Posttranscriptional regulation of soluble guanylyl cyclase expression in rat aorta

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running title: HuR regulates soluble guanylyl cyclase mRNA stability

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**Abstract**

We investigated the molecular mechanism of cyclic GMP-induced downregulation of soluble guanylylcyclase expression in rat aorta. 3-(5’-hydroxymethyl-2’-furyl)-1-benzyl indazole (YC-1)\(^1\), an allosteric activator of this enzyme, decreased the expression of soluble guanylylcyclase \(\alpha_1\) subunit mRNA and protein. This effect was blocked by the enzyme inhibitor 4\(H\)-8-bromo-1,2,4-oxadiazolo(3,4-d)benz(b)(1,4)oxazin-1-one (NS 2028)\(^2\), and by actinomycin D. Guanylylcyclase \(\alpha_1\) mRNA-degrading activity was increased in protein extracts from YC-1-exposed aorta and was attenuated by pretreatment with actinomycin D and NS 2028. Gelshift- and supershift-analysis using an AU\(^3\)-rich ribonucleotide from the 3’-untranslated region of the \(\alpha_1\) mRNA and a monoclonal antibody directed against the mRNA stabilizing protein HuR\(^4\) revealed HuR mRNA binding activity in aortic extracts, which was absent in extracts from YC-1-stimulated aortas. YC-1 decreased the expression of HuR, and this decrease was prevented by NS 2028. Similarly, downregulation of HuR by RNA interference in cultured rat aortic smooth muscle cells decreased \(\alpha_1\) mRNA and protein expression. We conclude that HuR protects the guanylylcyclase \(\alpha_1\) mRNA by binding to the 3’-untranslated region. Activation of guanylylcyclase decreases HuR expression, inducing a rapid degradation of guanylylcyclase \(\alpha_1\) mRNA and lowering \(\alpha_1\) subunit expression as a negative feedback response.

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\(^1\) YC-1: 3-(5’-hydroxymethyl-2’-furyl)-1-benzyl indazole

\(^2\) NS2028: 4\(H\)-8-bromo-1,2,4-oxadiazolo(3,4-d)benz(b)(1,4)oxazin-1-one

\(^3\) AU: adenylate-uridylate

\(^4\) HuR: "Human R" embryonic lethal abnormal visual (ELAV)-like RNA-binding protein
INTRODUCTION

The hemoprotein soluble guanylylcyclase (sGC)$^5$ is the predominant intracellular nitric oxide (NO)$^6$ receptor in vascular smooth muscle cells (1). The active enzyme exists as an obligate heterodimer, the most abundant isoform consisting of an $\alpha_1$ (76 - 81.5 kDa) and a $\beta_1$ (70 kDa) subunit (2). sGC mediates NO-signaling via formation of guanosine 3':5'-cyclic monophosphate (cGMP)$^7$, which induces, for instance, vascular smooth muscle relaxation by activating cGMP-dependent protein kinase, prevention of contractile agonist-elicited intracellular free Ca$^{2+}$ mobilization and dephosphorylation of myosin light chain kinase (3).

In addition to an acute activation of sGC the output of the NO-cGMP pathway can also be controlled at the level of sGC expression. Thus, a reduced vasodilator response to exogenous NO consistent with a downregulation of sGC has been observed in aortic tissue of aged spontaneously hypertensive rats (4). On the other hand, an upregulation of sGC expression was found in aortic tissue from nitroglycerin-tolerant rats (5) and from rats suffering from chronic heart failure (6), despite diminished vasodilator responses to NO. This apparently discrepant finding indicates that altered sGC expression does not necessarily translate into predictable changes in cGMP-dependent functional responses, but that other mechanisms, such as altered NO bio-availability, may overrun the influence of altered sGC expression.

These findings exemplify the need for understanding the molecular mechanisms accounting for regulation of sGC expression. There is evidence that expression of sGC is controlled by second messenger cyclic nucleotides via a post-transcriptional mechanism: in various cells cyclic AMP eliciting agonists decrease the expression of sGC mRNA and

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$^5$ sGC: soluble guanylylcyclase: GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2  
$^6$ NO: nitric oxide  
$^7$ cGMP: guanosine 3':5'-cyclic monophosphate
protein (7,8) by a destabilization of the sGC mRNA. This effect is mimicked by activation of the cGMP-signaling pathway, e.g. application of NO donors, stimulation of particulate guanylate cyclase by atrial natriuretic factor, and stimulation of cGMP-dependent protein kinase by the stable cGMP-analogue 8-chlorophenylthio-cGMP (9).

The objective of the present investigation was to characterize the mechanism accounting for sGCα1 mRNA destabilization induced by increased cGMP formation in isolated rat aorta. We observed that the elav family protein HuR (10) stabilizes the sGCα1 mRNA by binding to AU-rich elements (ARE)8 in its 3'-untranslated region (UTR)9, and that an increase in intracellular cGMP strongly decreases HuR expression and sGCα1 mRNA binding activity, leading to accelerated mRNA degradation.

