Human-Antigen R (HuR) Expression in Hypertension
Downregulation of the mRNA Stabilizing Protein HuR in Genetic Hypertension

Stephan Klöss, Daniela Rodenbach, Reingart Bordel, Alexander Mülsch

Abstract—In aged spontaneously hypertensive rats (SHR), vasorelaxant responses to NO are attenuated compared with normotensive control rats (Wistar-Kyoto [WKY]) because of a decreased expression of the important NO receptor soluble guanylyl cyclase (sGC). Because the expression of sGC subunits $\alpha_1$ and $\beta_1$ is controlled at the post-transcriptional level by the mRNA-binding protein human-antigen R (HuR), we now assessed whether or not altered expression of HuR could account for downregulation of sGC$\alpha_1$ and sGC$\beta_1$ in genetic hypertension. The expression of HuR (and sGC$\alpha_1$ and sGC$\beta_1$) in aortas from aged SHR was significantly decreased at the mRNA and protein level compared with age-matched WKY rats, whereas expression of HuR was not different in prehypertensive young (2 months of age) SHR and age-matched WKY rats. The mRNA-binding activity of HuR in native aortic protein extracts from aged SHR was markedly reduced compared with normotensive WKY rats, as detected by RNA electrophoretic mobility shift analysis, using biotin-labeled adenine and uracil (AU)–rich element (ARE)–containing RNA probes from the $3'-$untranslated region of sGC$\alpha_1$ and sGC$\beta_1$. In contrast, ARE-binding activity was not different between prehypertensive young and young WKY rats. In vitro RNA degradation assays using the same AU-rich sGC mRNA probes revealed an accelerated sGC$\alpha_1$ and sGC$\beta_1$ mRNA decay in the presence of native protein extract from hypertensive SHR, which was less rapid with aortic protein from normotensive WKY rats. These findings suggest that in this animal model of genetic hypertension, the reduced expression of sGC subunits is mediated by downregulation of the sGC mRNA–stabilizing protein HuR. (Hypertension. 2005;45:1200-1206.)

Key Words: rats, spontaneously hypertensive ■ aorta ■ gene regulation ■ hypertension, genetic

Chronic hypertension is associated with functional and morphological alterations of the vessel wall (ie, dysfunctional vascular endothelium and thickening of the smooth muscle layer). The pathomechanisms accounting for hypertension-induced vascular alterations are likely to be multifactorial. A major homeostatic factor in the vessel wall is NO, which is generated from L-arginine by endothelial NO synthase. NO reduces vascular tone by activation of soluble guanylyl cyclase (sGC) and stimulation of cGMP formation and cGMP-activated protein kinase-I (cGK-I). Activated cGK-I reduces vascular tone by interference with intracellular Ca$^{2+}$ mobilization and inhibition of contractile filament function.

In animal models of hypertension such as the spontaneously hypertensive rat (SHR), disturbances of the NO–cGMP system contribute to vascular dysfunction. Thus, increased oxidative stress interfering with endogenous NO bioavailability and reduced expression of sGC interfering with NO downstream signaling have been reported. The expression of sGC is subject to post-transcriptional regulation. The elav-like (embryonic-lethal abnormal vision) mRNA-binding protein human-antigen R (HuR) stabilizes the sGC$\alpha_1$ mRNA by binding to highly conserved AU-rich elements (AREs; AUUUA) in the $3'-$untranslated region (3'UTR). AREs are targeted for rapid mRNA decay, and thus the presence of AREs within the $3'-$untranslated region (3'UTR) of numerous mRNAs plays a critical role in regulating mRNA stability and degradation. Understanding of the regulation of HuR activity and expression has emerged only recently. Thus, we have shown that cGMP- and cAMP-eliciting agonists decrease HuR expression in rat aortic tissue and cultured rat aortic smooth muscle cells and, at the same time, decrease expression of sGC$\alpha_1$ and sGC$\beta_1$ subunits. Cyclic nucleotide-induced downregulation of HuR expression occurred via activation of Fos/activator protein-1 transcription factors. These findings indicate that HuR is not only an important factor controlling vascular gene expression, but is also subject to control by vasoactive factors that regulate cGMP and cAMP levels. In the current study, we wished to test whether or not the expression and function of HuR...
the mRNA-binding protein HuR in rat aorta is affected by genetic hypertension.

