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ORIGINAL ARTICLE

RNA-binding protein HuR enhances mineralocorticoid signaling in renal KC3AC1 cells under hypotonicity

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Abstract Mineralocorticoid receptor (MR) mediates the sodium-retaining action of aldosterone in the distal nephron. Herein, we decipher mechanisms by which hypotonicity increases MR expression in renal principal cells. We identify HuR (human antigen R), an mRNA-stabilizing protein, as an important posttranscriptional regulator of MR expression. Hypotonicity triggers a rapid and reversible nuclear export of HuR in renal KC3AC1 cells, as quantified by high-throughput microscopy. We also identify a key hairpin motif in the 3′-untranslated region of MR transcript, pivotal for the interaction with HuR and its stabilizing function. Next, we show that hypotonicity increases MR recruitment onto *Sgk1* promoter, a well-known MR target gene, thereby enhancing aldosterone responsiveness. Our data shed new light on the crucial role of HuR as a stabilizing factor for the MR transcript and provide evidence for a short autoregulatory loop in which expression of a nuclear receptor transcriptionally regulating water and sodium balance is controlled by osmotic tone.

Keywords Mineralocorticoid receptor · Aldosterone · Posttranscriptional regulation · Osmotic stress · Sodium transport · Nucleocytoplasmic trafficking

Abbreviations

MR	Mineralocorticoid receptor
HuR	Human antigen R
Sgk1	Serum and glucocorticoid-regulated kinase 1
Tis11b	Tetradecanoyl phorbol acetate inducible sequence 11b
RBP	RNA-binding protein
3′-UTR	3′-Untranslated region
ELAVL1	Embryonic lethal, abnormal vision, Drosophila homolog-like 1
EGF	Epidermal growth factor
T3	Triiodothyronine

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DCC	Dextran charcoal-coated
RNP-IP	Ribonucleoprotein immunoprecipitation
Luc	Luciferase
DNA-ChiP	DNA-chromatin immunoprecipitation
Iso	Isotonicity
Hypo	Hypotonicity
HTM	High-throughput microscopy
LMB	Leptomycin B
PKC	Protein kinase C
miRNA	MicroRNA
PIS	Preimmune serum
Ucp1	Uncoupling protein 1
MTA	Metastasis-associate 1
PTMA	Prothymosin

Introduction

In the distal nephron, Na^+ reabsorption is tightly regulated by aldosterone, a steroid hormone that activates the mineralocorticoid receptor (MR, *Nr3c2*). MR, a transcription factor, stimulates expression of ionic transporters or regulators, such as the α subunit of the epithelial Na^+ channel (*Scnn1a*) and the serum and glucocorticoid-regulated kinase 1 (*Sgk1),* which are involved in transepithelial Na^+ reabsorption [1]. A growing body of evidence suggests that modulation of MR expression greatly affects renal function. Indeed, overactivation of the mineralocorticoid signaling pathway leads to marked Na^+ retention, hypervolemia, high blood pressure, and subsequent renal damage [2, 3]. Conversely, abnormally low levels of mineralocorticoid signaling at birth lead to partial aldosterone resistance in human newborns reminiscent of the MR haploinsufficiency observed in autosomal dominant pseudohypoaldosteronism [4, 5]. These observations highlight the importance of fine-tuning modulation of MR levels; however, molecular mechanisms governing regulation of MR expression remain elusive.

In the nephron, MR expression levels are the highest in the cortex, notably in the distal convoluted tubules and the cortical collecting ducts, in which the tubular lumen is hypotonic (50 mOsm/kg), and lowest in the medulla [6], which is hypertonic (1200 mOsm/kg). Based on these observations, we investigated how MR expression could be modulated by variations in the extracellular fluid tonicity generated by the corticopapillary gradient [7]. Using both differentiated renal KC3AC1 cells and mouse models subjected to water deprivation or intoxication or in animals treated with diuretics, we previously demonstrated that hypertonicity greatly decreases renal MR levels, whereas conversely, MR transcript and protein levels increase in vitro and in vivo under hypotonicity [8]. We consequently elucidated a novel posttranscriptional mechanism based on the recruitment of Tis11b (tetradecanoyl phorbol acetate inducible sequence 11b), a RNA-binding protein (RBP) interacting with MR transcript 3′-untranslated region (3′-UTR), thus modulating mRNA turnover in response to osmotic stress [9].

Herein, we decipher for the first time the molecular events underlying hypotonicity-induced MR expression and identify HuR (human antigen R), another RBP member of the Hu family [10, 11] as a novel posttranscriptional regulator of MR expression. HuR, originally described as *ELAVL1* (embryonic lethal, abnormal vision, *Drosophila* homolog-like 1), and encoded by the *ELAVL1* gene in humans, was shown to modulate transcript stability and translation in adaptive responses to various stimuli, such as hypoxia, ATP depletion, and oxidative stress [10, 12]. HuR is considered as a master regulator of cell survival and proliferation [13], and its knockdown is lethal in mice [14], thus highlighting its fundamental role in key cellular functions. We now show that HuR is specifically and rapidly exported to the cytoplasm of renal cells under hypotonicity, where it interacts with a pivotal hairpin motif located within the MR 3′-UTR to stabilize and increase MR levels, thereby modulating MR signaling, as demonstrated by increased recruitment of MR onto *Sgk1* gene promoter. Overall, our study reveals a novel regulatory mechanism by which transient HuR nucleocytoplasmic shuttling facilitates rapid adaptive responses to osmotic stress in renal cells.