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8 ARE: AU-rich element
9 3'-UTR: 3'-untranslated region
MATERIALS AND METHODS

The polyclonal chicken antibody directed against the $\alpha_1$- and $\beta_1$-subunit of the rat lung sGC was produced by BioGenes GmbH (Berlin, Germany), which also provided the rabbit-anti-chicken antibody. For some experiments a sGC$\alpha_1$ specific peptide antibody was obtained from Dr. Stasch, Bayer AG, Leverkusen. The oligonucleotides for RT-PCR$^{10}$, in-vitro-transcription and gelshift analysis were synthesized by BioSpring GmbH and MWG-Biotech. 4H-8-bromo-1,2,4-oxadiazolo(3,4-d)benz(b)(1,4)oxazin-1-one (NS 2028) was from Neurosearch (Copenhagn, Denmark). 3-(5’-hydroxymethyl-2’furyl)-1-benzyl indazole (YC-1) was a kind gift from Aventis Pharma (Strassburg, France). The HuR-specific siRNA oligonucleotides were from Xeragon-Qiagen (Germantown, USA).

Rat aortic tissue

Male normotensive Wistar Kyoto rats (300 g) were obtained from Möllegaard (Skensved, Denmark) and were kept according to institutional guidelines, in compliance with German laws. The thoracic aorta was isolated from anaesthetised rats (200 mg/kg ketamin (Exalgon™), 100 mg/kg xylazin (Rompun™), cleaned from fat and connective tissue, and cut into rings of equal length (3 mm). The endothelium was removed by gentle forcing and rolling a glass rod through the lumen. The aortic rings were kept in culture dishes (6 well) in MEM$^{11}$ under a carbogen atmosphere (4.5% CO$_2$) at 37°C. The rings were exposed to YC-1, NS2028 and/or actinomycin D for different periods of time and were then snap-frozen in liquid nitrogen and stored at ~70°C.

Isolation of total RNA from rat aorta and RT-PCR

10 RT-PCR: reverse transcriptase-polymerase chain reaction
11 MEM: Modified Eagle’s Medium
Frozen tissue was ground in liquid nitrogen with porcelain mortar and a pestle. Total RNA was extracted by the modified guanidine isothiocyanate method of Chomczynski and Sacchi (11). The reverse transcriptase-polymerase chain reaction (RT-PCR) for the sGCα1 mRNA (product size 826 bp\(^{12}\)) and elongation factor II (225 bp) was performed exactly as described previously (4).

**Poly(A)+ RNA (mRNA) isolation from rat lung**

Poly(A)+ mRNA was purified from total RNA by means of the Messagemaker kit (Life Technologies, GIBCO-BRL). Total RNA (2 mg; 0.55 mg/ml) was denatured for 5 min at 65°C. The salt concentration was adjusted to 0.5 M NaCl. Subsequently the RNA was incubated with the oligo(dT) cellulose suspension and heated for 10 min at 37°C. After filtration the suspension was washed with 20 mM Tris/HCl pH 7.5, 0.5 M NaCl and then with 20 mM Tris/HCl pH 7.5, 0.1 M NaCl. The mRNA was eluted with RNase-free water.

**Northern blots**

The poly(A)+ RNA sample was denatured for 15 min at 65°C in 0.5x MOPS\(^{13}\)-buffer containing 25% formamide, 1.1 % formaldehyde, 1 % Ficoll 400, 0.02 % bromophenol blue (Na-salt). The mRNA was fractionated in a 1.2 % agarose-formaldehyde gel and blotted overnight onto nylon membrane (pore size: 0.45 µm, Biodyne B, Pall) in 10x saline-sodium citrate (SSC)\(^{14}\) buffer solution (1.5 M NaCl, 150 mM sodium citrate, pH 7.5). Membranes were washed in 2x SSC and the mRNA was fixed by UV-crosslinking. Subsequently the membranes were baked at 80°C for 2 h and then pre-hybridized for 2 h in 50 % formamide,

\(^{12}\) bp: base pairs
\(^{13}\) MOPS: 3-morpholino-propane sulfonic acid
\(^{14}\) SSC: saline-sodium citrate
0.8 M NaCl, 0.1 % sarkosyl, 0.1 % Ficoll 400, 0.1 % polyvinylpyrrolidone, 0.1 % bovine serum albumin (BSA)\textsuperscript{15}, 0.2 % SDS and 250 µg/ml sheared salmon sperm DNA at 42°C. Hybridization occurred at 42°C overnight in 10 % dextrane sulfate with biotinylated-DNA probes (5 ng/cm²) specific for elongation factor II and sGC α\textsubscript{1} mRNA. Blots were then washed twice at 65°C in 5x SSC, 0.5 % SDS, 30 minutes at 50°C in 0.1 x SSC, 1 % SDS and 1 min with TBS\textsuperscript{16}-Tween 20 (0.05 % Tween 20, 150 mM NaCl, 100 mM Tris/HCl pH 7.5). Afterwards membranes were blocked for 1 h at 65°C in TBS-Tween 20 containing 3 % BSA and incubated with a streptavidin-alkaline phosphatase conjugate (7 µl/100 cm²; 1:1000 in TBS-Tween 20) for 10 min at RT. The blots were washed twice in TBS-Tween 20 and then in 100 mM NaCl, 50 mM MgCl\textsubscript{2}, 100 mM Tris/HCl pH 9.5. Immunoreactive mRNA bands were visualized by chemiluminescence and exposure to x-ray film.