**Materials and Methods**

The antibodies, oligonucleotides, and chemicals for these studies can be found in an online supplement available at http://www.hypertensionaha.org.

**Animals**

Investigations were performed with isolated aortic rings from 2-month-old prehypertensive and aged (15 to 18 months) hypertensive (SHR) male rats and normotensive age-matched Wistar-Kyoto (WKY) rats (n = 4 to 6 in each group). For more details about these rats, see the online supplement.

**Preparation of the Total Protein From Nuclear and Cytosolic Extracts**

For more details on preparation of the total protein from nuclear and cytosolic extracts, see the online supplement.

**Construction and Purification of the Glutathione S-Transferase–HuR Fusion Protein**

The plasmid construct pGEX-HuR (generated from pGEX2T) was prepared as described. For the amplification and purification of the glutathione S-transferase (GST)–HuR fusion protein, see the online supplement.

**Western Blots**

Western blotting of total protein extracts for the detection of HuR, α-actin, and sGC subunits was performed as described previously.

**Reverse Transcription–Polymerase Chain Reaction**

The isolation of total RNA from rat aorta and the RT-PCR for HuR, sGCα1, and sGCβ1 subunits, and elongation factor II (ef II) mRNA were performed exactly as described previously.

**Synthesis of sGCα1 and sGCβ1 3′-UTR mRNA by In Vitro Transcription**

Total RNA of rat aortic tissue was used as a template for RT-PCR amplification of the 3′-UTR of sGCα1 and sGCβ1 cDNA regions as described. For more details, see the online supplement.

**Electrophoretic Mobility Shift Assay**

Electrophoretic mobility shift assays (EMSA) and supershift assays with total native protein extracts from rat aortic tissue were performed essentially as described. The oligonucleotide sequences can be found in the online supplement.

**In Vitro RNA Degradation Assay**

More details on the in vitro RNA degradation assay can be found in the online supplement.

**Northern Blots**

Total RNA samples were fractionated and electrophoresed as described previously. For more information, see the online supplement.

**Statistics**

Where appropriate, data were analyzed for significance of differences using ANOVA. A *P* value <0.05 was considered significant. When comparing multiple means, the Bonferroni correction was applied.

**Results**

**Influence of Chronic Hypertension on HuR Expression**

To study the influence of long-lasting genetic hypertension on expression of the mRNA-stabilizing protein HuR, endothelium-denuded isolated aortae of young (2 months of age) prehypertensive and old (15 to 16 months of age) hypertensive SHR and age-matched normotensive WKY rats were analyzed. An HuR immunoreactive band migrating at 34 kDa (Figure 1A) was identified in all aortae. The expression of HuR protein was significantly decreased (*P*<0.05; n = 6 to 7 rats; ANOVA) in old SHR compared with age-matched WKY rats (Figure 1A). As shown previously, chronic hypertension was also associated with a decrease of both sGCα1 and β1 subunits (Figure 1A) but did not alter the expression of α-actin (Figure 1A). The changes in HuR protein expression were accompanied by similar changes in HuR mRNA abundance (*P*<0.05; n = 3 rats; ANOVA), as shown by RT-PCR (Figure 1B) and Northern blotting (Figure 1C). In contrast, the protein content (Figure 2A) and mRNA expression rate (Figure 2B) of HuR were not different in young rats of either strain. However, the protein synthesis of HuR was markedly reduced in old SHR compared with young SHR (Figure 2C). Moreover, the content of ef II mRNA was not affected by hypertension (Figures 1B and 2C). This finding shows that chronic hypertension in SHR is associated with a significantly decreased expression of HuR in the aorta.