Materials and methods

Cell culture

HEK293T cells were cultured in DMEM medium supplemented with 2 mM glutamine, 20 mM HEPES, pH 7.4, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (Biowest). All reagents were from Life Technologies unless otherwise stated. KC3AC1 cells were cultured under isotonic conditions, at 300 mOsmol/l, as previously described [8]. Hypotonic conditions (150 mOsmol/l) were achieved by twofold dilution of the medium in sterile water. As stated in the corresponding figure legend, KC3AC1 cells were cultured on filters as previously described [8] to mimic asymmetrical conditions where renal cells are physiologically exposed to isotonic basolateral interstitial fluid and hypotonic luminal fluid ranging from 60 to 300 mOsmol/l. Minimal medium lacking dexamethasone (Sigma-Aldrich), epidermal growth factor (EGF, Peprotech), triiodothyronine (T3, Sigma-Aldrich), and dextran charcoal-coated (DCC) serum was used to study aldosterone action (Sigma-Aldrich).

Plasmid constructs

FL or truncated human MR (hMR)-3′-UTR sequences were obtained by PCR from genomic DNA. These sequences were inserted into pMiR-Report vector, downstream of the luciferase reporter gene, as previously described [9]. Human R2–3 MR 3′-UTR was generated by PCR with the R2 sense and R3 antisense primers. Site-directed mutagenesis of the R3 hairpin motif was performed with the QuikChange kit (Stratagene) with specific primers. All primer sequences are indicated in Table S2.

Transfection assays

HEK293T cells were transfected with luciferase constructs (1 ng) and with an empty or HuR-encoding pTarget vector (25 ng), in the presence of Lipofectamine 2000 (Life Technologies). Luciferase activities, normalized relative to *Renilla* luciferase activities, were measured 24 h posttransfection, as previously described [15].

Ribonucleoprotein immunoprecipitation (RNP-IP)

RNP-IP was performed as previously described [15] in HEK293T cells cotransfected with luciferase (Luc)-MR 3′-UTR- and HuR-encoding plasmids (500 and 100 ng, respectively) or in KC3AC1 cells exposed to hypotonicity. Luc-MR 3′-UTR, MR, or *Hprt* transcripts were amplified with the specific primers described in Table S2.

DNA-chromatin immunoprecipitation (DNA-ChiP)

Differentiated KC3AC1 cells were cultured in isotonic (Iso) or hypotonic (Hypo) medium for 18 h, and DNA-ChIP was then performed with the HighCell# ChIP kit (Diagenode), as previously described [16]. Sheared chromatin (1.3 mg protein) was resuspended in 500 µl of C1 buffer containing 7 µg of 39 N anti-MR antibody and 25 µl of protein A-coated beads, and incubated overnight at 4 °C in a rotating shaker. DNA fragments from the immunoprecipitated chromatin and from input samples (1% sheared chromatin) were eluted with DNA Isolation Buffer (Diagenode) supplemented with 1% proteinase K, and quantified by qPCR with specific genomic primers (see Table S2).

RNA interference

KC3AC1 cells were transfected twice (on days 6 and 7 of cell culture) with 50 nM HuR_siRNA or scrambled_siRNA (#s2090, and #4390843, Life Technologies), with the RNAimax transfection reagent (Life Technologies). Cells were then exposed to hypotonic conditions for various periods of time and MR expression was quantified by RT-qPCR.

Quantitative RT-PCR

Total RNA was isolated from cells or tissues with TRI Reagent (Euromedex) using a TissueLyser apparatus (Qiagen). RT-qPCR was performed as previously described [8]. The RNA was treated with DNAse I (New England Biolabs), and 1 µg of total RNA was then reverse-transcribed with the High Capacity cDNA reverse transcription kit. Relative gene expression was analyzed on a QuantStudio™ 6 Flex Real-Time PCR System (Life Technologies), by qPCR with the Power SYBR® Green PCR Master Mix (Life Technologies) and the specific primers described in Table S2. Relative expression in a given sample was calculated as amol of the specific gene/fmol of *18S*, or amol of *36b4 or Hprt* mRNA.

Immunocytochemistry

KC3AC1 cells were seeded in 4-well tissue culture chambers (Sarstedt). After 24 h, the cells were incubated under isotonic (Iso) or hypotonic (Hypo) condition during the indicated time period and fixed with 4% paraformaldehyde (Electron Microscopy Sciences). Cells were analyzed by immunocytochemistry with a goat Alexa Fluor 555-coupled secondary antibody raised against the mouse HuR antibody, as previously described [17]. Detailed information about the antibodies is provided in Table S3. Nuclear counterstaining was performed with 0.5 µg/ml DAPI. Cells were observed with the Olympus BX61 and images were acquired at 40× using a Retiga-2000R monochrome camera (Q Imaging).

Automated high-throughput microscopy (HTM)

Cytoplasmic and nuclear fluorescence intensities were quantified with an Arrayscan VTI (ThermoFisher Scientific). The Molecular Translocation V4 Bioapplication (vHCS Scan, version 6.3.1) was used to calculate the ratio of nuclear-to-cytoplasmic fluorescence, as previously described [18]. Briefly, nuclear and cytoplasmic masks were created (ring width = 35 and fixed threshold = 55) and the ratio of nuclear-to-cytoplasmic fluorescence within these masks was determined from the mean values obtained (>5000 cells per condition). A cell surface calculation of KC3AC1 cells exposed to isotonic or hypotonic condition was performed by HTM, to demonstrate renal cell surface increase.