**Preparation of sGCα\textsubscript{1} transcripts by in-vitro transcription**

Total RNA of rat lungs was used as a template for RT-PCR amplification of the 3-untranslated region (3-UTR) of sGCα\textsubscript{1} cDNA regions. A Pfu-DNA-Polymerase (\textit{Pyrococcus furiosus} DSM3638, 92 kDa, Promega) with 3 → 5 exonuclease (proofreading) activity was used for the PCR reaction. The 5’-sense primer contained the T7 promoter sequence 5’-CCAAGCTTCTAATACGACTCACTATAGGGAGA-3’ (\textit{T7}). For generating the 3UTRSK2\textsuperscript{17} (424 bp) template, sense primer (5’- \textit{T7}CCAGCTACATCTTTGTGCC-3’) and antisense primer (5’-ACTGTCCTCTACAGTAGGGAGTGGC-3’), corresponding to positions

\textsuperscript{15} BSA: bovine serum albumine  
\textsuperscript{16} TBS: Tris-buffered saline  
\textsuperscript{17} 3UTRSK2: truncated 3’UTR of sGC ±1 mRNA
3049 to 3067 and 3423 to 3441 of the sGCα1 cDNA were used (GenBank™ accession number U60835). Subsequently PCR fragments encompassing the 3-UTR of sGCα1 mRNA were synthesized bearing a T7 sequence at the 5-end. Biotinylation of transcripts was performed with the North2South™ Biotin in-vitro transcription kit from Pierce (Rockford, USA).

**RNA-protein binding reactions and supershift assays**

Electrophoretic mobility shift assays (EMSA)\(^1\) were carried out by a modification of the method of Wang (12). The oligoribonucleotide (3UTRSK2, 50 - 200 ng) was incubated with 40 - 100 µg native nuclear extract (prepared according to (13)) from endothelium-denuded rat aorta, and a 10x reaction buffer (15 mM Hepes pH 7.9, 600 mM KCl, 10 mM DTT, 50 % glycerol, 30 mM MgCl₂, 2 U/µl RNase-inhibitors [40 U/µl, RNaseOUT, Life Technologies, GIBCO], 200 ng/ml total RNA) for 30 min at RT. Complexes were resolved by native 2 % TAE\(^1\)-agarose gelelectrophoresis (40 mM Tris pH 8.5, 0.1 % acetic acid, 2 mM EDTA) for 2 h and blotted onto a nylon membrane (Biodyne B, Pall) overnight in 10 x SSC. Blocking and detection of biotin-labeled bands was performed as described for northern blots. For supershifts, 4-15 µg of the monoclonal HuR-antibody was incubated with the native nuclear extract for 1 h on ice before the specific riboprobe was added; all subsequent steps were performed as described for native gels.

**Immunodetection of the sGCα1 subunit**

Total protein was precipitated (1.5 ml 100 % isopropanol) from the phenol-ethanol supernate (Trizol-method) of the RNA extraction, and the precipitate dissolved in 1 % SDS. The

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\(^1\) EMSA: electrophoretic mobility shift assay

\(^1\) TAE: 40 mM Tris pH 8.5, 0.1 % acetic acid, 2 mM EDTA
protein (40 µg per lane) was fractionated on Lämmli gels and electro-blotted onto nitrocellulose filters (Protrans; Schleicher & Schuell). The blots were blocked at 4°C over night and then incubated for 2 hours at RT with either a polyclonal chicken antibody (IgY) or a rabbit antibody (IgG) directed against the α₁-subunits of sGC (1:100 dilution in blocking buffer). The blots were washed and then developed either with a peroxidase A-conjugated anti-chicken IgY (IgG, rabbit, 1:5,000 in blocking buffer), or anti rabbit IgG (goat, 1:10,000). Immunoreactive peptides were visualized by chemiluminescence and exposure to x-ray film. The autoradiographs were analyzed by scanning densitometry. Equal protein loading and blotting was verified by α-actin immunostaining.

**Design of HuR-specific siRNA**

A HuR specific siRNA (HuR-siRNA) was designed by selecting a target region from base position 163 - 183 relative to the start codon, which fulfilled the specific sequence requirements: (AA(N₁₉)dTdT; N is any nucleotide; 21-nt sense and 21-nt antisense strand; approximately 50% G/C content and a symmetric 2-desoxythymidine 3’ overhang. Sense and antisense oligonucleotides were synthesized by Xeragon Oligonucleotides (Xeragon-Qiagen). The lyophilised siRNA was dissolved in sterile annealing buffer (100 mM potassium acetate, 30 mM Hepes-KOH, 2 mM magnesium acetate, pH 7.4) to obtain a 20 µM solution. Then oligonucleotides were heated to 90°C for 1 min followed by 1 hour at 37°C. In addition, we also used a HuR-siRNA targeting base position 564-584 relative to the start codon (kindly provided by S. Sengupta) for cell transfections. Lyophilised or dissolved siRNAs were stored at -20°C.

