE and transferred to nitrocellulose membrane (Hybond ECL). Proteins were then detected as described in Paukku *et al.* (16) or with infrared technology using Odyssey Blocking Buffer (Li-Cor), polyclonal HuR (Upstate), GAPDH (Trevigen), p100 (Santa Cruz) and AUF1 (Upstate) antibodies and Alexa Fluor 680 Goat anti-Mouse IgG (Invitrogen) or Goat anti-Rabbit IRDye (Li-Cor) secondary antibodies. The membrane was scanned with Odyssey Infrared Imaging System (Li-Cor).

RNP-IP and real-time PCR (qPCR)

This assay was performed essentially as described in Backlund *et al.* (17). Briefly, cytoplasmic lysates prepared from 10^7 of HEK293 cells or coronary artery VSMCs were first precleared with protein G-agarose beads and then IP with 2 μg of HuR, p100, GAPDH or AUF1 antibodies. The coprecipitated mRNA was then extracted, and subjected to RT-PCR and qPCR with AT1R specific oligos.

Cross-linking of AT1R mRNA complexes

Coronary artery VSMC were cross-linked by 0.5% formaldehyde. The reaction was terminated by 0.25 M glycine. Fixed cells were resuspended in RIPA buffer containing protease inhibitors. The cells were lysed by sonication. Then IP was performed as described earlier. Resuspended beads were incubated at 70°C for 45 min to reverse the crosslinks. The RNA is extracted from these samples using Trizol according to the manufacturer's protocol. The coprecipitated mRNA was then extracted, and subjected to RT-PCR and qPCR with AT1R specific oligos.

RNA affinity purification

For producing polyadenylated RNA probes 30 bases long polyA tail was included in the 3'-oligos described earlier. ~2 μg of *in vitro* transcribed polyadenylated RNA probes were incubated with polystyrene latex beads with $dC_{10}T_{30}$ oligonucleotides covalently linked to the surface (Oligotex, Qiagen), in a buffer containing 20 mM Tris–HCl, pH 7.5, 1 M NaCl, 2 mM EDTA and 0.2% SDS. After two washes in a wash buffer 10 mM Tris–HCl, pH 7.5, 150 mM NaCl and 1 mM EDTA, the RNA-coated beads were incubated with 100 μg cytoplasmic extracts (NE-PER, Pierce) in the RNA binding buffer [5 mM Hepes (pH 7.9), 7.5 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 0.1 mg/ml yeast tRNA, 0.1 mg/ml bovine serum albumin (BSA). Poly-T beads were saturated with bait RNA and thus the endogenous mRNA in the lysates gives only minor background. After incubation for 10 min at 30°C, the beads were extensively washed with RNA binding buffer without BSA. Laemmli sample buffer was then added to the beads and proteins bound to the RNA probe were resolved on 4–20% SDS–PAGE and subjected to silver staining according to the manufacturer's instructions (Silver Stain Plus, Bio-Rad).

Mass spectrometry

Silver stained protein bands of interest were cut out of the polyacrylamide gel and 'in-gel' digested essentially as described by Shevchenko *et al.* (18). Proteins were reduced with DTT and alkylated with iodoacetamide before digestion with trypsin (Sequencing Grade Modified Trypsin, V5111, Promega). The recovered peptides were, after desalting using Millipore C18 ZipTip™, subjected to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometric analysis. MALDI-TOF mass spectra for mass fingerprinting and MALDI-TOF/TOF mass spectra for identification by fragment ion analysis were acquired using an Ultraflex TOF/TOF instrument (Bruker-Daltonik GmbH, Bremen, Germany). Protein identification with the generated data was performed using Mascot® Peptide Mass Fingerprint and MS/MS Ion Search programs (http://www.matrixscience.com).

Immunofluorescence microscopy

VSMCs grown on glass coverslips were serum starved for 16 h and subsequently treated with 0.1 μl/ml insulin only, with insulin and 10 ng/ml LMB or left untreated for 16 h. All the subsequent procedures were performed at room temperature. Culture medium was removed and the cells were washed briefly with PBS. Cells were fixed with 4% paraformaldehyde for 15 min and washed twice with PBS. Cells were permeabilized with 0.25% Triton X-100 (Bio-Rad) in PBS for 10 min and washed three times with PBS. To reduce non-specific binding of the antibodies, the cells were blocked with 3% BSA (Sigma); 0.3 M glycine (Bio-Rad) and 5% normal goat serum (Millipore) in PBS for 1 h. Cells were incubated with primary antibody (anti-HuR, Upstate) diluted 1:500 in immunolabeling buffer (1% BSA and 0.1% Tween-20) (Amresco) in PBS for 1 h. Cells were washed three times with PBS and incubated for 1 h. with Alexa Fluor 594 goat-anti rabbit secondary antibody (Invitrogen) diluted 1:1000 in immunolabeling buffer. Cells were washed three times with PBS and the nuclei were stained with 2 μg/ml Hoechst 33258 (Sigma) in PBS for 1 min. Cells were washed twice with PBS and the coverslips were rinsed with purified water before mounting to microscope slides with Fluoro Mount (Sigma). Images were obtained by Zeiss Axioplan 2 fluorescence microscope.

Statistical analysis

Data are presented as means ± SE. Statistical analysis was performed using Student's paired one-tailed *t*-test. $P < 0.05$ were considered significant.