Western blot analysis

Differentiated KC3AC1 cells were lysed in lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1% Triton X-100, and 1% protease inhibitors). The debris were removed by centrifugation and the resulting protein extracts were subjected to SDS-PAGE and processed for the multiplex detection of HuR or MR protein, together with α-tubulin or β-actin protein as a loading control. Signal fluorescence intensity was determined with an Odyssey® Fc (Li-Cor). Detailed information about the antibodies used is provided in Table S3.

Statistical analysis

The data are presented as mean ± SEM. One-way ANOVA test or Mann–Whitney *U* test was used, as appropriate, to assess the significance of differences (Graphpad Prism software). A p value lower than 0.05 was considered significant: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Results

HuR mediates hypotonicity-induced increase in MR expression

Hypotonicity significantly increased MR expression in renal KC3AC1 cells (Fig. 1a–c), confirming our earlier observations [8]. A fourfold increase in MR mRNA levels was observed and maintained under hypotonicity even in the presence of the transcription inhibitor DRB (Fig. S1), suggesting that hypotonicity modulates MR levels via an increase in mRNA stability. Likewise, MR expression increased with synthetic hypotonic media, demonstrating that increase of MR abundance is exclusively due to a reduced extracellular osmolarity, rather than other mechanisms, such as energy depletion (Fig. S2). We hypothesized that HuR, a RNA-binding protein known to regulate short-lived mRNA [10, 19], might be involved in such tonicity-mediated regulation. KC3AC1 cells were transfected with specific HuR siRNA (HuR_siRNA) to knockdown endogenous HuR as revealed by western blotting (Fig. 1d). Consistent with the stabilizing role of HuR, HuR depletion significantly prevented the hypotonicity-induced increase in MR steady-state levels (Fig. 1e). As HuR regulates its targets by interacting with their 3′-UTR, we assumed a possible interaction between HuR and MR transcript in KC3AC1 cells. Ribonucleoprotein immunoprecipitation (RNP-IP), followed by reverse transcription-PCR, indicated that endogenous HuR and MR transcripts form a ribonucleoprotein complex only under hypotonicity (Fig. 1f), thus supporting a role of HuR in MR mRNA stability.

Nuclear export of HuR is rapidly and reversibly induced by hypotonicity

HuR shuttles between nucleus and cytoplasm to modulate mRNA turnover in response to cellular stress or mitogens [12, 20, 21]. We investigated HuR trafficking in response to hypotonicity in KC3AC1 cells. Figure 2a showed that hypotonicity induces a clear relocalization of HuR to the cytoplasm. Quantification of the nuclear/cytoplasmic ratio (N/C ratio) using automated high-throughput microscopy (HTM) [17] revealed that hypotonicity leads to a robust decrease in N/C ratio, consistent with the rapid export of HuR to the cytoplasm, within as little as 5 min (Fig. 2b). This nuclear export of HuR was not observed in human fibroblasts, consistent with a renal cell-specific effect (Fig. S3). As expected, hypotonicity also induced a rapid swelling of the cell, as demonstrated by the 29% increase in renal cell surface area calculated by HTM (from 1186 ± 12 μm^2, $n = 900$, under isotonic conditions to 1530 ± 16 μm^2, $n = 1650$, after 15 min of exposure to hypotonicity, $*p < 0.001$), corresponding to ~46% increase in cell volume. Nuclear export of HuR was rapidly reversible, as shown by the decrease in HuR labeling in the cytoplasm, whereas nuclear HuR gradually increased when isotonic conditions were restored (Fig. 2c). This was accompanied by a significant increase in N/C ratio (Fig. 2d).

We also provide evidence that HuR nuclear export under hypotonicity is a Chromosomal Maintenance 1 (CRM1, also known as Exportin 1)-independent mechanism as HuR shuttling was not affected by Leptomycine B, an inhibitor of this export pathway (Fig. S4a and S4c). This result was confirmed by HTM quantification, since both conditions led to a significant decrease in N/C ratio (Fig. S4b and S4d).

HuR functionally interacts with a pivotal hairpin motif located in MR 3′-UTR

HuR was shown to interact with various RNA motifs consisting of $^{U}_{A}$UUUA pentamers (AU-rich sequences) or U-rich stretches. It was also suggested that hairpin RNA secondary structures contribute to the specificity of HuR binding [22–24]. We found more than 50 AU-rich or U-rich motifs along the ~2.8 kb length of the MR 3′-UTR (Table S1; Fig. 3). To identify binding sites for HuR, we used plasmid constructs that we previously generated [9], in which the full length (FL) human MR 3′-UTR or its truncated regions R1, R2, R3, and R4 (originally referred to as hΔ1, hΔ2, hΔ3 and hΔ4) were inserted downstream of the luciferase reporter gene (Fig. 3a). KC3AC1 cells