**Interaction of Recombinant HuR With the 3′-UTR of the sGCα1 and sGCβ1 mRNA**

As shown in the scheme on the left side of Figure 3, the 3′-UTR of the rat sGCα1 and sGCβ1 mRNA contains several conserved AREs (indicated by ▴), which are targeted for a rapid mRNA degradation by specific endonucleases. Although we demonstrated previously an interaction of endogenous HuR present in cell and tissue extracts with the 3′-UTR of the rat sGCα1 mRNA, we now assessed the interaction of GST-tagged recombinant purified HuR with rat sGCα1 and sGCβ1 mRNA. Biotin-labeled riboprobes from the 3′-UTR of sGCα1 (3UTRSK1) and sGCβ1 (β1-UTR) were incubated for 30 minutes with either GST–HuR fusion protein or recombinant GST (control), and HuR–riboprobe complex formation was detected by RNA EMSA. Both probes migrated as single bands at the bottom (front; Figure 3A and 3B). In the presence of purified GST–HuR fusion protein, the riboprobes were shifted upward (Figure 3, shift), indicating a complex formation between HuR and sGCα1 mRNA. Preincubation of HuR with a 100-fold excess of an unlabeled synthetic ARE, [AUUUA]4, prevented the complex formation between HuR and 3UTRSK1, indicating a competition between the synthetic ARE and the truncated 3′-UTR of sGCα1 mRNA (Figure 3A). mRNA-binding activity was not observed with pure GST (Figure 3A; n = 3). Furthermore, the extent of the sGCβ1 mRNA band shift seen in Figure 3B was high at low concentrations of HuR and decreased with an increasing amount of HuR added. We assume that an excess of fusion protein shields the biotin label, thereby preventing the streptavidin–horseradish peroxidase reaction for riboprobe detection, or that a nuclease contamination of the GST–HuR preparation destroys the riboprobe at higher concentrations. In summary, HuR-binding activity was detected for both 3′-UTRs of the rat sGCα1 and sGCβ1 mRNA.
Mutagenesis Analysis of Conserved AREs Within 3'-UTR of sGCα and sGCβ mRNA

From the preceding experiments, it was still unclear which nucleotide sequences were responsible for a specific interaction between HuR and these 3'-UTR transcripts. To identify specific HuR-binding sites, we chose highly conserved ARE and CU-rich element (CRE) regions of the 3'-UTR from sGCα and sGCβ mRNA and analyzed the interaction with HuR by EMSA. The riboprobes DRαGC3UTR2 (Figure 4A) and βGC3UTR1 (Figure 4B) were incubated for 15 to 30 minutes at 4°C with purified GST and GST–HuR protein. As shown in Figure 4, both riboprobes were shifted
by low doses of HuR (ie, 200 ng HuR) induced a detectable shift with DR\textsubscript{1}GC3UTR2 (Figure 4A) and 30 to 60 ng HuR with DR\textsubscript{2}GC3UTR1 (Figure 4B). In contrast, purified GST did not interact with both RNA transcripts (Figure 4). In control experiments, even a high dose of HuR (400 ng) failed to induce a shift of mismatch riboprobes with mutation of the specific ARE/CRE motifs to GC-rich motifs DR\textsubscript{1}GC3UTR2MIS (Figure 4A) and DR\textsubscript{2}GC3UTR1MIS (Figure 4B). The latter probe showed 2 bands, presumably monomeric and dimeric RNA, independent of the presence or absence of HuR (Figure 4B). In summary, the highly conserved AREs/CREs of both 3'-UTRs from sGC\textsubscript{α1} and sGC\textsubscript{β1} mRNA are responsible for a specific binding of HuR.