RESULTS

Insulin increases AT1R expression by stabilizing AT1R mRNA

The present study was prompted by the discovery that insulin regulates AT1R expression. In keeping with previous studies (11), stimulation of primary culture of coronary artery VSMC by 75 nM insulin for 24 h increases AT1R expression (Figure 1A) and AT1R mRNA (Figure 1B). An increase in mRNA could be due to increased stability of AT1R mRNA. To test this possibility, cells were exposed to actinomycin D (2 μg/ml) and total cellular RNA then was prepared at the times indicated and subjected to RT-qPCR to assess the half-life of AT1R mRNA. As shown in Figure 1C, the half-life of AT1R mRNA in coronary artery VSMC stimulated by insulin was markedly longer (10 h) than that observed in unstimulated cells. In contrast, the half-life of negative control GAPDH mRNA was not altered. These results supported the view that insulin stabilized the AT1R mRNA. Independent assessment of the mRNA stabilizing and inducing effect of insulin on AT1R expression was sought through the creation and analysis of luciferase reporter constructs linked to the full-length AT1R 3'-UTR. Following transient transfection with the parent vector pGL3, luciferase activity in insulin treated HEK293 cells was no different than that measured in untreated populations. By contrast, Luc(3'-UTR) expressing a chimeric mRNA encoding luciferase and the full-length AT1R 3'-UTR, exhibited a readily inducible luciferase activity that was 2.5-fold
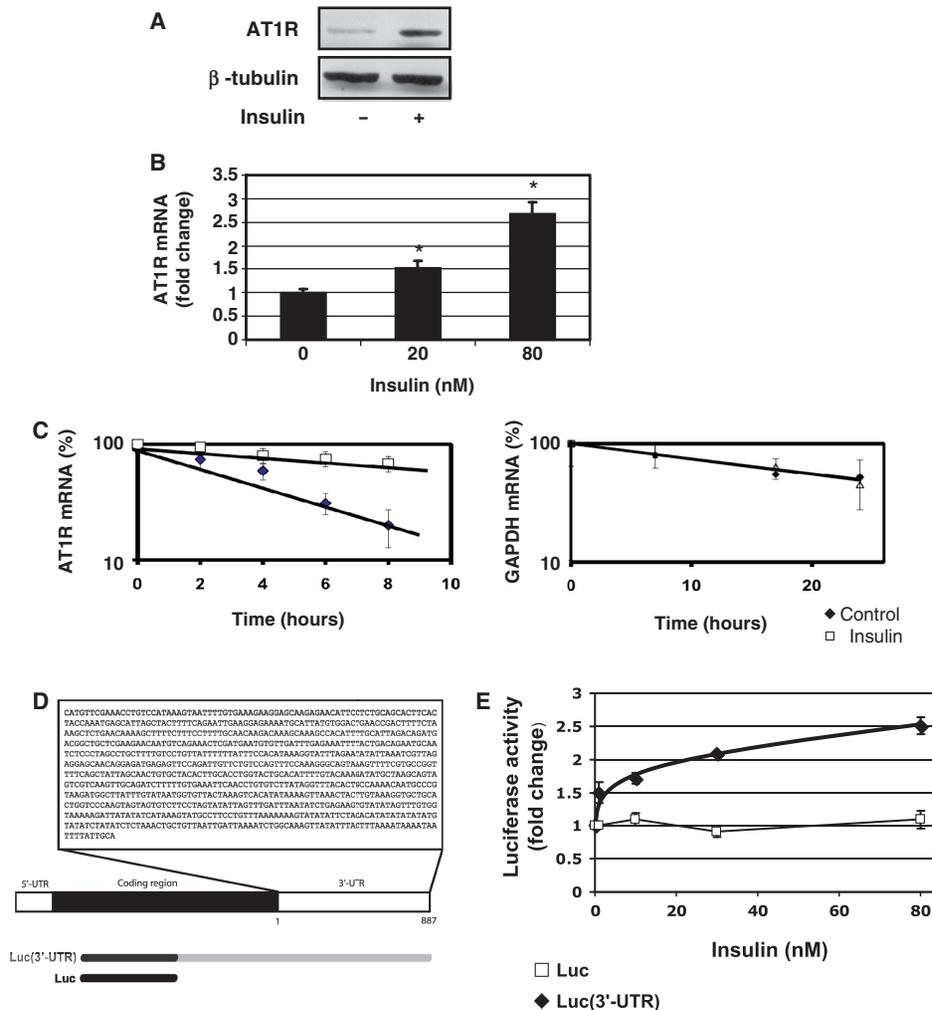
Figure 1. Insulin upregulates AT1R expression by stabilizing AT1R mRNA. (**A**) Twenty-four hours after insulin or vehicle stimulation of coronary artery VSMCs, lysates were prepared to assess AT1R and loading control GAPDH expression by western blot. (**B**) RNA isolated from cells described for panel (A) was subjected to qPCR to assess the mRNA levels of AT1R and of loading control GAPDH. The western blotting and real-time qPCR data are representatives at least of three or more experiments. (**C**). Cells described for panel (A) were exposed to actinomycin D (2 μg/ml), whereupon the cellular RNA was isolated at the times indicated and subjected to real-time qPCR to assess the half-life of AT1R mRNA. Results represent AT1R mRNA normalized to the expression of the construct at time 0 and are shown as a linear fit. Results represent the means of an average of three independent experiments. (**D**) The structure of the full-length AT1R mRNA, indicating the coding and untranslated regions (5'- and 3'-UTRs). (**E**) AT1R 3'-UTR confers responsiveness of luciferase to insulin stimulation. Twenty-four hours after insulin stimulation of HEK293 cells transfected with luciferase with or without AT1R 3'-UTR and renilla luciferase plasmids as a transfection control, lysates were prepared to assess fire-fly and renilla luciferase activities. Data were normalized to the activity of luciferase construct lacking the 3'-UTR and represent means ± SD. *$P < 0.05$ versus luciferase without AT1R 3'-UTR.

higher after insulin stimulation than that in untreated cells of the same transfection group (Figure 1D). In summary, insulin upregulates AT1R expression by stabilizing AT1R mRNA in a 3'-UTR dependent manner.

Identification of HUR as an insulin-sensitive regulator of AT1R mRNA

Insulin induces AT1R mRNA levels by increasing AT1R mRNA half-life. Since stabilization of mRNAs is known to involve binding of proteins that recognize certain mRNA sequences, we examined whether proteins present in lysates from coronary artery VSMCs bound to AT1R mRNA. We assayed the existence of insulin-regulated RBPs by employing affinity purification.