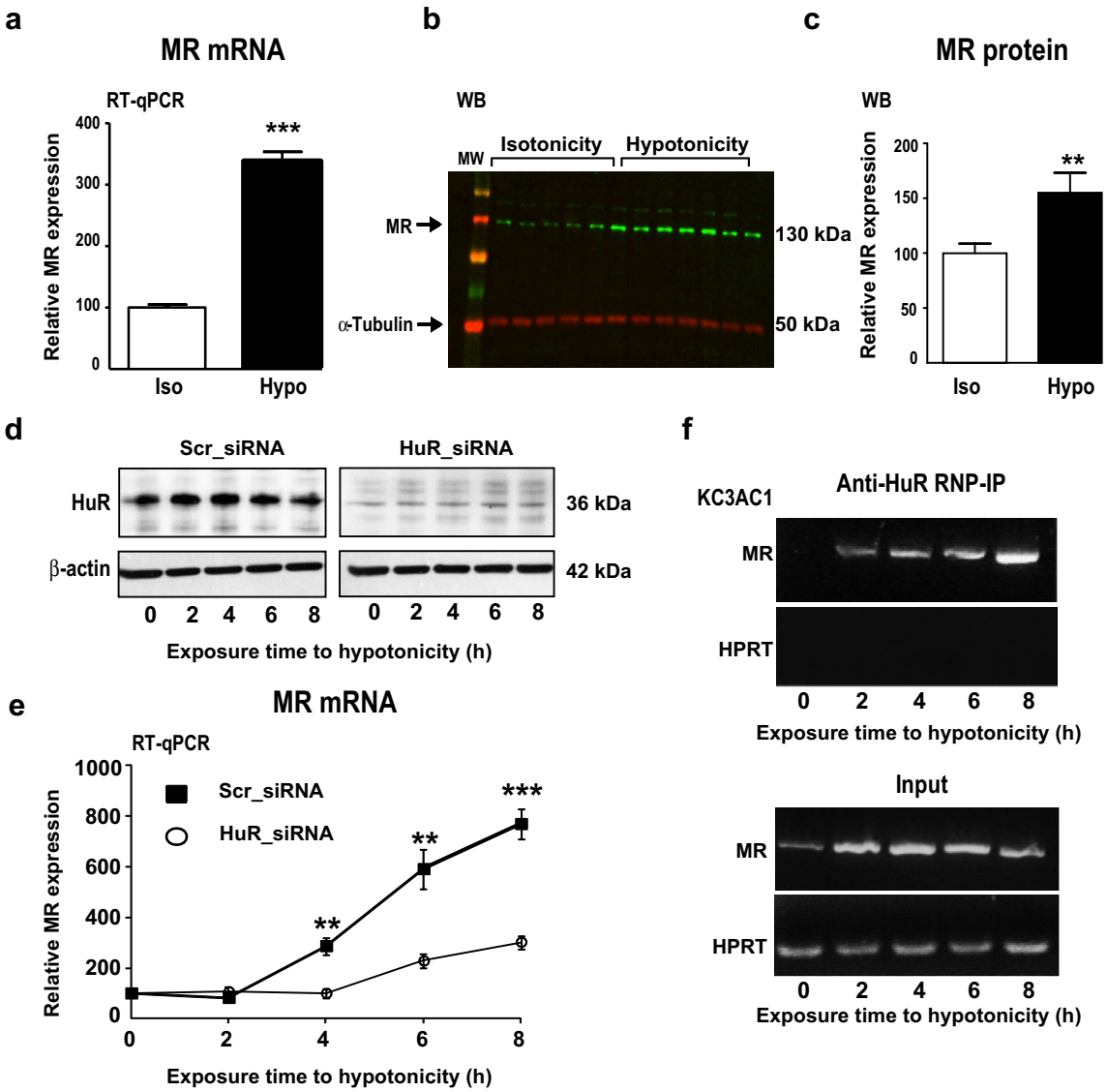


Fig. 1 HuR mediates hypotonicity-induced increase in MR expression in renal cells. KC3AC1 cells were exposed to isotonicity (Iso) or hypotonicity (Hypo) for 6 h for RT-qPCR (**a**) or 18 h for WB (**b**, **c**). Transcript and protein levels were normalized relative to *18S* rRNA and α-tubulin, respectively, and are expressed as a percentage of MR mRNA or MR protein levels in cells exposed to Iso conditions. Data are mean ± SEM of three independent experiments (**$p < 0.01$; ***$p < 0.001$). Analysis of HuR protein (**d**) and MR mRNA levels (**e**) on KC3AC1 cells transfected with scrambled (Scr) or HuR_siRNA and exposed to hypotonic conditions. HuR protein and MR mRNA were normalized, respectively, to β-actin and *18S* rRNA levels. Data are mean ± SEM of three independent experiments (**$p < 0.01$; ***$p < 0.001$). **f** RNP-IP were performed on KC3AC1 cells exposed to hypotonic conditions (0–8 h). Specific RNP-IP complexes of HuR and MR mRNA are represented (*upper panel*); the *lower panel* (input) shows amplification of the MR and hypoxanthine–guanine phosphoribosyltransferase (*Hprt*) transcripts in 10% non-immunoprecipitated lysates. One experiment representative of three independent experiments carried out is shown

were cotransfected with each of these luciferase constructs along with HuR expression vector. In the presence of HuR, a significant 1.5-fold increase in luciferase activity was observed with the FL MR 3′-UTR and with R3 truncated region, whereas no significant change in luciferase activity was detected with the R1, R2, and R4 constructs (Fig. 3a), demonstrating that this R3 region is pivotal for stabilizing function of HuR.

