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Post-transcriptional Regulation of Soluble Guanylyl Cyclase Expression in Rat Aorta*

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We investigated the molecular mechanism of cyclic GMP-induced down-regulation of soluble guanylyl cyclase expression in rat aorta. 3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1), an allosteric activator of this enzyme, decreased the expression of soluble guanylyl cyclase α_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 (NS2028) and by actinomycin D. Guanylyl cyclase α_1 mRNA-degrading activity was increased in protein extracts from YC-1-exposed aorta and was attenuated by pretreatment with actinomycin D and NS2028. Gelshift and supershift analyses using an adenylate-uridylate-rich ribonucleotide from the 3′-untranslated region of the α_1 mRNA and a monoclonal antibody directed against the mRNA-stabilizing protein HuR 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 NS2028. Similarly, down-regulation of HuR by RNA interference in cultured rat aortic smooth muscle cells decreased α_1 mRNA and protein expression. We conclude that HuR protects the guanylyl cyclase α_1 mRNA by binding to the 3′-untranslated region. Activation of guanylyl cyclase decreases HuR expression, inducing a rapid degradation of guanylyl cyclase α_1 mRNA and lowering α_1 subunit expression as a negative feedback response.

The hemoprotein soluble guanylyl cyclase (sGC)[1] is the predominant intracellular nitric oxide (NO) receptor in vascular smooth muscle cells (1). The active enzyme exists as an obligate heterodimer, the most abundant isoform consisting of an α_1 (76–81.5 kDa) and a β_1 (70 kDa) subunit (2). sGC mediates NO signaling via formation of guanosine 3′:5′-cyclic monophosphate (cGMP), 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 down-regulation of sGC has been observed in aortic tissue of aged spontaneously hypertensive rats (4). On the other hand, an up-regulation 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 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 guanyl 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 sGCα_1 mRNA destabilization induced by increased cGMP formation in isolated rat aorta. We observed that the *elav* family protein HuR (10) stabilizes the sGCα_1 mRNA by binding to AU-rich elements (ARE) in its 3′-untranslated region (UTR), and that an increase in intracellular cGMP strongly decreases HuR expression and sGCα_1 mRNA binding activity, leading to accelerated mRNA degradation.

MATERIALS AND METHODS

The polyclonal chicken antibody directed against the α_1 and β_1 subunits of the rat lung sGC was produced by BioGenes GmbH (Berlin, Germany), which also provided the rabbit anti-chicken antibody. For some experiments an sGCα_1-specific peptide antibody was obtained from Dr. Stasch, Bayer AG, Leverkusen. The oligonucleotides for RT-PCR, *in vitro* transcription, and gelshift analysis were synthesized by BioSpring GmbH and MWG-Biotech. 4*H*-8-Bromo-1,2,4-oxadiazolo(3,4-*d*)benz(*b*-1,4)oxazin-1-one (NS2028) was from

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[1] The abbreviations used are: sGC, soluble guanylyl cyclase, GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole; NS2028, 4*H*-8-bromo-1,2,4-oxadiazolo(3,4-*d*)benz(*b*-1,4)oxazin-1-one; AU, adenylate-uridylate; HuR, "Human R" embryonic lethal abnormal visual (ELAV)-like RNA-binding protein; NO, nitric oxide; ARE, AU-rich element; UTR, untranslated region; RT, reverse transcriptase; MEM, modified Eagle's medium; MOPS, 3-morpholinopropanesulfonic acid; TBS, Tris-buffered saline; 3UTRSK2, truncated 3′-UTR of sGC α_1 mRNA; EMSA, electrophoretic mobility shift assay; Act D, actinomycin D; RASMC, rat aortic smooth muscle cells; ANOVA, analysis of variance; nt, nucleotide(s); siRNA, short interfering RNA.

Neurosearch (Copenhagen, Denmark). 3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1) was a kind gift from Aventis Pharma (Strasbourg, France). The HuR-specific siRNA oligonucleotides were from Xeragon-Qiagen (Germantown, MD).