A probe corresponding to bases 1–847 of the 3'-UTR of the AT1R was transcribed. Due to problems in transcribing the full-length RNA construct, the polyadenylated RNA transcript used in the affinity purification did not include the last 40 bp in 3'-end of the 3'-UTR. Cells, treated with either insulin or vehicle, were lysed and incubated with poly-A containing RNA transcripts that were attached to poly-T beads. Figure 2A is a silver-stained gel showing the protein pattern of fractions eluted from AT1R 3'-UTR probe. The most abundant band migrated at 36 kDa. Binding of this protein was more prominent in reactions where insulin-stimulated cell lysates were used. Protein was excised from the gel and subjected to mass spectrometric analysis and the protein was identified as HuR.
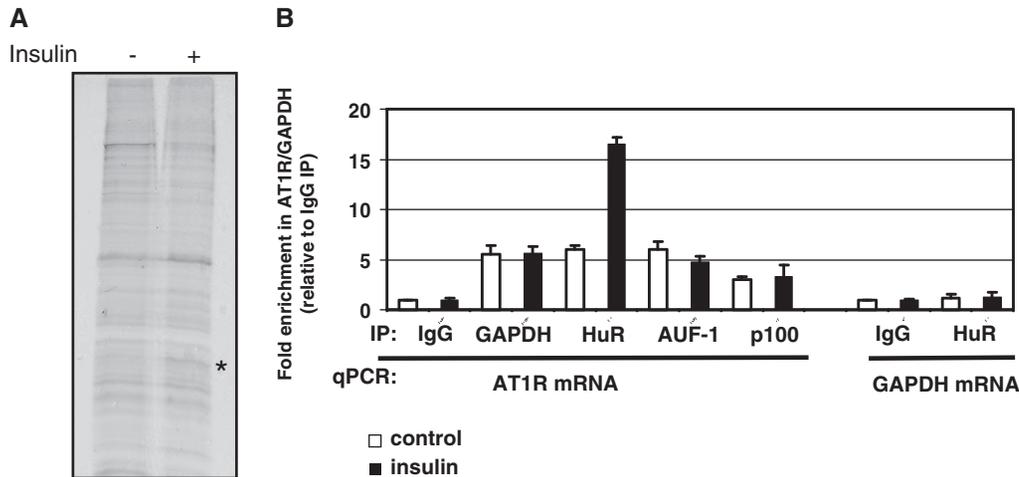
Figure 2. Insulin increases the interaction of HuR with AT1R 3′-UTR. (**A**) Twenty-four hours after insulin or vehicle stimulation of coronary artery VSMCs, lysates were prepared to affinity purify proteins by using AT1R 3′-UTR mRNA as a bait. A silver stained SDS–PAGE gel shows a differential expression of a 36-kDa protein (asterisk) which was then excised, digested and recognized by mass spectrometry. The details of the purification are given under "Experimental procedures". (**B**) Proteins isolated from cells described for panel (A) was subjected to IP by HuR-, GAPDH-, AUF1-, p100, or as a control preimmuno IgG antibodies followed by RNA extraction and qPCR of either AT1R or GAPDH as a control. Results represent the means ± SD of an average of three independent experiments performed as a triplicate for each construct. *$P < 0.05$ versus control stimulation.

Our laboratory has previously reported that the 3′-UTR of AT1R mRNA is a target of GAPDH, AUF1 and p100 (16,17), and now we identified HuR as such as well. We next sought to test whether these proteins capable of forming a complex with the endogenous AT1R 3′-UTR are involved in the insulin-mediated AT1R regulation. Therefore, we performed a RNP-IP with specific antibodies and with cytoplasmic lysates from unstimulated and insulin-stimulated coronary artery VSMCs. qPCR with AT1R specific oligos was used to detect protein-associated endogenous AT1R mRNA. Among several RBPs that bind to AT1R mRNA, HuR showed increased levels of association with AT1R mRNA following insulin treatment (2.8-fold), while GAPDH, AUF1 and p100 did not show marked differences in binding (Figure 2B). Thus, the association of endogenous AT1R mRNA with HuR is regulated by insulin. We performed the same experiment with formaldehyde-treated cells and found essentially identical results (data not shown). Therefore, the data suggests that HuR–AT1R interaction takes place in the living cells.

Time course of insulin effect

The evidence presented thus far indicates that insulin enhances the formation of cytoplasmic HuR-AT1R mRNA complexes. Detailed mechanisms by which HuR protects its target mRNAs are still incompletely understood but it is thought to involve nucleo-cytoplasmic shuttling of HuR and its subsequent translocation with associated mRNA (19). To explore the time course of insulin effect, Luc(3′-UTR) was transfected to HEK293 cells and luciferase activities were then measured at 6, 12 and 24 h after insulin stimulation. Insulin stimulation increased luciferase activity in cells transfected with Luc(3′-UTR) maximally ∼2.5-fold as compared to Luc (Figure 3A). To examine this possibility, we traced HuR

localization by cell fractionation. In a western blot, we observed a translocation of HuR from the nucleus to the cytoplasm that became visible after 6 h of insulin treatment, and stronger signal at 12 and 24 h (Figure 3B). Similarly, affinity purification of HuR with AT1R 3′-UTR showed increased HuR signal in western blot after longer insulin stimulations (Figure 3B). To further confirm the time dependent effect, we assessed HuR binding to AT1R 3′-UTR with REMSA. A probe-consisting nucleotides 1–847 of AT1R 3′-UTR showed a shift with cytoplasmic lysate in which HuR was overexpressed (Figure 3C). This band was abolished by ∼10-fold excess of unlabeled probe and also decreased in lysates prepared from cells in which HuR was depleted. To confirm the presence of HuR in gel shift band, we assayed the ability of antibodies recognizing HuR to supershift the AT1R RNA–protein complexes on native gels. As shown (Figure 3C), HuR indeed forms part of the complexes, as a prominent band of slower electrophoretic mobility was detected when the anti-HuR antibody was added to protein complexes. Analogously to affinity purification data, gel shift experiment showed that insulin enhances HuR binding to AT1R 3′-UTR probe. These results indicate that insulin-induced translocation of HuR into the cytoplasm leads to enhanced HuR binding to AT1R 3′-UTR.