To determine whether the R3 region harbors RNA secondary structures, we used the mFold prediction software (http://unafold.rna.albany.edu/?q=mfold) [25]. A highly conserved hairpin motif containing the UUUAAU U sequence similar to those described for the *PTMA* and *MTA1* genes [22, 26] was identified in this region (Fig. S5). We examined the role of this hairpin motif in the binding of HuR to the R3 region, by mutating the

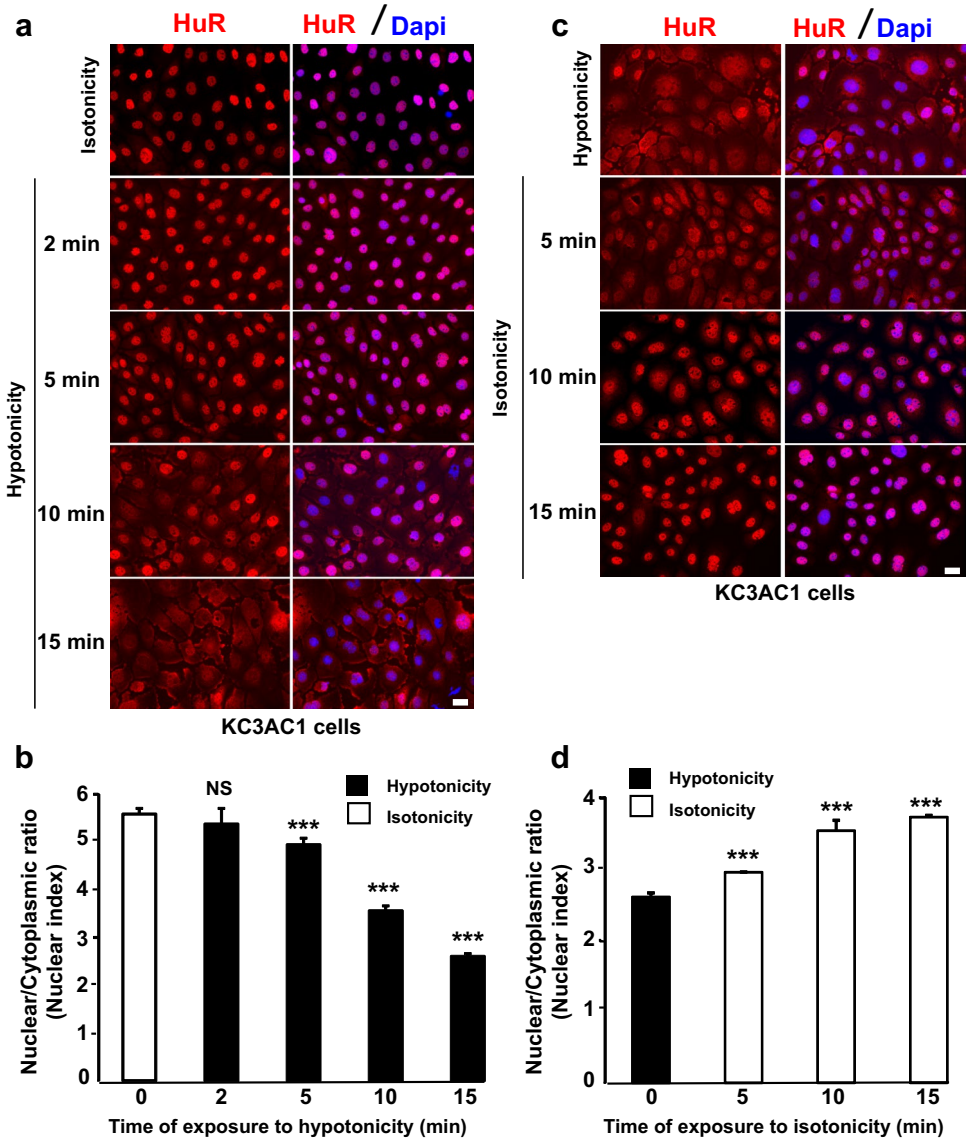


Fig. 2 Nuclear export of HuR is under hypotonicity. **a**, **b** HuR immunostaining in KC3AC1 cells exposed to isotonicity or hypotonicity. The nuclei were stained with DAPI. **a** Pattern of HuR subcellular localization was determined with quantification by HTM. **b** Results are presented as the ratio of nuclear-to-cytoplasmic fluorescence (***$p < 0.001$, $n > 5000$) cells for each set of conditions. **c**, **d** KC3AC1 cells were cultured for 15 min in hypotonic medium (hypotonicity, *black*) and then were returned to isotonic medium (isotonicity, *white*) for the indicated times. Cellular distribution of HuR was quantified by HTM as in **b**

uridine (U) at position +1651 (+1 relative to the stop codon TGA) to cytosine (C) (see arrow in Fig. 3b). This U-to-C substitution totally abolished this RNA secondary structure (Fig. 3b, compare R3 and mutR3). In addition, RNP-IP assays in KC3AC1 cells showed that HuR strongly interacts with MR 3′-UTR as well as with the R3 region (Fig. 3c). In contrast, only weak binding of HuR to the R1 and R2 regions was detected and no specific signal was obtained with R4 region. The U-to-C substitution led to a complete disruption of the mutR3/HuR complexes (Fig. 3c) and abolished mutR3-driven luciferase activity (Fig. 3d), demonstrating the requirement of this hairpin motif for the efficient binding and stabilizing function of HuR.

Antagonism between HuR and Tis11b for the regulation of MR mRNA turnover

Several studies reported competition between RBP for binding to the same target transcripts and their corresponding motifs [21, 27]. To assess potential antagonism between HuR and Tis11b, we generated a luciferase construct encompassing the R2–3 region, which harbors both the key ARE_3 and ARE_4 motifs (R2 region) responsible for the destabilizing effect of Tis11b [9] and the hairpin motif, required for the stabilizing effect of HuR (R3 region, Fig. 3e). Exposure to hypotonic stress for 2 h induced a significant 1.6-fold increase in the R2–3 driven luciferase activity, whereas, as expected, hypertonic stress decreased

the luciferase activity of the R2–3 construct (Fig. 3f). When KC3AC1 cells were exposed to hypotonicity and hypertonicity successively, a large decrease in luciferase activity was still observed, suggesting that HuR-mediated stabilization of MR transcript may be counteracted by the destabilizing action of hypertonicity-induced Tis11b. These results indicated that HuR binding to MR 3′-UTR does not impair subsequent Tis11b binding and that MR mRNA steady-state levels are regulated through the balance between antagonistic actions of stabilizing HuR and destabilizing Tis11b effects on MR mRNA in response to osmotic stresses.