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 anesthetized rats (200 mg/kg ketamine (Exalgon™), 100 mg/kg xylazine (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 (six well) in MEM under a CO_2-enriched 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—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 sGCα_1 mRNA (product size 826 bp), 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 (Invitrogen). 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 0.5× MOPS buffer containing 25% formamide, 1.1% formaldehyde, 1% Ficoll 400, 0.02% bromphenol blue (sodium 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 10× saline-sodium citrate (SSC) buffer solution (1.5 M NaCl, 150 mM sodium citrate, pH 7.5). Membranes were washed in 2× SSC, and the mRNA was fixed by UV-cross-linking. Subsequently the membranes were baked at 80 °C for 2 h and then pre-hybridized for 2 h in 50% formamide, 0.8 M NaCl, 0.1% Sarkosyl, 0.1% Ficoll 400, 0.1% polyvinyl pyrrolidone, 0.1% bovine serum albumin, 0.2% SDS, and 250 μg/ml sheared salmon sperm DNA at 42 °C. Hybridization occurred at 42 °C overnight in 10% dextran sulfate with biotinylated-DNA probes (5 ng/cm^2) specific for elongation factor II and sGC α_1 mRNA. Blots were then washed twice at 65 °C in 5× SSC, 0.5% SDS, washed for 30 min at 50 °C in 0.1× SSC, 1% SDS, and washed for 1 min with TBS-Tween 20 (0.05% Tween 20, 150 mM NaCl, 100 mM Tris/HCl, pH 7.5). Afterward membranes were blocked for 1 h at 65 °C in TBS-Tween 20 containing 3% bovine serum albumin and incubated with a streptavidin-alkaline phosphatase conjugate (7 μl/100 cm^2; 1:1000 in TBS-Tween 20) for 10 min at room temperature. The blots were washed twice in TBS-Tween 20 and then in 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris/HCl, pH 9.5. Immunoreactive mRNA bands were visualized by chemiluminescence and exposure to x-ray film.

Preparation of sGCα_1 Transcripts by in Vitro Transcription—Total RNA from rat lungs was used as a template for RT-PCR amplification of the 3′-UTR of sGCα_1 cDNA regions. A *Pfu* DNA polymerase (*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′ (**T$_7$**). For generating the 3UTRSK2 (424 bp) template, sense primer (5′-**T$_7$**CCAGCTACATCTTTGTGCC-3′) and antisense primer (5′-ACTGTCCTCTACAGTAGGC-3′), corresponding to positions 3049–3067 and 3423–3441 of the sGCα_1 cDNA, were used (GenBank™ accession number U60835). Subsequently, PCR fragments encompassing the 3′-UTR of sGCα_1 mRNA were synthesized bearing a T_7 sequence at the 5′-end. Biotinylation of transcripts was performed with the North2South™ Biotin *in vitro* transcription kit from Pierce (Rockford, IL).

RNA-Protein Binding Reactions and Supershift Assays—Electrophoretic mobility shift assays (EMSA) were carried out by a modification of the method of Wang (12). The oligoribonucleotide (3UTRSK2, 50–200 ng) was incubated with 40–100 μg of native nuclear extract (prepared according to Ref. 13) from endothelium-denuded rat aorta, and a 10× reaction buffer (15 mM Hepes, pH 7.9, 600 mM KCl, 10 mM dithiothreitol, 50% glycerol, 30 mM $MgCl_2$, 2 units/μl RNase inhibitors (40 units/μl, RNaseOUT, Invitrogen), 200 ng/ml total RNA) for 30 min at room temperature. Complexes were resolved by native 2% TAE-agarose gel electrophoresis (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× 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 sGCα_1 Subunit—Total protein was precipitated (1.5 ml of 100% isopropanol) from the phenol-ethanol supernatant (TRIzol method) of the RNA extraction, and the precipitate was dissolved in 1% SDS. The protein (40 μg per lane) was fractionated on Laemmli gels and electroblotted onto nitrocellulose filters (Protrans; Schleicher & Schuell). The blots were blocked at 4 °C overnight and then incubated for 2 h at room temperature with either a polyclonal chicken antibody (IgY) or a rabbit antibody (IgG) directed against the α_1 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—An HuR-specific siRNA (HuR-siRNA) was designed by selecting a target region from base position 163 to 183 relative to the start codon, which fulfilled the specific sequence requirements as follows: AA(N_{19})dTdT (N is any nucleotide); 21-nt sense and 21-nt antisense strand; ~50% G/C content; and a symmetric 2-desoxythymidine 3′ overhang. Sense and antisense oligonucleotides were synthesized by Xeragon Oligonucleotides (Xeragon-Qiagen). The lyophilized 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 h 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. Lyophilized or dissolved siRNAs were stored at −20 °C.