**Transfection of cultured vascular smooth muscle cells (VSMC)**

Cultured smooth muscle cells from rat aortas were grown in minimal essential medium
(MEM, Gibco) containing 10% FCS and 1% penicillin and streptomycin at 37 °C and 5% CO₂. SMCs were trypsinized, mixed with fresh MEM (+ antibiotics) without FCS and seeded on 6-well plates (2 ml per well). 24 h later the cells had reached 80 -90 % confluence and were transfected with siRNA (final concentration 100 nM) using the TransMessenger Transfection Reagent (TMTR; Qiagen). Therefore, 6 µg of siRNA was diluted with 12 µl of Enhancer R (ratio RNA [µg] : Enhancer R [µl] was 1:2) and 168 µl Buffer EC-R (Qiagen) and mixed by vortexing for 10 s. After incubation (5 min, RT) 24 µl of TMTR (ratio RNA [µg] : TMTR [µl] was 1:4) was added to the RNA-Enhancer R mixture. During this incubation (10 min, 15 - 25°C) SMCs were washed with 2 ml (per well) sterile PBS Dulbecco’s (GibcoBRL). Then the RNA-transfection mixture was diluted in 1.78 ml MEM (+ antibiotics) and added drop-wise to the cells (2 ml transfection complex per well). The cells were cultured for 2 days at 37°C (5% CO₂). Thereafter the HuR and sGC mRNA and protein expression was assayed by RT-PCR and western blot experiments.
RESULTS

Influence of YC-1 on the expression of sGCα₁ mRNA and protein in rat aorta

To assess the effect of increased cGMP formation on the sGCα₁ subunit expression in rat aorta freshly isolated endothelium-denuded aortic rings from WKY rats were kept under organ culture conditions, either in the absence (control +/- 0.2 %DMSO) or presence of the sGC activator molecule YC-1 (10 µM), and the specific sGC inhibitor NS 2028 (10 µM). After 24 h the vascular tissue was snap-frozen and homogenized in liquid nitrogen, then further processed for sGCα₁ subunit mRNA and protein expression by RT-PCR (Fig 1A) and western blot (Fig 1B), respectively. According to densitometric analysis of the RT-PCR product (Fig. 1A) and the immunoreactive protein (α₁ = 82 kD, Fig. 1B) the abundance of sGCα₁ subunit mRNA and protein was markedly lower in YC-1 exposed aorta compared to controls (α₁-mRNA = 93% lower, α₁-protein = 56% lower vs. controls; bar graphs; Fig. 1A and B), while the levels of elongation factor II mRNA (Fig. 1A) and α-actin protein (Fig. 1B) were not affected by YC-1. In the presence of NS2028 the ability of YC-1 to decrease sGC subunit gene expression was almost completely blocked (Fig. 1A and B). These findings show that long-lasting activation of sGC in the rat aorta decreases sGCα₁ subunit expression at the mRNA and protein level.

YC-1 decreases the sGCα₁ mRNA expression in rat aorta by a mechanism requiring transcription

To assess whether YC-1 affects the stability of sGCα₁ mRNA we investigated the time

20 DMSO: dimethylsulfoxide
course of sGCα1 mRNA expression after inhibition of cell transcription by actinomycin D (Act D)\textsuperscript{21}. The aortic rings were held in organ culture for 3, 6 or 9 h, in presence of YC-1 (10 µM), or

\textsuperscript{21} Act D: actinomycin D
Act D (10 µM), or both. The tissue level of sGCα₁ mRNA was estimated by semi-quantitative RT-PCR. As illustrated by the fluorographs in Fig. 2A and the densitometric analysis shown below the level of sGCα₁ mRNA did not decrease in Act D-exposed aortic rings for up to 9 h, indicating that half-life of this mRNA exceeds 9 h when sGC is not activated. In contrast, in the presence of YC-1 and the absence of Act D the sGCα₁ mRNA levels decreased with a half-life of about 6 h. Preincubation (45 min) of the aortic rings with Act D prevented the YC-1-induced decrease of sGCα₁ mRNA abundance (Fig.2A). The mRNA levels of elongation factor II remained stable for up to 9 h and were not affected by Act D and YC-1 (Fig. 2A). These results suggest that YC-1 decreases the stability of sGCα₁ mRNA by a mechanism requiring transcriptional activation of an unknown factor.

The time course of YC-1-induced sGCα₁ mRNA decay was mirrored by a quite similar time course of sGCα₁ protein expression, as assessed by western blots analysis (Fig. 2B). In contrast, the expression of α-actin was constant for the same period of time (Fig. 2.B).