HuR is required for the stabilization of AT1R mRNA by insulin

Further supporting evidence for the role of HuR in insulin-induced AT1R regulation was obtained from the reporter gene assays. To determine whether cytoplasmic HuR, GAPDH, AUF1, or p100 plays a causal role in insulin effect on AT1R, siRNA against these proteins were utilized to reduce intracellular protein levels. Cells expressing either normal or knockdown HuR levels
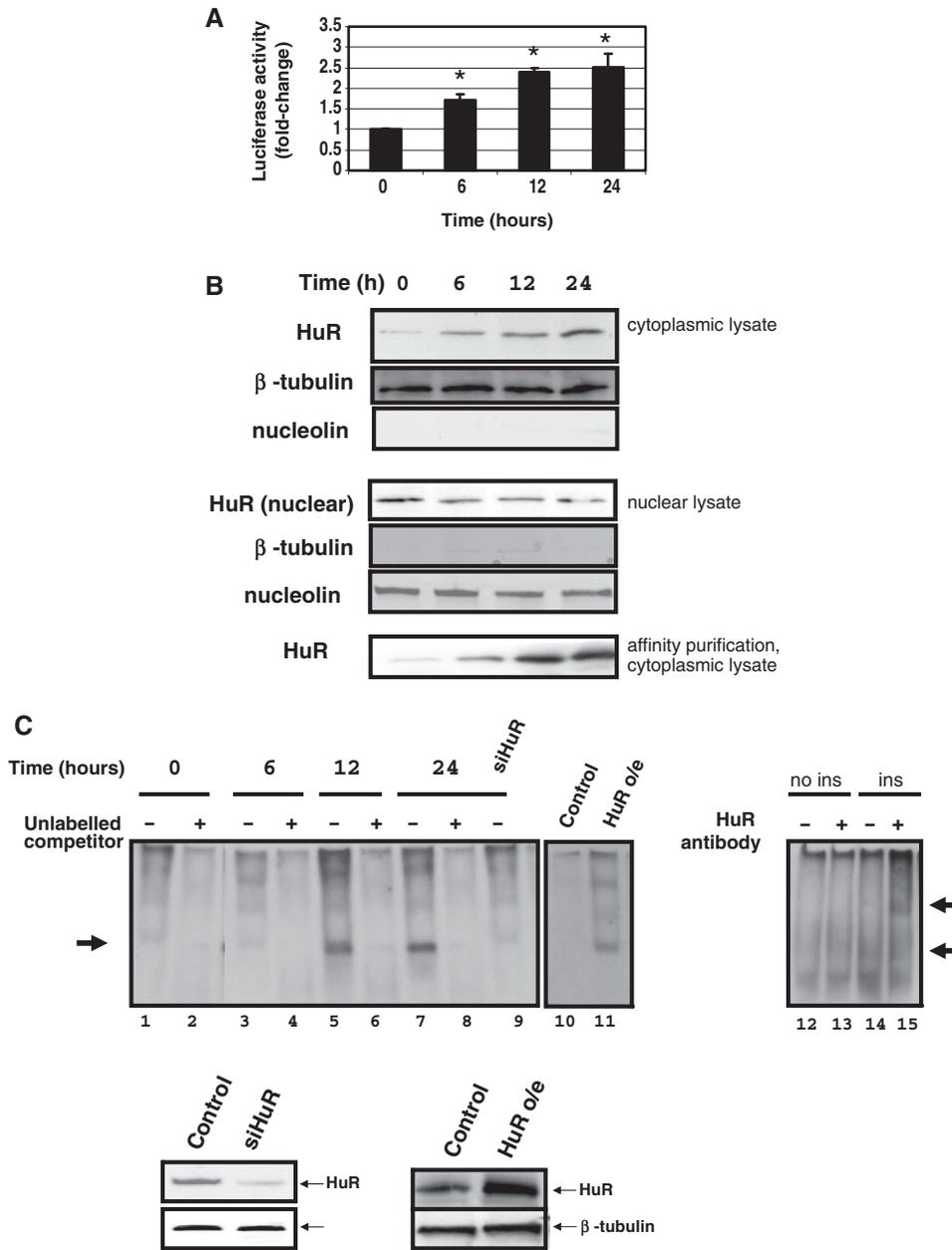
Figure 3. Increased cytoplasmic HuR after exposure to insulin. (**A**) At 0, 6, 12 and 24 h after insulin stimulation of HEK293 cells transfected with luciferase with or without AT1R 3′-UTR, lysates were prepared to assess luciferase activity. Luminometer measurements for this experiment are expressed as fold of promoter control at time 0. The results represent the means ± SD of an average of three independent experiments. *$P < 0.05$ versus without insulin stimulation. (**B**) Proteins isolated from cells described for panel (A) were fractionated into cytoplasmic and nuclear lysates and then subjected to western blot or to affinity purification using AT1R 3′-UTR as probe. β-tubulin and nucleolin were detected in the lysates to monitor for the purity of the fractionation. Data representative of three independent experiments is shown. (**C**) To demonstrate an increased binding of HuR to AT1R 3′-UTR in response to insulin, REMSA assays were performed using lysates from cells described in panel (A). Cytoplasmic lysates were assayed in REMSA with labeled RNA probe corresponding to nucleotides 1–847 of AT1R 3′-UTR. After binding, the samples were RNAase treated to reduce background due to non-specific binding. Lanes 1–8 show a time course of insulin effect on HuR binding to AT1R 3′-UTR. Lanes 2, 4, 6 and 8 were treated with a 10-fold excess of unlabeled 1-847 probe. The cells used to prepare lysates for a gel shift shown in Lanes 1–8 were untransfected, whereas in Lane 9 cells were transfected with siHuR. In Lanes 10 and 11, same probe 1-847 was used but either with control (10) or HuR overexpression lysates were used (11). In insulin-stimulated cells (lanes 14,15) incubation of the cytoplasmic lysates with HuR-specific antibody caused a gel shift (lane 15). The western below blots document the effect of HuR silencing and overexpression by densitometry.