Transfection of Cultured Vascular Smooth Muscle Cells—Cultured smooth muscle cells from rat aortas were grown in minimal essential medium (MEM, Invitrogen) containing 10% fetal calf serum and 1% penicillin and streptomycin at 37 °C and 5% CO_2. SMCs were trypsinized, mixed with fresh MEM (plus antibiotics) without fetal calf serum, and seeded onto six-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: micrograms of RNA to micrograms of Enhancer R, 1:2) and 168 μl of Buffer EC-R (Qiagen) and mixed by vortexing for 10 s. After incubation (5 min, room temperature) 24 μl of TMTR (ratio: micrograms of RNA to microliters of TMTR, 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) of sterile Dulbecco's phosphate-buffered saline (Invitrogen). Then the RNA-transfection mixture was diluted in 1.78 ml of MEM (plus antibiotics) and added dropwise to the cells (2 ml of transfection complex per well). The cells were cultured for 2 days at 37 °C (5% CO_2). 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 sGCα_1 mRNA and Protein in Rat Aorta—To assess the effect of increased cGMP formation on the sGCα_1 subunit expression in rat aorta, freshly isolated endothelium-denuded aortic rings from Wistar Kyoto rats were kept under organ culture conditions, either in the absence (control ± 0.2% Me_2SO) or presence of the sGC activator molecule YC-1 (10 μM), and the specific sGC inhibitor NS2028 (10 μM). After 24 h the vascular tissue was snap-frozen and homogenized in liquid nitrogen, then further processed for sGCα_1 subunit mRNA and protein expression by RT-PCR (Fig. 1*A*) and Western blot (Fig. 1*B*), respectively. According to densitometric analysis of the RT-PCR product (Fig. 1*A*) and the immunoreactive protein (α_1 = 82 kDa, Fig. 1*B*) the abundance of sGCα_1 subunit mRNA and protein was markedly lower in YC-1-exposed aorta compared with controls (α_1-mRNA = 93%