**YC-1-induced sGCα₁ poly(A)+RNA-destabilizing activity in the native protein extract from rat aorta**

To further corroborate our finding of a YC-1-induced destabilization/accelerated degradation of sGCα₁ mRNA in the rat aorta, we assessed the effect of a protein extract from YC-1-exposed rat aorta on the rate of sGCα₁ mRNA degradation. Therefore, total native protein was isolated from a part of the aortic rings used in the previous experiments (Figs. 1 and 2) and 20 µg protein was incubated at 37°C with 1 µg enriched poly(A)+RNA isolated from rat
lungs (cf. methods). After different periods of time (10 – 50 min (Fig. 3A) or 15 – 45 min (Fig. 3B) an aliquot of the incubation mixture was probed for sGCα₁ and elongation factor II mRNA by northern blotting (see methods). In the absence of aortic protein (control) the sGCα₁ mRNA was stable for up to 50 min under these assay conditions (Fig. 3A). In the presence of protein from aortas exposed to 0.2% DMSO (solvent control) a moderate time-dependent decrease in sGCα₁ mRNA abundance was observed (Fig. 3A and B, lanes "DMSO"). The rate of sGCα₁ mRNA decay was considerably accelerated by protein isolated from YC-1-exposed aortas (Fig. 3A,B, lanes "YC-1"). In contrast, elongation factor II mRNA was quite stable even in the presence of protein from YC-1-exposed aorta (Fig. 3A and B, lower autoradiographs), indicating that YC-1 specifically induced factors which led to accelerated decay of sGCα₁ mRNA. The formation of these factors was apparently prevented by a pre-incubation of the aortas with Act. D (Fig. 3A) or NS2028 (Fig. 3B), since under these conditions the aortic protein extract exhibited markedly less sGCα₁ mRNA degrading activity.

**Identification of HuR as a sGCα₁ mRNA-binding protein in rat aorta**

The 3-UTR of the rat sGCα₁ mRNA bears several AUUUA-motifs (AU-rich elements, AREs)⁸ (Fig. 4A), which target the mRNA for rapid degradation by specific endonucleases (14) and enable regulation of the mRNA stability by trans-acting factors (15). One specific protective factor is the RNA-binding protein HuR (34-38 kDa) (10). To investigate whether HuR can interact with 3-UTR of sGCα₁ mRNA we synthesised a biotin-labelled oligoribonucleotide comprising bases 3049 to 3441 of the sGCα₁ mRNA (3UTRSK2; 424 bp;
Fig. 4) containing several AREs. This probe was incubated with native protein extracted from rat aorta. RNA-protein complex formation was assessed by electrophoretic mobility shift assays (EMSA). The free probe migrated in two bands at the bottom (front) (Fig. 5, lane 1), very likely representing monomeric and oligomeric forms. In the presence of aortic protein the probe was retarded (shifted upwards), indicating interaction with a protein present in the extract. The extent of this shift was increased with increasing amount of protein added (Fig. 5, lanes 2 and 3). Addition of an 100 fold excess of an unlabeled synthetic ARE, [AUUUA]4, to the RNA/protein mixture prior to electrophoresis prevented the probeshift, indicating competition between the synthetic ARE and the truncated 3'-UTR of sGCα1 mRNA (Fig. 5, lanes 4 and 5). When the aortic protein was preincubated (45 min, 4°C) with a monoclonal HuR-antibody the RNA-protein band was further retarded (supershifted), demonstrating, that HuR forms a complex with the ARE containing sequence of sGCα1 mRNA (Fig. 5, lanes 6 and 7).

**Activation of sGC by YC-1 decreases HuR-ARE-binding activity**

In order to clarify whether activation of sGC decreases HuR-like binding activity, endothelium-denuded rat aortic segments were kept for 12 h under organ culture conditions (cf Methods), either in the absence or presence of YC-1 (100 µM), and NS 2028 (100 µM), or the solvent control (0.2 % DMSO). Nuclear protein extracts were prepared from the vascular tissue and the expression of HuR-like ARE-binding activity was assessed by RNA-EMSA using the 3UTRSK2 probe. In the presence of protein (80 µg) from control aortas a similar bandshift as shown in Fig. 5 was observed (Fig. 6, lane 2+3). The protein extract from DMSO-treated aorta induced a quite similar shift (Fig. 6, lanes 4+5). In contrast, with protein
from YC-1-exposed aorta the shifted band markedly decreased (Fig. 6, lanes 6+7). This effect of YC-1 was prevented by concomitant exposure of the aorta to the sGC inhibitor NS 2028 (Fig. 6, lanes 8+9). Addition of the monoclonal HuR antibody induced a strong supershift, which under these chromatographic conditions unfortunately superimposed with the shift (Fig. 6, lane 10). These findings indicate that YC-1 either induces a reduction of the HuR affinity for the 3'-UTR of GCα1 mRNA, or that it downregulates HuR expression.

**Activation of sGC in rat aorta by YC-1 decreases expression of HuR**

By western blot analysis we assessed whether YC-1 affected HuR expression. Protein from rat aorta incubated with YC-1 and NS 2028 as shown in Fig. 6 was loaded on a SDS-PAGE, blotted and immuno-probed for HuR. The protein from the control and DMSO-treated aortas showed a marked HuR positive band at 34 kD (Fig. 7A, lanes 1-4 from left), similar to the nuclear extract from Hela cells applied as a positive control (+contr. lane 9). This band was significantly reduced (P > 0.05, ANOVA) in YC-1-treated aortas (lanes 5,6, and densitometric analysis shown below in Fig. 7B). The sGC inhibitor NS 2028 prevented the downregulation of HuR expression by YC-1 (lanes 7,8).

**HuR gene knockdown by RNA interference decreases expression of sGC±1**

RNA interference allows targeted genes to be easily and efficiently "switched off", using short stretches of double-stranded RNA that contain the same sequence as mRNA transcribed from the target gene (16). We used this approach to assess whether specific gene knockdown of HuR in cultured rat aortic smooth muscle cells (RASMC)22 affects expression of sGC.