were treated with insulin, and the changes in luciferase activity were monitored. HuR silencing did not cause cell death over the course of these experiments based on lactate dehydrogenase release. The activity of Luc (3′-UTR) was reduced in HuR-silenced cells. No insulin response was detected in the siHuR-treated cells [Figure 4A (left)]. Contrary to HuR silencing, GAPDH, AUF1, or p100 knockdown had no effect on insulin response (Figure 4A). In order to investigate HuR effects on AT1R mRNA, we subjected cells to
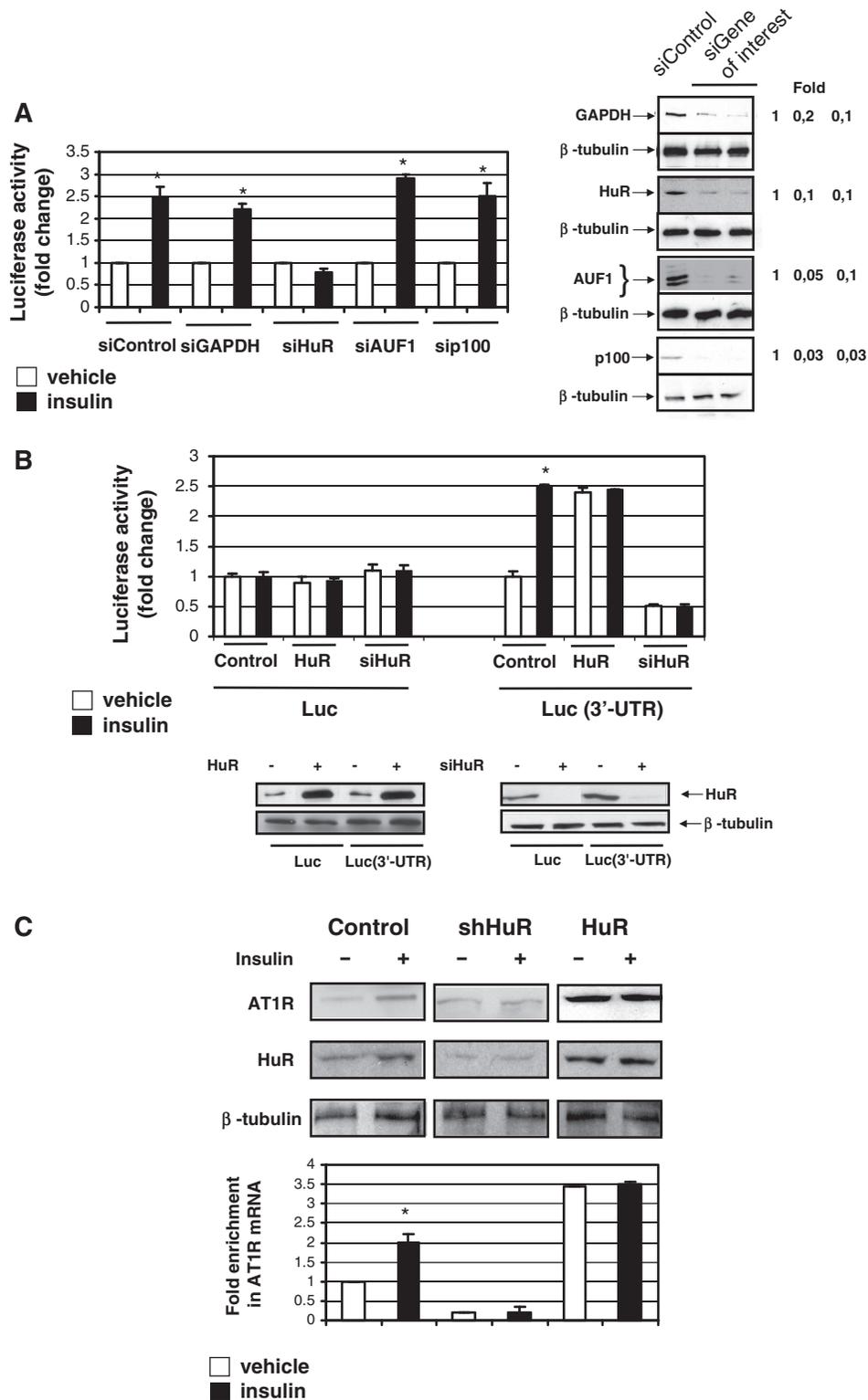
Figure 4. HuR is required for the insulin response. (**A**) Left panel: HEK293 cells were transfected with Luc(3′-UTR) and siRNA against either GAPDH, HuR, AUF-1, or p100 and then stimulated by 75 nM insulin for 24 h followed measurement of luciferase activity in lysates. Luciferase measurements are expressed as fold of unstimulated control. Results represent the means ± SD of an average of three independent experiments. Lower panel: Western blots controlling the effect of gene-specific siRNA on target-protein expression and β-tubulin control blots. (**B**) HEK293 cells transfected with either Luc or Luc(3′-UTR) were had their HuR expression increased or decreased by transfection of HuR or siHur, respectively, and luciferase activity was measured in cell lysates. Results were normalized to unstimulated control transfection. Results represent the means ± SD of an average of three independent experiments. *$P < 0.05$ versus unstimulated cells. Lysates were prepared and subjected to western blot analysis to monitor the expression of HuR. β-tubulin levels were monitored to assess the evenness of loading. (**C**) Coronary artery VSMC were transduced with lentiviral expression vectors of HuR and shHuR, stimulated with 75 nM insulin or vehicle for 24 h followed by western blot analysis of the protein lysates by AT1R, HuR and β-tubulin antibodies and qPCR analysis of mRNA. Results represent the means ± SD of an average of three independent experiments. Right panel: qPCR of HuR in overexpression lysates or shHuR lysates.
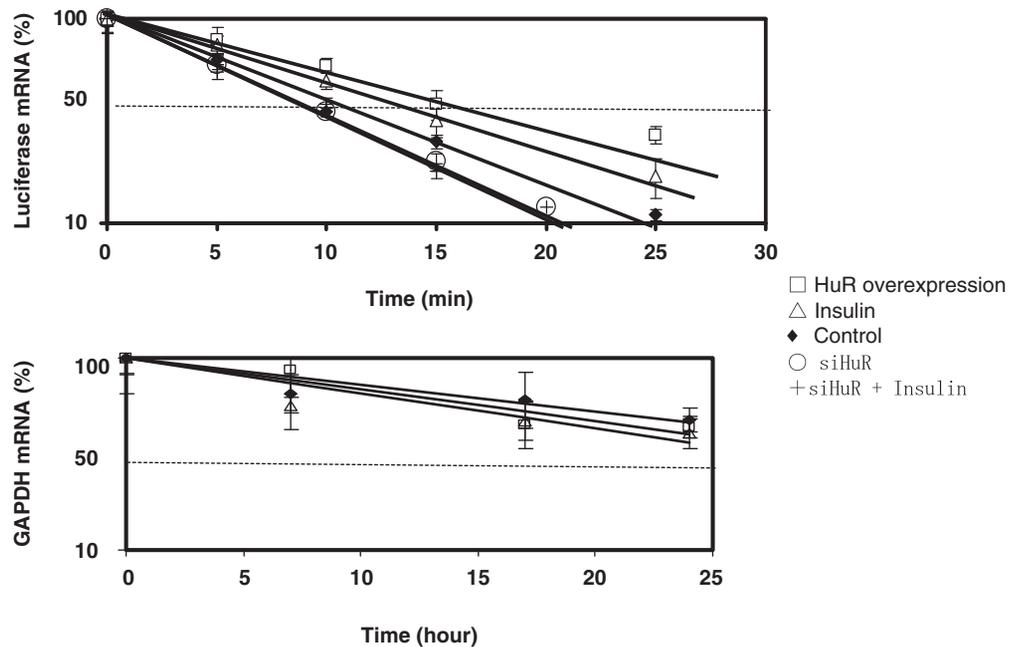
Figure 5. The ectopic modulation of HuR expression alters the insulin effect on AT1R 3′-UTR half-life. HEK293 transfected with Luc(3′-UTR) had their cytoplasmic HuR levels altered by cotransfection of either HuR expression vector or siHuR or stimulation by insulin. Cells were exposed to actinomycin D (2 μg/ml), whereupon the cellular RNA was isolated at the times indicated and subjected to real-time qPCR to assess the half-life of Luc mRNA. In the upper panel, luciferase qPCR. Lower panel: GAPDH qPCR. Results represent Luc or GAPDH mRNA normalized to the expression of the construct at time 0. Results are shown as a linear fit. Results represent the means ± SD of an average of three independent experiments.