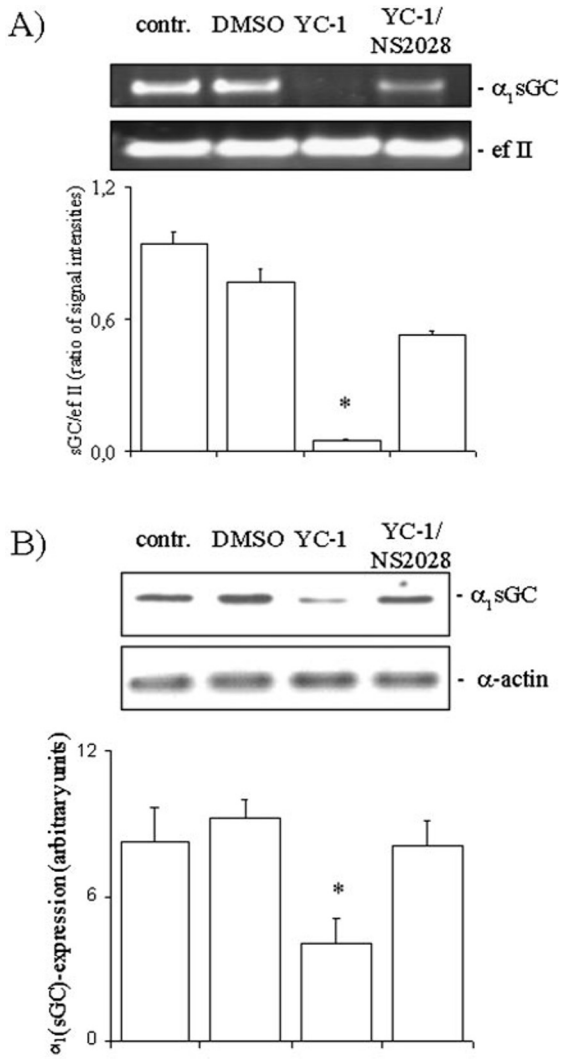

FIG. 1. **Influence of YC-1 on sGCα_1 subunit expression in rat aorta.** *A*, RT-PCR analysis of mRNA isolated from rat aorta kept for 24 h in organ culture (control), in the presence of 0.2% Me_2SO, 10 μM YC-1, or YC-1 and 10 μM NS2028. The *upper fluorographs* show ethidium bromide-stained agarose gels containing RT-PCR products of the sGCα_1 mRNA and elongation factor (*ef*) II mRNA amplified from 2 μg of total RNA. The *bar graph* below shows a densitometric analysis of the sGCα_1 mRNA intensity, normalized by ef II mRNA intensity. Summarized data (mean value ± S.E.) from four rats. *, significantly different from control (*contr.*), Me_2SO (*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 sGCα_1 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 sGCα_1-specific band, normalized by α-actin-staining. Summarized data (mean value ± S.E.) from four rats. *, significantly different from control (*contr.*), Me_2SO (*DMSO*), and YC1/NS2028 ($p < 0.05$, ANOVA).

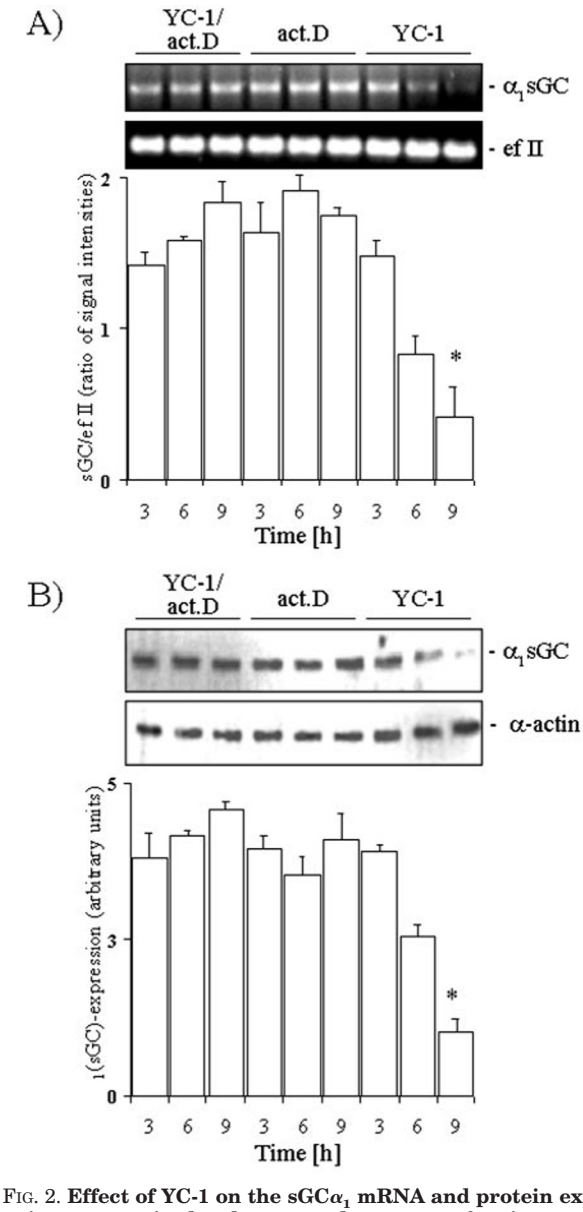

FIG. 2. **Effect of YC-1 on the sGCα_1 mRNA and protein expression in rat aorta in the absence and presence of actinomycin D.** Rat aortic rings were exposed in organ culture to 10 μM YC-1 and/or 10 μM actinomycin D for 3, 6, and 9 h, frozen, and processed for RT-PCR and Western blot analysis of sGCα_1. *A*, the *upper fluorographs* show ethidium bromide-stained agarose gels containing RT-PCR products of the sGCα_1 mRNA amplified from 2 μg of total RNA as well as of elongation factor (*ef*) II mRNA. The sGCα_1 RT-PCR product intensities were normalized for ef II intensities. Summarized data (mean value ± S.D.) from three rats. *, significantly different from *YC1/act.D* (9 h) and *act.D* (9 h) ($p < 0.05$, ANOVA). *B*, Western blot. The protein from the same aorta as used for RT-PCR analysis was separated by SDS-PAGE, and the sGCα_1 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 sGCα_1-specific band, normalized by α-actin staining. Summarized data (mean value ± S.D.) from three rats. *, significantly different from *YC1/act.D* (9 h) and *act.D* (9 h) ($p < 0.05$, ANOVA).