Comparison of the HuR-Binding Activity in Old SHR and WKY Rats

We then analyzed whether the reduced expression of HuR in hypertensive SHR translated into reduced HuR-binding activity in native protein from rat aorta, using EMSA with sGC mRNA riboprobes DR\textsubscript{1}GC3UTR2 (Figure 5A) or DR\textsubscript{2}GC3UTR1 (Figure 5B) as an HuR specific activity assay. In the presence of protein from old WKY rats, a specific band shift signal was observed for DR\textsubscript{1}GC3UTR2 (Figure 5A), and 2 signals were detected for DR\textsubscript{2}GC3UTR1 (Figure 5B). In contrast, with aortic protein from old SHR, the band shift signals with both riboprobes decreased (Figure 5A and 5B). Addition of a monoclonal HuR antibody to aortic protein from WKY rats (and from young SHR; data not shown) induced a supershift for DR\textsubscript{1}GC3UTR2 (Figure 5A, lane 8) and DR\textsubscript{2}GC3UTR1 (Figure 5B, lane 10), indicating the presence of HuR in the shifted bands. These results show that the downregulation of the HuR expression in hypertensive SHR correlates with a decrease in the HuR binding for conserved AREs in the 3'-UTR of sGC\textsubscript{α1} and sGC\textsubscript{β1} mRNA.

Comparison of Consensus ARE-Like–Binding Activity of HuR in SHR and WKY Rats

To clarify whether the decreased HuR-binding activity for sGC-specific AREs in aged SHR translated total HuR-binding activity, we performed EMSAs with a consensus ARE, [AUUUA]\textsubscript{4}. Incubations without protein served as a control for unbound [AUUUA]\textsubscript{4}. As shown in Figure 6, the consensus ARE-binding activity of HuR in aortic extracts from old SHR was significantly reduced compared with old WKY rats (Figure 6A). In contrast, [AUUUA]\textsubscript{4}-binding activity was not different between young prehypertensive SHR and young WKY rats (Figure 6B) or between young and old WKY rats (n=4; data not shown). Consequently, reduced...
expression of HuR in old SHR translated into reduced HuR binding to a conserved ARE.

Degradation of sGCα1 and sGCβ1 mRNA by Native Extracts of Aortas From Old SHR and WKY Rats

We then assessed by an in vitro RNA degradation assay whether reduced HuR expression and sGCα1 and sGCβ1 mRNA-binding activity in hypertensive SHR accounts for decreased sGC mRNA stability. Total native protein extracts of endothelium-denuded rat aortas were incubated at 30°C with either biotin-labeled oligoribonucleotide DRαGC3UTR2 or βGC3UTR1. After 15, 30, and 45 minutes, RNA degradation was stopped, RNA was separated by denaturing 20% polyacrylamide gel electrophoresis (PAGE)/7 mol/L urea and electroblotted onto nylon membranes (see Materials and Methods). The decay rate of the DRαGC3UTR2 (Figure 7A) and βGC3UTR1 (Figure 7B) mRNA was significantly enhanced by aortic protein from old SHR compared with WKY rats (Figure 7A and 7B). In contrast, in vitro RNA degradation of the mismatched ARE-mutated riboprobes DRαGC3UTR2MIS and βGC3UTR1MIS was not different between old SHR and WKY rats (data not shown). In summary, these experiments demonstrate an accelerated degradation of ARE-containing and HuR-protected mRNA probes from the 3′UTR of sGC subunits by aortic extracts from old SHR.

Discussion

In aged SHR, the NO–vasodilator responsiveness of the aorta is reduced.3 We recently provided an explanation for this arterial dysfunction by showing that α1 and β subunits of the NO receptor sGC are downregulated in these animals.5 We now set out to investigate the molecular mechanism underlying this phenomenon. We decided to focus on a specific post-transcriptional mechanism, which we identified previously to account for cyclic nucleotide–induced downregulation of sGCα1 and sGCβ1 subunits in aortic vascular smooth muscle cells and aortic tissue from normotensive rats.7,9 This mechanism is based on regulation of mRNA stability by the elav-like protein HuR,8 which binds to AREs present in the 3′-UTR of sGCα1 and sGCβ1 mRNA. HuR protects ARE-containing mRNAs from degradation, presumably by preventing the attack of specific endonucleases that are targeted to AREs.12 We reported that cGMP and cAMP destabilize sGC mRNA by downregulation of HuR expression.7,9