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22 RASMC: rat aortic smooth muscle cells
Incubation of RASMC for 24 h with two different HuR siRNA oligonucleotides decreased HuR expression at the protein (Fig. 8A "siRNA") and mRNA level (Fig. 8B "siRNA"). In the same cell extracts the expression of sGC±1 protein and mRNA was decreased as well (Fig. 8A,B), compared to controls. Expression of actin protein (Fig. 8A) and elongation factor II mRNA (Fig. 8B) was not affected by HuRsiRNA. This finding clearly shows that specific knockdown of HuR decreases sGC±1 expression in vascular smooth muscle cells.
Discussion

The heterodimeric hemoprotein and NO receptor sGC is a key component of the NO/cGMP signal transduction pathway in vascular smooth muscle and other tissues. In addition to an acute regulation by positive (NO) or negative (superoxide radical) input signals (1) the activity of this pathway can also be controlled at the level of sGC expression (4). Previous studies have shown a feedback-inhibition of sGC expression by its product cGMP (9, 17, 18). This finding was related to an accelerated decay of the sGCα1 and β1 mRNA (17).

We set out to reveal the mechanism accounting for the downregulation of the sGCα1 subunit expression in response to sGC activation. Our rationale for studying sGCα1 was that in preliminary studies we found that this subunit was less expressed in rat vascular tissues than the β1 subunit and therefore formation of the NO-sensitive α1β1 holoenzyme would be limited by the α1 subunit. A NO-independent activator of sGC, YC-1 (19), was chosen here to avoid possible interference by cGMP-independent effects of NO on gene expression (20,21).

The 3-UTR of the rat sGCα1 mRNA bears several AUUUA-motifs (AU-rich elements, AREs), which are targeted by trans-acting factors for regulation of mRNA stability (15, 22). One of these factors is the ubiquitous 34 kDa protein HuR, which binds to AREs with high affinity and selectivity (10), thereby protecting the respective mRNA from accelerated decay (12). By western blot analysis we were able to show for the first time that HuR is constitutively expressed in the rat aorta. We provide evidence by in vitro mRNA degradation assay and RNA-EMSA that HuR protects the rat sGCα1 mRNA by binding to ARE present in the 3’-UTR. Furthermore, we could demonstrate that prolonged (12 h) sGC-activation by
YC-1 decreases the expression of HuR protein and HuR binding activity for sGCα₁ mRNA. Consequently, the expression of the sGCα₁ subunit was decreased at the mRNA and protein level. All these effects could be blocked by an inhibitor of YC-1-stimulated sGC activity, NS 2028 (23), indicating that they were caused by an increased sGC activity/cGMP formation. In this regard, sGC is not just another HuR-regulated gene, but also a regulator of HuR expression, linking increased cGMP levels to depression of HuR activity and lower sGC expression. Though we did not investigate whether lowering of resting cGMP-levels will increase HuR expression, our findings suggest the existence of a negative feedback loop formed by sGC and HuR.

To confirm the hypothesis that a decrease in HuR expression induces a decrease in sGC expression we used the RNA knockdown (RNA interference) technique (16). RNA interference is a gene silencing mechanism that uses double-stranded (ds) RNA as a signal to trigger the degradation of the targeted mRNA. 48 h after transfection of cultured rat aortic smooth muscle cells with a 21mer ds RNA homologous to base position 163 - 183 relative to the start codon of the HuR message (HuRsiRNA) we observed a strong decrease in HuR as well as in sGCα₁ expression at the mRNA and protein level (Fig. 8). This experiment proves that downregulation of sGC mRNA is a consequence of decreased HuR expression.

The signaling cascade accounting for cGMP-dependent downregulation of HuR could not be revealed in this study. Since concomitant application of Act D during exposure of the rat aorta to YC-1 prevented the downregulation of HuR expression and binding activity it is likely that cGMP induces the transcriptional activation of (unknown) factors which decrease HuR expression. Our preliminary data indicate that a cGMP-activated proteinkinase and the transcription factor AP-1 are involved in downregulation of HuR by sGC activators (S.
Kloess, A. Mülsch, unpublished), but an in depth study is required. AP1 sites are present in
the mouse HuR promoter region (24). Interestingly, CREB sites were also found in this
promoter region (24). Agents which increase intracellular cyclic AMP decrease sGC subunit
mRNA levels and cellular cGMP formation in response to NO-donor compounds (7, 8). We
observed that cyclic AMP-eliciting agonists decrease expression of HuR in rat aortic smooth
muscle cells as well (Kloess and Mülsch, unpublished results), suggesting that HuR also
mediates the down-regulation of sGC in response to increased cAMP levels. It appears that
HuR can integrate cyclic nucleotide second messenger signaling and translate changes in
cAMP and cGMP levels in altered gene expression. This underlines the increasing importance
of mRNA stability regulation for gene expression (25), as compared to transcriptional
regulation. In addition to sGC, other components of the NO/cGMP pathway are also regulated
by altered mRNA stability. In human mesangial cells, which exhibit a smooth muscle cell-
like phenotype, the expression of the cytokine-inducible NO synthase II is also
downregulated by NO and cGMP. Part of this negative modulation is caused by decreased
mRNA stability (26). The 3′-UTR of NO synthase II also bears AREs, and HuR was shown
to stabilize the NOS III mRNA by binding to several of these AREs. The expression of HuR
in cytokine-exposed DLD1 cells (human intestinal epithelium) decreased concomitantly with
enhanced NOS III-derived NO formation (27). Furthermore, an increase or decrease in HuR
expression brought about by stable transfection with HuR-sense or -antisense vectors
increased or decreased NO synthase II expression. Collectively, these examples and our
present findings emphasize that several major components of the NO/cGMP pathway are
controlled at a post-transcriptional level by HuR in a negative feedback manner. Future
studies will have to reveal the relative importance of HuR-regulated mRNA stability versus
transcriptional processes for NO/cGMP dependent gene expression.
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Fig. 1: Influence of YC-1 on sGCα1 subunit expression in rat aorta