perturbations that increased or decreased HuR levels. Following HuR overexpression, Luc(3′-UTR) activity was significantly increased as compared to control Luc (Figure 4B). In line with this, the knockdown of HuR reduced Luc(3′-UTR) activity but not that of control Luc (Figure 4B).

We sought to directly examine the role of HuR in the regulation of endogenous AT1R. Coronary artery VSMC were transduced with lentiviral vectors overexpressing either HuR shRNA or HuR and stimulated these cells with insulin. AT1R expression was measured by western blot in cells expressing normal, increased, or reduced HuR levels. Increased HuR resulted in higher AT1R expression and conversely lower HuR decreased AT1R expression. In VSMCs, AT1R expression was higher after insulin treatment, whereas HuR knockdown resulted in impairment in the cell's responsiveness to insulin and the increase was attenuated (Figure 4C). Together, these observations support the notion that HuR is necessary for the regulation of AT1R by insulin.

Both HuR and insulin increase AT1R mRNA stability via 3′-UTR

Both insulin and HuR alter AT1R mRNA steady-state levels and we proceeded to test the hypothesis that these effects are mediated by changes in mRNA half-lives since HuR has been found to stabilize many target mRNAs. To ascertain if the increase in the Luc(3′-UTR) mRNA levels was due to changes in mRNA stability, the mRNA half-life ($t_{1/2}$) was analyzed following treatment with actinomycin D to inhibit with *de novo* transcription

(Figure 5) after both HuR overexpression or insulin stimulation. A construct without the AT1R 3′-UTR did not respond to either HuR overexpression or insulin treatment (data not shown). Treatments that increased cytoplasmic HuR levels, HuR overexpression and insulin, stabilized Luc(3′-UTR) mRNA. Reduction in HuR levels by siHuR decreased Luc(3′-UTR) mRNA half-life consistent with steady-state AT1R mRNA levels. In summary, the mechanisms of insulin and HuR effects on AT1R mRNA appear to be the same.

Mapping of HuR binding sites and the insulin and HuR responsive regions within the 3′-UTR

Next, we wanted to identify the AT1R 3′-UTR regions involved in association with HuR. The observations from affinity purification supported a binding scheme on the AT1R 3′-UTR in which HuR associates with 300–847 region of the 3′-UTR (Figure 6A). As binding and response sequences are not necessarily the same, we proceeded to map the insulin and HuR responsive elements within the 3′-UTR. A series of reporter constructs containing different fragments of 3′-UTR were designed to disrupt the insulin response. These constructs were transfected into HEK293 cells. As shown in Figure 6B, insulin stimulation increased the activity of luciferase constructs containing at least 637 first nucleotides of 3′-UTR, whereas the activity of constructs containing shorter 3′-UTR sequences remained effectively unchanged. Sensitivity to the effect of HuR overexpression was mapped to the same region than insulin effect, Figure 6C.

A

1-847	
1-100	
100-300	
300-600	
600-887	

1-100 100-300 300-600 500-700 600-800 1-847

← HuR

B

Luc(3'-UTR)
Luc(1-637)
Luc(1-607)
Luc(1-600)
Luc(1-577)
Luc(1-300)
Luc(1-100)
Luc

Luc(1-100)
Luc(1-300)
Luc(1-577)
Luc(1-600)
Luc(1-607)
Luc(1-637)   *
Luc(3'UTR)   *
Luc

0   0.5   1   1.5   2   2.5   3

**Luciferase activity
(fold change)**

C

Luc(1-100)
Luc(1-300)
Luc(1-577)
Luc(1-600)
Luc(1-607)
Luc(1-637)   *
Luc(3'-UTR)   *
Luc

0   0.5   1   1.5   2   2.5

**Luciferase activity
(fold change)**

Luc(1-100) Luc(1-300) Luc(1-577) Luc(1-600) Luc(1-607) Luc(1-637) Luc(3'-UTR) Luc