HuR Expression and Activity in Chronic Hypertension

We now show that in aortic tissue from aged SHR, the expression of HuR is decreased by >50% at the protein and mRNA level compared with normotensive age-matched WKY rats (Figure 1). We also confirmed our previous findings that expression of sGCα1 and sGCβ1 is decreased to a similar degree in aged SHR (Figure 1). In contrast, HuR expression was not different between prehypertensive young SHR and young WKY rats (Figure 2). By EMSA, we ascertained that the reduced expression of HuR in aged SHR translated into a similarly reduced binding of HuR present in native aortic protein extract to an ARE-containing 40-nt-sized riboprobe from the 3′-UTR of sGCα1 and to a mixed

Figure 5. Interaction of sGCα1 and sGCβ1 3′-UTR riboprobes with aortic HuR of old SHR and WKY rats. Biotin-labeled riboprobes DRαGC3UTR2 (A) or βGC3UTR1 (B, 15 ng) were incubated (30 minutes at 4°C) with aortic protein (20 μg) from old SHR and WKY rats. C indicates riboprobe incubated without protein (lane 1); +AB, supershift induced by preincubation (45 minutes at 4°C) of 7.5 μg HuR antibody with aortic protein from WKY rats (A, lane 8; B, lane 10). Representative of 3 experiments performed with aortas from 5 SHR and 5 WKY rats.

Figure 6. EMSA showing HuR-like ARE-binding activity in aorta from old and young SHR and age-matched WKY rats. A biotin-labeled model ARE, [AUUUA]₄ (100 ng), was incubated (30 minutes) with total protein extracts (40 μg) of rat aortic tissue from old (A) and young (B) SHR and age-matched WKY (control) rats. The position of free [AUUUA]₄ and the HuR–[AUUUA]₄ complex is indicated. [AUUUA]₄ incubated without protein served as a negative control (C). Representative data from 3 independent experiments performed with aortic protein from 5 young and old SHR and WKY rats.
ARE/CRE containing ribprobe from the 3′-UTR of sGCβ1 (Figure 5). Using a model ARE, [AUUUA]₉, we could also show that HuR-binding activity was not different in aortic extract from young SHR and WKY rats (Figure 6), in accordance with equal HuR expression in these normotensive rats. Because decreased HuR expression does not necessarily translate into decreased protection of HuR-targeted mRNAs, we assessed the influence of aortic protein on sGC mRNA stability in vitro using the same riboprobes as for EMSA. Indeed, the in vitro degradation of AU-rich sGC mRNA–specific riboprobes during 45 minutes at 30°C was accelerated in the presence of native protein from aortas of aged SHR compared with WKY rats (Figure 7). Altogether, these findings suggest that chronic hypertension induces changes in HuR expression and activity, which account for decreased sGC expression and activity in the aorta of hypertensive rats.

**Specificity of the Interaction of HuR With the 3′-UTR of sGCα1 and sGCβ1 mRNA**

Using affinity-purified GST-tagged recombinant HuR in EMSA experiments, we confirmed our previous observation performed with cell and tissue extracts that HuR binds to a 1.1-kb fragment of the 3′-UTR of sGCα, mRNA containing 6 AREs and to the full-length 3′-UTR of the β1 mRNA containing 7 AREs (Figure 3). Furthermore, we could demonstrate a specific interaction of recombinant HuR with an ARE-containing 40-nt ribprobe from the 3′-UTR of sGCα1 mRNA and with an mixed ARE/CRE–containing probe from the 3′-UTR of sGCβ1 mRNA (Figure 4). Mutation of these AREs and CREs resulted in complete loss of HuR binding, indicating the specific requirement of these sequence motifs for HuR recognition of these truncated mRNAs. CREs lacking the AUUUA pentamer were described recently as new HuR targets and were classified as type III AREs. sGCβ1 seems to represent another example in which interaction of HuR with a CRE (in addition to interaction with AREs) stabilizes the respective mRNA.