A) RT-PCR analysis of mRNA isolated from rat aorta kept for 24 h in organ culture (control), in the presence of 0.2 % DMSO, 10 µM YC-1, or YC-1 and 10 µM NS 2028. The upper fluorographs show ethidium bromide-stained agarose gels containing RT-PCR products of the sGCα1 mRNA and elongation factor (ef) II mRNA amplified from 2 µg total RNA. The bar graph below shows a densitometric analysis of the sGCα1 mRNA intensity, normalized by ef II mRNA intensity. Summarized data (mean value ± SEM) from 4 rats. *: significantly different from "control", "DMSO", and "YC1/NS2028" (P < 0.05, ANOVA).

B) Western blot. The protein from the same aorta as used for RT-PCR analysis was separated by SDS-PAGE, and the sGCα1 subunit (82 kDa) was identified by a polyclonal antibody raised in rabbit. The blot was then stripped for α-actin (47 kDa) to verify equal loading and blotting efficiency. The bar graph below shows a densitometric evaluation of the sGCα1-specific band, normalized by α-actin-staining. Summarized data (mean value ± SEM) from 4 rats. *: significantly different from "control", "DMSO", and "YC1/NS2028" (P < 0.05, ANOVA).

Fig. 2: Effect of YC-1 on the sGCα1 mRNA and protein expression in rat aorta in the absence and presence of actinomycin D.

Rat aortic rings were exposed in organ culture to 10 µM YC-1 and/or 10 µM actinomycin D for 3, 6 and 9 hours, frozen and processed for RT-PCR and western blot analysis of sGCα1.

A) The upper fluorographs show ethidium bromide-stained agarose gels containing RT-PCR products of the sGCα1 mRNA amplified from 2 µg total RNA as well as of elongation factor
II mRNA. The sGCα₁ RT-PCR product intensities were normalized for ef II intensities. Summarized data (mean value ± SD) from 3 rats. *: significantly different from "YC1/act.D (9 hours)" , and "act.D (9 hours)" , (P < 0.05, ANOVA).  

B) Western blot. The protein from the same aorta as used for RT-PCR analysis was separated by SDS-PAGE, and the sGCα₁ subunit (82 kDa) was detected by a polyclonal antibody raised in chicken (IgY). The blot was stripped and probed for α-actin. The bar graph below shows a densitometric evaluation of the sGCα₁-specific band, normalized by α-actin-staining. Summarized data (mean value ± SD) from 3 rats. *: significantly different from "YC1/act.D (9 hours)" , and act.D (9 hours)" (P < 0.05, ANOVA).  

Fig. 3: Effect of native protein from YC-1-, NS2028- and Act D- treated rat aorta on the sGCα₁ mRNA stability.  
Representative Northern blots of sGCα₁ subunit (5.5 kb) and ef II mRNA. Native protein (20 µg) isolated from the same aortas as used in the experiments shown in Figs. 1 and 2 was incubated at 37° C with 1 µg of poly(A)+RNA isolated from rat lung, and the amount of sGCα₁ and ef II mRNA remaining after different periods of time (A: 10 – 50 min; B: 15. - .45 min) was assessed by northern blotting. Two different experiments yielded qualitatively similar results.  

Fig. 4: AU-rich elements (AREs) present in the 3’-UTR of rat sGCα₁ mRNA.  
The oligonucleotides (black arrows) for the PCR synthesis of 3UTRSK2 (424 bp) are marked by a gray background. Sequence data taken from GenBank accession No. U60835.
Fig. 5: HuR present in rat aortic nuclear extract binds to the 3’-UTR of sGCα1 mRNA.

RNA electrophoretic mobility shift analysis (RNA-EMSA) representative of 2 experiments. A biotin-labeled RNA probe (3UTRSK2; 424 bp; 250 ng) was incubated for 30 min with nuclear protein (40 and 80 µg) extracted from freshly isolated endothelium-denuded rat aorta (cf Methods), then loaded and electrophoresed on a 6 % TBE-acrylamide gel. The protein-RNA-complexes were electro-blotted on a nylon membrane and visualized by chemiluminescence, as described (cf Methods). Lanes (from left to right) 1: free probe; 2+3: probe-shift induced by 40 and 80 µg rat aortic protein; 4+5: specific shift blocked by unlabelled competitor [AUUUA]₄ probe (25 µg); 6+7: super-shift induced by a monoclonal HuR antibody (HuR-AB; 7.5 and 15 µg); 8: negative control with aortic protein and HuR antibody.