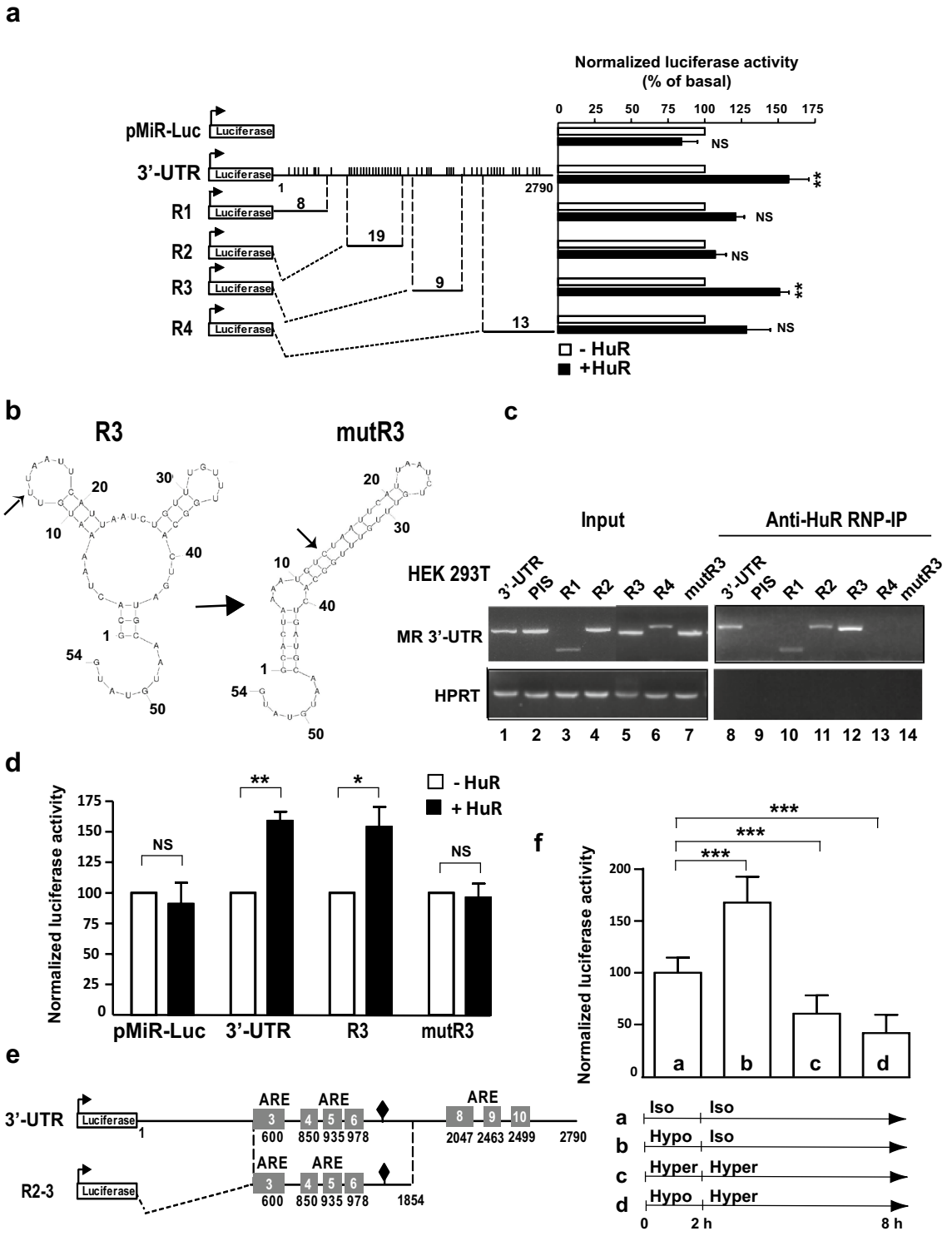
Hypotonicity enhances renal MR signaling

Cortical collecting duct cells are physiologically exposed to isotonic basolateral interstitial fluid and hypotonic luminal fluid ranging from 60 to 300 mOsmol/kg. To mimic such asymmetrical conditions, KC3AC1 cells were cultured on filters as previously described [8]. The application of a hypotonic medium onto the apical surface of the cells led to a threefold increase of MR transcript levels (Fig. 4a). We further investigated the effect of this increase in MR levels on hormone responsiveness, by performing DNA-ChIP experiments on KC3AC1 cells. We found that hypotonicity-induced increase in MR expression enhanced aldosterone-stimulated MR recruitment onto the promoter of *Sgk1* nine times higher than those in isotonic conditions (Fig. 4b). MR was not recruited to the promoter of the *Ucp1* gene used as a negative control. One hour treatment of KC3AC1 cells with aldosterone induced a significant 1.3-fold increase in *Sgk1* mRNA levels under isotonicity, whereas *Sgk1* levels doubled when the cells were bathed apically with hypotonic medium (Fig. 4c). These results suggested that renal cells respond to luminal hypotonicity by promoting the recruitment of MR to the promoter regions of MR target genes, thereby enhancing aldosterone responsiveness.