← HuR

← β-tubulin

HuR overexpression   − +   − +   − +   − +   − +   − +   − +   − +

Figure 6. Insulin and HuR responsive sites are localized within the same region in the AT1R 3'-UTR (**A**) Upper panel: Schematic representation of the AT1R 3'-UTR mRNA fragments prepared for affinity purification. Lower panel: Mapping of the HuR binding site within AT1R 3'-UTR. RNA affinity purification was performed with different transcripts and HuR binding was measured by western blotting. (**B**) Mapping the insulin-responsive element within the AT1R 3'-UTR. HEK293 cells were transfected with control Luc, Luc(3'-UTR) and different deletions of 3'-UTR, treated with 75 nM insulin, and for 24 h, luciferase activity was measured in the lysates. (**C**) Upper panel: Mapping of HuR-responsive element within the AT1R 3'-UTR. HEK293 cells were transfected with luciferase vectors as described for panel (B) and cotransfected with HuR expression vector. RNA isolated from cells described for panel (B) was subjected to real-time qPCR to assess the mRNA levels of AT1R and loading control. Results were normalized to Luc. The results represent the means ± SD of an average of three independent experiments. *$P < 0.05$ versus Luc. Lower panel: Control blots for HuR overexpression.
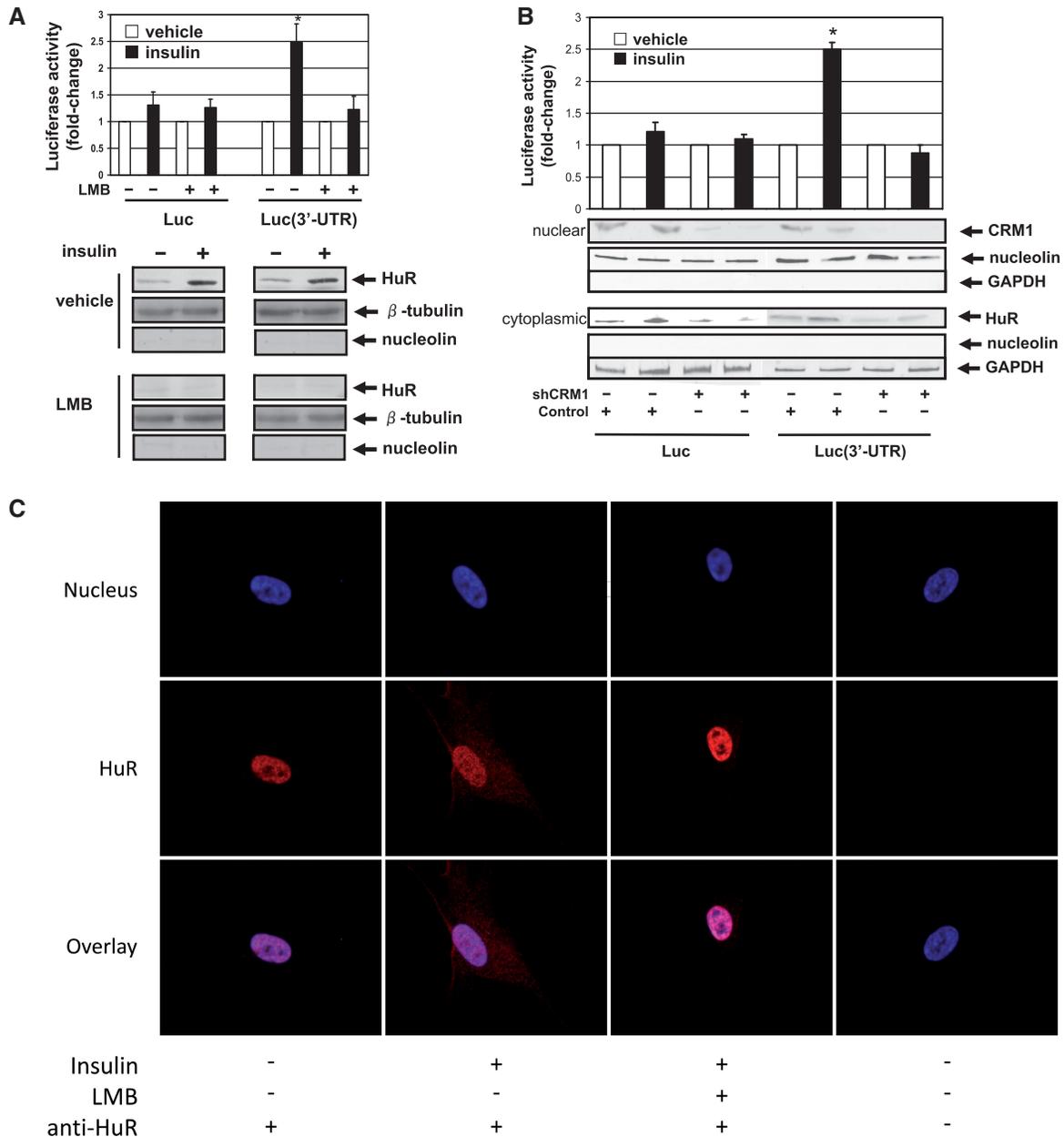
Figure 7. Translocation of HuR mediates insulin effect on AT1R. (**A**) Upper panel: HEK293 cells transfected with Luc or Luc(3′-UTR) were stimulated with insulin in the presence or absence of LMB for 12 h and lysates were measured for luciferase activity. Luciferase results are expressed as fold of promoter control without 3′-UTR in relative light units of firefly luciferase. Results represent the means ± SD of an average of three independent experiments. Lower panel: Protein lysates from cells described for panel (**A**) were extracted and probed for the expression of HuR, nucleolin and β-tubulin. (**B**) Upper panel: HEK293 cells transfected with Luc or Luc(3′-UTR) and cotransfected with either shCRM1 or shControl. Insulin stimulation (75 nM) for 24 h followed by determination of luciferase activity on the cell lysates. Luciferase results are expressed as fold of promoter control without 3′-UTR in relative light units of firefly luciferase. Results represent the means ± SD of an average of three independent experiments. Lower panel: Protein lysates from cells described for panel (**B**) were extracted and probed for the expression of CRM1, HuR, nucleolin and GAPDH. (**C**) Immunofluorescence analysis of HuR. HuR signals (red) in either untreated or insulin treated (75 nM for 12 h) coronary artery VSMC cells. Red, HuR fluorescence; blue, DAPI staining to visualize nuclei; overlay, overlap of the two signals. Note the distinct overlap of DAPI and HuR signals in untreated cells; while insulin-stimulated cells also exhibit abundant nuclear HuR, the treatment causes a substantial increase in the cytoplasmic HuR signal, not seen in untreated cells. Exposure to LMB prevented the insulin effect.