Fig. 6: HuR-binding activity in rat aorta is decreased by sGC activation.

RNA-EMSA performed with protein from two rat aortas. Rings of endothelium-denuded rat aorta were kept for 12 h in organ culture without additions (control), with solvent control (0.2 % DMSO), with YC-1 (100 µM) or with YC-1 and NS 2028 (both 100 µM) (cf Methods), then frozen and homogenized. Biotin-labeled sGC±1 RNA from the 3’UTR (3UTRSK2; 250 ng) was incubated for 30 min with aortic protein (80 µg) and the EMSA was performed as in Fig. 6, except that a 8 % TBE-AA gel was used. Lanes (from left to right) 1: free probe; 2+3: protein from control aortas; 4+5: protein from solvent (0,2 % DMSO)-treated aortas; 6+7: protein from YC-1-exposed aortas; 8+9: protein from YC1/NS2028-exposed aortas; 10: supershift induced by addition of 5 µg HuR antibody to aortic protein from control aortas. Representative data from 3 rats.
**Fig. 7: Activation of sGC in rat aorta decreases expression of HuR.**

A. Representative Western blot with aortas from 2 rats. Endothelium-denuded rat aortic segments were incubated for 12 h as described in Fig. 6. Total native protein (40 µg) was probed for HuR using a monoclonal antibody. Lanes (from left to right) 1+2: control aortas; 3+4: solvent (0.2 % DMSO)-treated aortas; 5+6: YC-1-exposed aortas; 7+8: YC1/NS2028-exposed aortas; 9: positive control (Hela nuclear extract); 10: marker proteins. Equal protein loading was verified by immunostaining for α-actin (47 kDa) shown below.

B: Densitometric analysis of the HuR-specific bands, normalized by α-actin-staining. Summarized data (mean value ± SD) from 3 rats. *: significantly different from "control", "DMSO", and "YC1/NS2028" (P < 0.05, ANOVA).

**Fig. 8: HuR RNA silencing by siRNA transfection decreases sGCα1 expression**

RASMC (16th to 18th passage) were transfected with two different double-stranded HuR-specific siRNAs (final concentration: 100 nM; 2nd lane from left: siRNA from S. Sengupta; 4th lane: siRNA as described in methods) and cultured for 48 h at 37°C. As a control RASMCs were incubated without siRNA (C) or with with the transfection-reagent only (TR). A) Western blot. Total native protein (10 µg) was probed for HuR (34 kDa) and sGCα1 subunit using specific antibodies. Equal protein loading was verified by immunostaining for smooth muscle actin (47 kDa). B) RT-PCR analysis of mRNA isolated from RASMC. The graph shows ethidium bromide-stained agarose gels containing RT-PCR products of the HuR, sGCα1 and elongation factor II (ef II) mRNA amplified from 2 µg total RNA. Representative data out of 2 experiments.
Fig. 3

A)

|       | control | DMSO | YC-1 | YC-1/act. D |
|-------|---------|------|------|-------------|
| min   | 10      | 20   | 30   | 40          | 50   |

$\alpha_1 sGC$ - (5.5 kb)

B)

|       | control | DMSO | YC-1 | YC-1/NS2028 |
|-------|---------|------|------|-------------|
| min   | 15      | 30   | 45   | 15          | 30   | 45   |

$\alpha_1 sGC$ - (5.5 kb)

ef II -
Fig. 4
Fig. 5

|   | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |
|---|----|----|----|----|----|----|----|----|
| + | +  | +  | +  | +  | +  | +  |  + | 3UTRSK2 |
| - | -  | -  | -  | 7.5| 15 | 7.5| HuR-AB [μg] |
| - | 40 | 80 | 40 | 80 | 80 | 80 | aortic protein |
| - | -  | +  | +  | -  | -  | -  | [AUUUA]₄ |

Supershift

\[ \text{3'}\text{-UTR/HuR/} \]

HuR-AB

Unspec.

Probe-shift

\[ \text{[3'}\text{-UTR/HuR]} \]

Free probe

Free probe
|       |       |       |       |       | 5     | HuR-mAB (µg) |
|-------|-------|-------|-------|-------|-------|--------------|
|       | control | DMSO  | YC-1 | YC-1/NS2028 |       | treatment of aorta |
|       | 80 80 | 80 80 | 80 80 | 80 80 | 80 80 | aortic protein (µg) |

| lane |
|------|
| 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   |

![Image of gel electrophoresis](image)

- **unspec.**
- **probe-shift**
- **3′-UTR/HuR**
- **supershift**
- **3′-UTR/HuR/HuR-AB**
- **free probe**
Fig. 7

A) Table and gel image showing protein expression:

| Rat# | control | DMSO | YC-1 | YC-1+ NS2028 |
|------|---------|------|------|--------------|
| 1    |         |      |      |              |
| 2    |         |      |      |              |

HuR (34 kDa)

α-actin (47 kDa)

B) Graph showing HuR expression:

- contr.  
- DMSO  
- YC-1  
- YC-1/NS2028

HuR expression (arbitrary units)

Contr. DMSO YC-1 YC-1/NS2028

Graph with HuR expression levels for different conditions.