lower, α_1-protein = 56% lower *versus* controls; *bar graphs*; Fig. 1, *A* and *B*), whereas the levels of elongation factor II mRNA (Fig. 1*A*) and α-actin protein (Fig. 1*B*) 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. 1, *A* and *B*). These findings show that long lasting activation of sGC in the rat aorta decreases sGCα_1 subunit expression at the mRNA and protein level.

YC-1 Decreases the sGCα_1 mRNA Expression in Rat Aorta by a Mechanism Requiring Transcription—To assess whether YC-1 affects the stability of sGCα_1 mRNA, we investigated the time course of sGCα_1 mRNA expression after inhibition of cell transcription by actinomycin D (Act D). The aortic rings were held in organ culture for 3, 6, or 9 h, in the presence of YC-1 (10 μM), or Act D (10 μM), or both. The tissue level of sGCα_1 mRNA was estimated by semi-quantitative RT-PCR. As illustrated by the fluorographs in Fig. 2*A* and the densitometric analysis shown below, the level of sGCα_1 mRNA did not decrease in Act D-exposed aortic rings for up to 9 h, indicating that the 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

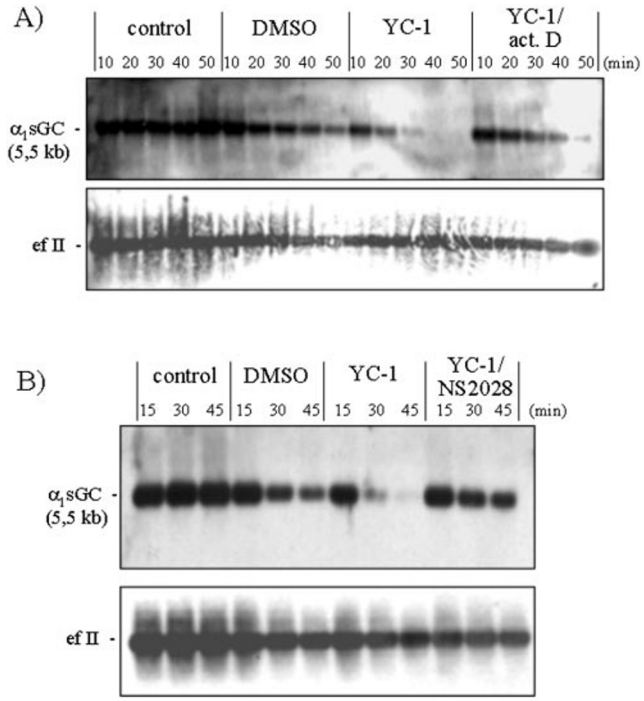

FIG. 3. **Effect of native protein from YC-1-, NS2028-, and Act D-treated rat aorta on the sGCα_1 mRNA stability.** Representative Northern blots of sGCα_1 subunit (5.5 kb) and ef II mRNA. Native protein (20 μg) isolated from the same aortas as used in the experiments shown in Figs. 1 and 2 was incubated at 37 °C with 1 μg of poly(A)$^+$ RNA isolated from rat lung, and the amount of sGCα_1 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.