Discussion

HuR is known to regulate levels of short-lived mRNA, in an adaptive response to various stress stimuli [10]. This RNA-binding protein is mostly located in the nucleus and its export to the cytoplasm is a key prerequisite for stabilization of target transcripts [28]. We find that HuR export in renal cells is rapidly induced by hypotonicity within minutes and is reversible upon return to isotonic conditions. We also demonstrate that leptomycin B (LMB), an inhibitor of the CRM1-dependent export pathway, is unable to inhibit the nuclear export of HuR, whereas LMB was previously shown to inhibit the TGFβ1-induced accumulation of HuR in the cytoplasm of hepatic cells [29], suggesting that HuR is exported from the nucleus in a CRM1-independent manner in renal cells. Interestingly, Fan et al. identified a shuttling sequence in the hinge region of the HuR protein referred to as "HuR nucleocytoplasmic shuttling" sequence [30] which contains key pivotal amino acids phosphorylated by kinases, such as the cell-cycle checkpoint kinase 2, the p38 MAP kinase, or the members of the protein kinase C (PKC) family [12]. Similarly, Doller et al. demonstrated that angiotensin II promoted nuclear export of HuR, which was blocked by the PKC inhibitor rottlerin [31]. It remains to be determined whether these kinases are involved in HuR export in KC3AC1 cells. The subcellular distribution of HuR seems to be highly dependent on cell type, given that HuR accumulates in the cytoplasm under hypotonicity in renal cells but not in human fibroblasts. Overall, our data indicate that hypotonicity should be considered as an external stimulus capable of inducing the nuclear export of HuR, together with UV radiation, hypoxia, energy deprivation, and oxidative stress [12, 32, 33].

Furthermore, we identify a hairpin RNA secondary structure in the R3 region (Fig. S5) similar to that already described in the 3′-UTR of the *PTMA* and *MTA1* transcripts [22, 26], which mediates the physical interaction with HuR. This RNA motif plays a key role in the binding of HuR to the MR transcript and in the stabilization of this transcript, because a U-to-C substitution completely disrupted R3/HuR complexes and abolished R3-driven luciferase activity. However, the functional importance of the secondary structure remains a matter of debate. Indeed, some studies showed that HuR binds to its RNA target with no preference for a hairpin RNA secondary structure [23], whereas others have shown that this secondary structure enhances the recognition of mRNA targets by HuR [34]. Furthermore, we recently showed that Tis11b interacts with simple primary sequences corresponding to the ARE_3 and ARE_4 motifs to exert its destabilizing activity [9]. We and others have also reported the ability of HuR and other RBP to compete for the same recognition sequences. For instance, HuR and AUF1 were shown to bind the same sequence in the 3′-UTR of the androgen receptor [35], whereas Tis11b and HuR had antagonistic effects on the VEGF 3′-UTR in vitro [21]. ARE_3 and ARE_4 are not located in the close vicinity of the hairpin secondary structure, but we could not rule out the possibility of competition between HuR and Tis11b, due to the non-linear structure of mRNA. We, therefore, generated the R2–3 plasmid construct and demonstrated the recruitment of both endogenous HuR and Tis11b to MR 3′-UTR following exposure to hypotonicity and hypertonicity in KC3AC1 cells, thus providing direct evidence for antagonistic effects of both RBP on MR mRNA stability in renal cells. MicroRNA (miRNA), another class of posttranscriptional regulators, has also been shown to interact physically



and functionally with HuR. Combinatorial or cooperative interplay between HuR and miRNA on shared target mRNA molecules may typically result in the enhancement or repression of gene expression [36]. Identification of miRNA regulating MR expression should provide new insights into the mechanisms involved in physiological or pathophysiological situations, such as renal fibrosis in diabetic nephropathy [37]. Finally, we propose a model in which HuR is mostly present in the nucleus of renal cells under isotonicity (Fig. S6) and behaves as a pivotal stabilizing factor following hypotonic stress-induced nuclear export of HuR to the cytoplasm of renal cells, where it

◂**Fig. 3** HuR increases MR-3′-UTR driven luciferase activity through a specific U-rich sequence. **a** Schematic representation of the luciferase (Luc) reporter gene fused to the FL hMR 3′-UTR or to the truncated regions R1–R4 of the hMR-3′-UTR. The number of U-rich motifs (*vertical bars*) is indicated for each mutant. HEK 293T cells were transiently transfected and the luciferase activities of each construct were measured in the absence (*white*) or presence (*black*) of pTarget-HuR plasmid. Data are mean ± SEM of three independent experiments (**$p < 0.01$). **b**, *left* Model generated with mFold Web server for the secondary structure of the last 54 bases of the R3 RNA sequence (positions +1638 to +1692 relative to the stop codon TGA). **b**, *right* Secondary structure of the mutated R3 sequence. The *arrow* indicates the U-to-C mutation. **c** RNP-IP experiments were performed with anti-HuR antibodies or preimmune serum (PIS) as a control, on total cell lysates from HEK 293T cells cotransfected with the pTarget-HuR and pMIR-Luc-MR 3′-UTR vectors. RT-PCR analysis of *Hprt* mRNA (*left panel*) and RNP-IP (*right panel*). Amplification of the MR 3′-UTR or truncated mutants transcripts in inputs (*lanes 1–7*) and in RNP-IP complexes from HuR-transfected cells (*lanes 8, 10–13*). **d** Luciferase activities in HEK 293T cells transfected with empty or pTarget-HuR vectors together with Luc, Luc-MR 3′-UTR, Luc-MR R3, or Luc-MR R3 mutant (mutR3) constructs. Data are mean ± SEM of three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, *NS* non significant). **e** Schematic representation of the Luc-MR-3′-UTR and Luc-MR R2–3 constructs. Luc-MR R2–3 construct harbors both the ARE_{3-6} (Tis11b targets) and the R3 hairpin motif (HuR target) at nucleotide 1639 (*black diamond*). **f** Competition assays between Tis11b destabilizing function and HuR stabilizing function on KC3AC1 cells transfected with the R2–3 construct and pSV-βgal plasmid, and exposed to an extracellular tonicity challenge for 8 h. Luciferase activities normalized relative to β-galactosidase activities are mean ± SEM of three independent experiments (***$p < 0.001$)