**Significance of Downregulation of HuR in Chronic Hypertension**

Our present findings suggest that downregulation of sGC expression by downregulation of HuR fulfills intracellular signaling requirements for adaptation of the arterial vascular wall to chronic hypertension. In SHR, vascular remodeling is a complex process leading to different morphological phenotypes in early and late hypertension and in different vascular beds. In the aorta, hypertrophic growth is prevailing in late hypertension. HuR regulates progression of the cell cycle and sGC activation was shown to block progression of the cell cycle in vascular smooth muscle cells. Therefore, it is conceivable that downregulation of HuR and sGC is required for hypertrophic remodeling of the aortic wall. This hypothesis is also supported by the observation that sGC is down-regulated during neointima formation in rat carotid artery in response to balloon injury and that adenoviral sGCα1 and sGCβ1 gene transfer plus NO donor application prevented neointima formation in this model. Furthermore, altered expression of other HuR-regulated proteins, such as matrix metalloproteinase-9 (MMP-9), the β2-adrenergic receptor, angiotensin II type 2 (AT2) receptors, and others, may contribute to this adaptation to chronic hypertension. For instance, MMP-9 was found to be slightly reduced in hearts for hypertensive SHR, and the β2-adrenergic and AT2 receptors are downregulated in several vessel types in chronic hypertension.

However, it must be kept in mind that HuR is not the sole **trans**-acting factor regulating mRNA stability by **cis**-regulatory elements. A prominent opponent of HuR is the mRNA-destabilizing protein AU-rich RNA binding factor-1 (AUF1), which competes with HuR for the same AREs in target mRNAs and facilitates degradation of ARE-targeted mRNAs by specific endonucleases. By Western blot analysis, we detected all 4 AUF1 isoforms (p45, p42, p40, and p37) in aortic tissue from old SHR and WKY rats (S. Kloess et al, unpublished results, 2003). We also found that AUF1 proteins can bind to the 3′-UTR of sGCα1 and sGCβ1,
However, expression of these AUFl isosforms was not different between both rat strains (S. Klöss et al, unpublished results, 2003). According to these findings, we may speculate that the ratio between HuR and AUFl activity controls sGC mRNA stability and that HuR is the dominating factor in normotensive rat aorta. However, when this ratio decreases, such as in long-lasting hypertension, AUFl-mediated mRNA degradation may take over and lead to sGC mRNA destabilization. Further studies will have to clarify the molecular mechanism accounting for decreased HuR expression in this condition.

**Perspectives**

We show here that the decrease in sGCα and sGCβ protein expression in aged SHR very likely results from a shorter half life of sGC subunit mRNAs in aged SHR because of downregulation of the mRNA-stabilizing protein HuR. The molecular mechanism underlying reduced HuR expression in this animal model is worth being resolved. We made some suggestions.7,9 Our present finding bears multiple implications. Obviously, a consequence of decreased HuR expression is the reduced nitrovasodilator responsiveness observed in the aorta of aged SHR. However, it is conceivable that other cGMP-dependent processes in vascular smooth muscle cells (and other cells) may be affected as well, such as proliferation, hypertrophic remodeling, angiogenesis, and apoptosis, which we did not study here. On the other hand, because HuR controls the mRNA stability of a variety of cardiovascular targets, all these will potentially be affected in this animal model, and the respective alterations in gene expression will constitute the adaptive response to chronic hypertension. This has to be verified in the human system, too. Furthermore, our finding bears on other situations in which HuR and sGC expression are altered in concert, such as in embryonal and postnatal development of different organ systems, and in cell cycle progression. Altogether, this underpins that HuR is a main player in regulation of gene expression at the post-transcriptional level.

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**References**


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