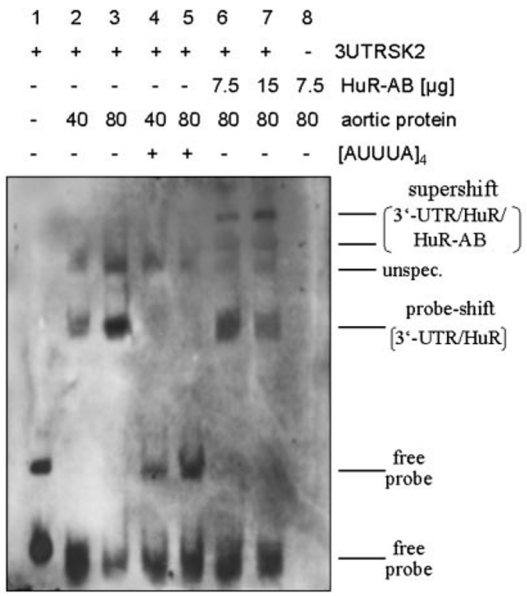

FIG. 5. **HuR present in rat aortic nuclear extract binds to the 3′-UTR of sGCα_1 mRNA.** RNA electrophoretic mobility shift analysis (RNA-EMSA) representative of two experiments. A biotin-labeled RNA probe (3UTRSK2, 424 bp, 250 ng) was incubated for 30 min with nuclear protein (40 and 80 μg) extracted from freshly isolated endothelium-denuded rat aorta (see "Materials and Methods") then loaded and electrophoresed on a 6% TBE-acrylamide gel. The protein-RNA complexes were electroblotted onto a nylon membrane and visualized by chemiluminescence, as described under "Materials and Methods." *Lanes* (from *left* to *right*): *1*, free probe; *2* and *3*, probe-shift induced by 40 and 80 μg of rat aortic protein; *4* and *5*, specific shift blocked by unlabeled competitor [AUUUA]$_4$ probe (25 μg); *6* and *7*, supershift induced by a monoclonal HuR antibody (HuR-AB; 7.5 and 15 μg); and *8*, negative control with aortic protein and HuR antibody.

FIG. 4. **AU-rich elements (AREs) present in the 3′-UTR of rat sGCα_1 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 number U60835.

sGCα_1 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 sGCα_1 mRNA abundance (Fig. 2*A*). 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. 2*A*). These results suggest that YC-1 decreases the stability of sGCα_1 mRNA by a mechanism requiring transcriptional activation of an unknown factor.

The time course of YC-1-induced sGCα_1 mRNA decay was mirrored by a quite similar time course of sGCα_1 protein expression, as assessed by Western blots analysis (Fig. 2*B*). In contrast, the expression of α-actin was constant for the same period of time (Fig. 2*B*).

YC-1-induced sGCα_1 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 sGCα_1 mRNA in the rat aorta, we assessed the effect of a protein extract from YC-1-exposed rat aorta on the rate of sGCα_1 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 of protein was incubated at 37 °C with 1 μg of enriched poly(A)$^+$ RNA isolated from rat lung (see "Materials and Methods"). After different periods of time (10–50 min (Fig. 3*A*) or 15–45 min (Fig. 3*B*)) an aliquot of the incubation mixture was probed for sGCα_1 and elongation factor II mRNA by Northern blotting (see "Materials and Methods"). In the absence of aortic protein (control) the sGCα_1 mRNA was stable for up to 50 min under these assay conditions (Fig. 3*A*). In the presence of protein from aortas exposed to 0.2% Me_2SO (solvent control), a moderate time-dependent decrease in sGCα_1 mRNA abundance was observed (Fig. 3, *A* and *B*, "*DMSO*" *lanes*). The rate of sGCα_1 mRNA