binds MR 3′-UTR on a specific hairpin secondary structure. As a consequence, renal MR expression and signaling are enhanced. Importantly, we also demonstrate that this hypotonic stress-induced increase in MR expression is accompanied by increased MR transcriptional activity, thus enhancing and potentiating renal aldosterone responsiveness. These results confirm those of Taruno et al. who showed that hypotonicity increases *Sgk1* mRNA levels in *Xenopus laevis* renal A6 cells, through an intracellular Ca^{2+}-dependent mechanism [38]. HuR appears, therefore, as a physiological regulator of renal MR abundance. It remains to be determined whether such posttranscriptional events occur in vivo, in other nephronic segments or in other MR-expressing tissues. These mechanisms may be relevant to pathophysiological situations in which the renal corticopapillary gradient is altered during osmotic diuresis, such as in nephrogenic diabetes insipidus [39].

Of interest and along with our hypothesis, it is noteworthy that in nephrogenic diabetes insipidus patients, hypernatremia with inappropriately diluted urine [39] is fully consistent with the proposed mechanism in which relative hypoosmotic stress and HuR intervention in the collecting ducts stimulate MR expression and thereby increase sodium reabsorption through enhanced aldosterone signaling. Moreover, it is interesting to note that HuR

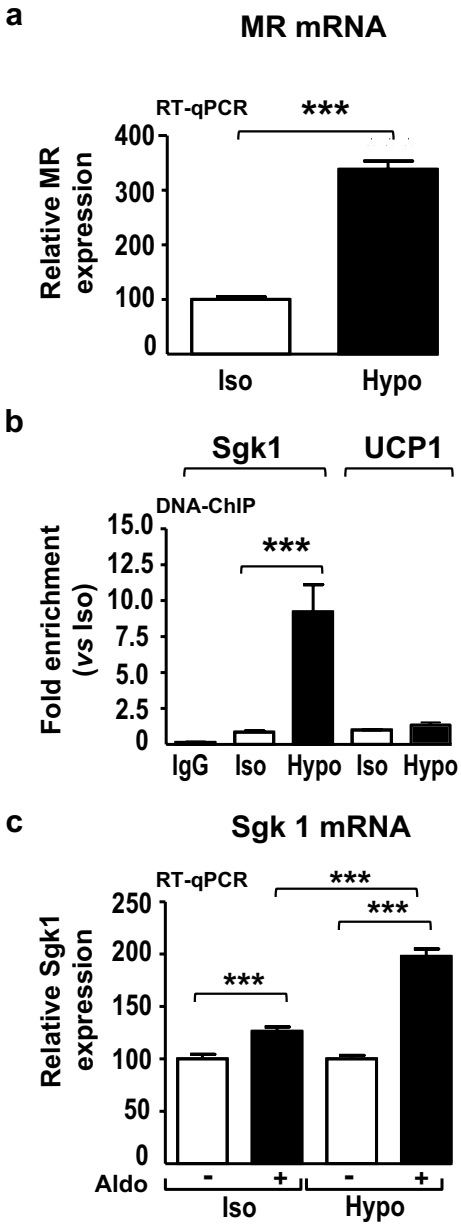


Fig. 4 Hypotonicity potentiates aldosterone responsiveness in renal cells. **a** MR expression analysis by RT-qPCR on KC3AC1 cells cultured on filters subjected or not to hypotonic conditions at their apical surface for 6 h. Data are mean ± SEM of two independent experiments. ***$p < 0.001$. **b** Analysis of MR recruitment onto *Sgk1* promoter by DNA-ChIP. KC3AC1 cells under isotonic (Iso) or hypotonic (Hypo) conditions were stimulated with 100 nM aldosterone for 1 h. DNA was quantified by qPCR targeting the *Sgk1* promoter region. Data expressed as DNA fold-enrichment in hypoconditions relative to isoconditions are mean ± SEM of three independent experiments (***$p < 0.001$). The promoter region of the uncoupling protein 1 (*Ucp1*) gene was used as a negative control. **c** KC3AC1 cells grown on filters were cultured for 24 h in minimal medium before their exposure or not to hypotonicity at their apical face for 18 h. The following day, cells were stimulated or not with 10 nM aldosterone for 1 h in minimal medium. MR expression was analyzed by RT-qPCR and normalized relative to *36b4* mRNA. Data are mean ± SEM of three independent experiments (***$p < 0.001$)

is also involved in diabetic nephropathy by inducing NOD2 expression and mRNA stability [40].

Collectively, our results unveil a novel and original regulatory mechanism in which HuR constitutes an important regulator enabling rapid adaptive responses of renal cells to osmotic stress. These findings provide new insights into the molecular events underlying the kidney specific, MR-mediated aldosterone responsiveness, and may, therefore, have major implications for the pathogenesis of renal dysfunction, sodium retention, and mineralocorticoid resistance. Beside MR and renal physiology, such a posttranscriptional control of gene expression might bring important new conceptual advance highlighting a novel autoregulatory loop in which expression and function of a transcription factor are likely controlled by feedback inputs.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no competing interests.

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