LMB prevents insulin-induced HuR translocation into cytoplasm and insulin response

To address if the nuclear export of HuR is required for insulin response, HEK293 cells expressing Luc(3′-UTR) were exposed to LMB, a CRM1 inhibitor which has been proposed to affect HuR translocation through the nuclear envelope (20). We treated control Luc- or Luc(3′-UTR)-transfected HEK293 cells for 12 h either with 10 ng/ml LMB or vehicle, followed by insulin stimulation and measurement of luciferase activity. Pre-treatment of LMB potently inhibits the effect of insulin measured by luciferase activities (Figure 7A,

upper panel) and prevents HuR translocation to cytoplasm (Figure 7A, lower panel). To verify the effective inhibition of CRM1 by LMB, HEK293 cells were either treated with LMB or left untreated, and whole-cell, nuclear and cytoplasmic lysates were subjected to western blot analysis. As shown in Figure 7A (lower panel), exposure of cells to LMB reduced cytoplasmic HuR levels. Moreover, LMB inhibited insulin-induced HuR increase in the cytoplasm. As controls for loading and purity of subcellular fractionation, β-tubulin and nucleolin were measured. To further confirm the role of CRM1 on insulin effect, insulin effect was assessed in CRM1-silenced cells. As shown in Figure 7B, transfection of HEK293 cells with a vector expressing CRM1 shRNA inhibited CRM1 levels by ∼85% and attenuated the insulin response compared with what was observed in control shRNA-transfected cells. Immunofluorescence analysis verified the cytoplasmic increase in HuR levels in coronary artery VSMC and the inhibition of HuR translocation by LMB (Figure 7C). This data is consistent with the shuttling of HuR to the cytoplasm having a key role in the signaling of insulin-triggered AT1R upregulation.

DISCUSSION

In this study, we set out to explore the mechanisms underlying the regulation of AT1R by insulin. The starting point for this investigation was the discovery that insulin upregulates AT1R expression by stabilizing its mRNA. We found this effect to be transferable to a reporter gene by including 3′-UTR of AT1R after luciferase gene. Next, we searched for insulin-regulated RBPs interacting with AT1R 3′-UTR and identified HuR as such a protein. Several lines of evidence support the role of HuR as a mediator of insulin effect. HuR and insulin both increase AT1R expression and mRNA levels via stabilization of AT1R mRNA and they share functional elements in the same region within the 3′-UTR. Insulin induces translocation of HuR into cytoplasm. Inhibition of HuR translocation prevents the effect of insulin on AT1R.

HuR is likely to regulate AT1R expression in blood vessels and myocardium in common cardiovascular disorders. In the VSMC of normal coronary arteries, HuR expression is low and nuclear whereas in atherosclerotic arteries HuR is high and cytoplasmic, potentially a contributing mechanism underlying increased AT1R expression (21,22). Similarly, in endothelial cells pro-atherosclerotic conditions increase HuR expression whereas anti-atherosclerotic treatments decrease HuR and AT1R expression (23). After myocardial infarction, both inhibition of AT1R and knockdown of HuR expression reduce the severity of pro-inflammatory responses and contribute to improved left ventricular function and remodeling (24).

HuR is primarily localized in the nucleus and can shuttle between the nucleus and cytoplasm by virtue of its shuttling signal, HNS (HuR nucleocytoplasmic shuttling sequence) located in the hinge region between its second and third RNA recognition motifs (19).

Several factors increase the cytoplasmic levels of HuR suggesting that the nuclear export of HuR has a role in the regulation of mRNA stability. For example, under stress, the increased cytoplasmic HuR level led to the stabilization of p21CIP1 mRNA (25). Similarly, during the cell division cycle, HuR stabilized mRNAs encoding cell cycle regulatory genes in accordance with its fluctuating presence in the cytoplasm (26). Investigation into the mechanism of insulin-mediated regulation of AT1R mRNA by HuR showed that HuR was translocated to cytoplasm in response to insulin. In addition, the effects of insulin on AT1R were attenuated by a reduction in the cytoplasmic levels of HuR either by HuR siRNA or by inhibition of HuR translocation. Based on these findings, we propose that insulin regulates AT1R expression by promoting the nuclear export of HuR and consequently by increasing the stability of AT1R mRNA.

In conclusion, our study presents evidence for the importance of HuR as a downstream mediator of insulin-induced AT1R regulation. These results are consistent with the proposed models of HuR function in mRNA stabilization. The dynamic recruitment of varying RBPs to individual mRNAs determines the fate of each transcript in respect to splicing, nuclear export, stability and ultimately translation. HuR has effects on large number of genes and currently we do not know whether the insulin-induced increase in HuR has general effects on gene expression, or rather exerts effects restricted to AT1R transcription. It is tempting to speculate that at least some deleterious effects of hyperinsulinemia could be modified by targeting HuR. Key questions remain how HuR, a widely expressed protein with broad target specificity, can regulate specific mRNAs. HuR has been reported to act in concert with other RBPs like AUF1 (27), T-cell intracellular antigen 1 (TIA1) (28) and tristetraprolin (TTP) (29) and thus protein–protein interaction enable HuR to have transcript specific effects. Studies to address if other RBPs or miRNAs are involved in the regulation of AT1R expression in concert with HuR are underway in our laboratory. While additional details of this regulatory pathway await further experimental demonstration, the findings presented here underscore the role of HuR in the post-transcriptional regulatory process of AT1R mRNA.

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