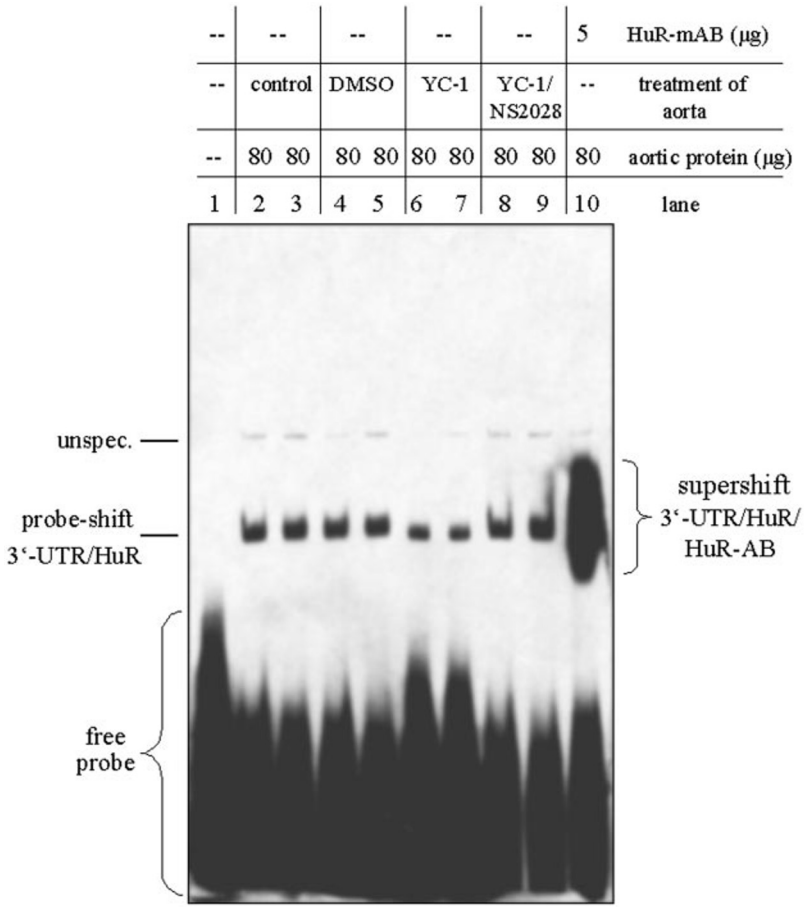

FIG. 6. **HuR binding activity in rat aorta is decreased by sGC activation.** RNA-EMSA performed with protein from two rat aortas. Rings of endothelium-denuded rat aorta were kept for 12 h in organ culture without additions (control), with solvent control (0.2% Me_2SO), with YC-1 (100 μM), or with YC-1 and NS2028 (both 100 μM) (see "Materials and Methods") then frozen and homogenized. Biotin-labeled sGCα_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 an 8% TBE-AA gel was used. *Lanes* (from *left* to *right*): *1*, free probe; *2* and *3*, protein from control aortas; *4* and *5*, protein from solvent (0.2% Me_2SO)-treated aortas; *6* and *7*, protein from YC-1-exposed aortas; *8* and *9*, protein from YC1/NS2028-exposed aortas; *10*, supershift induced by addition of 5 μg of HuR antibody to aortic protein from control aortas. Representative data from three rats.

decay was considerably accelerated by protein isolated from YC-1-exposed aortas (Fig. 3, *A* and *B*, "*YC-1*" *lanes*). In contrast, elongation factor II mRNA was quite stable even in the presence of protein from YC-1-exposed aorta (Fig. 3, *A* and *B*, *lower autoradiographs*), indicating that YC-1 specifically induced factors that led to accelerated decay of sGCα_1 mRNA. The formation of these factors was apparently prevented by a preincubation of the aortas with Act D (Fig. 3*A*) or NS2028 (Fig. 3*B*), because under these conditions the aortic protein extract exhibited markedly less sGCα_1 mRNA degrading activity.

Identification of HuR as a sGCα_1 mRNA-binding Protein in Rat Aorta—The 3′-UTR of the rat sGCα_1 mRNA bears several AUUUA motifs (AU-rich elements, AREs) (Fig. 4*A*), 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 sGCα_1 mRNA we synthesized a biotin-labeled oligoribonucleotide comprising bases 3049–3441 of the sGCα_1 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 (EMSAs). 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 probe shift, indicating competition between the synthetic ARE and the truncated 3′-UTR of sGCα_1 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 sGCα_1 mRNA (Fig. 5, *lanes 6* and *7*).

Activation of sGC by YC-1 Decreases HuR-ARE-binding Activity—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 (see "Materials and Methods"), either in the absence or presence of YC-1 (100 μM), and NS2028 (100 μM), or the solvent control (0.2% Me_2SO). 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, *lanes 2* and *3*). The protein extract from Me_2SO-treated aorta induced a quite similar shift (Fig. 6, *lanes 4* and *5*). In contrast, with protein from YC-1-exposed aorta the shifted band markedly decreased (Fig. 6, *lanes 6* and *7*). This effect of YC-1 was prevented by concomitant exposure of the aorta to the sGC inhibitor NS2028 (Fig. 6, *lanes 8* and *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 GCα_1 mRNA or down-regulates 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 NS2028 as shown in Fig. 6 was loaded for SDS-PAGE analysis, blotted, and immunoprobed for HuR. The protein

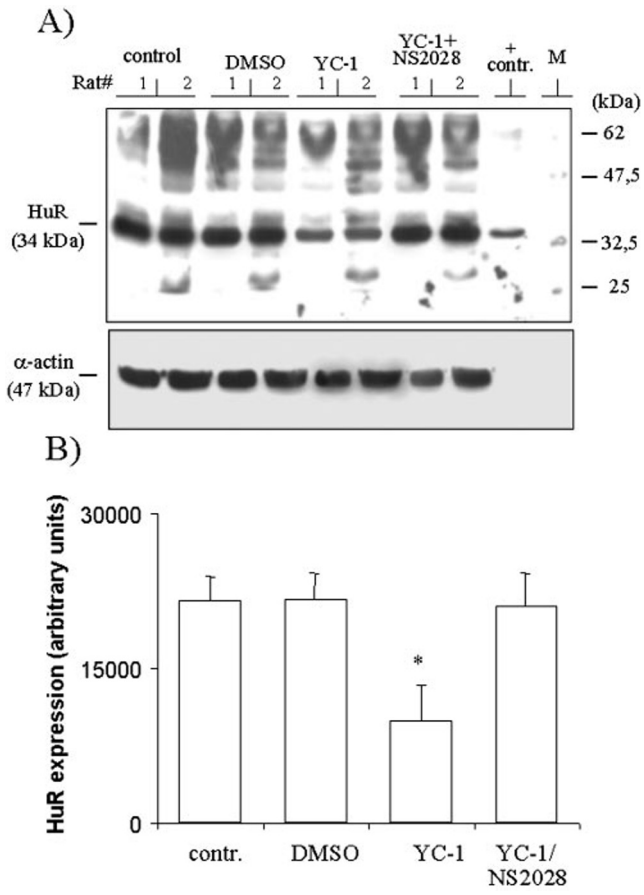

FIG. 7. **Activation of sGC in rat aorta decreases expression of HuR.** *A*, representative Western blot with aortas from two rats. Endothelium-denuded rat aortic segments were incubated for 12 h as described in Fig. 6. Total native protein (40 μg) was probed for HuR using a monoclonal antibody. *Lanes* (from *left* to *right*): *1* and *2*, control aortas; *3* and *4*, solvent (0.2% Me_2SO)-treated aortas; *5* and *6*, YC-1-exposed aortas; *7* and *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 ± S.D.) from three rats. *, significantly different from control (*contr.*), Me_2SO (*DMSO*), and YC1/NS2028 ($p < 0.05$, ANOVA).

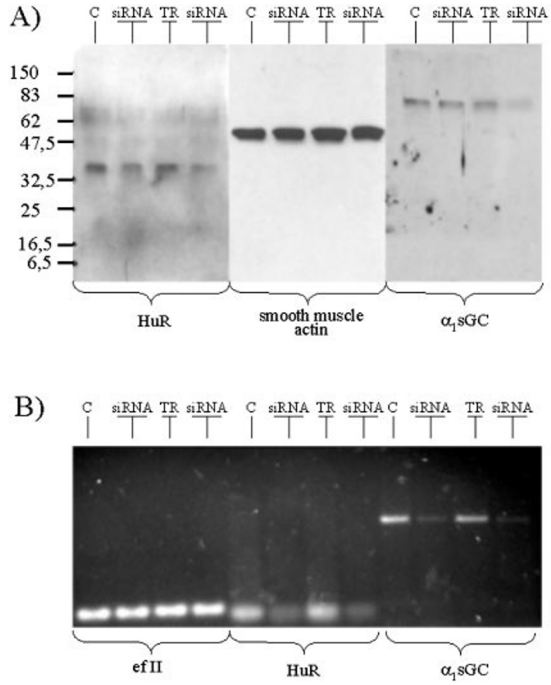

FIG. 8. **HuR RNA silencing by siRNA transfection decreases $sGC\alpha_1$ expression.** RASMC (16th to 18th passage) were transfected with two different double-stranded HuR-specific siRNAs (final concentration: 100 nM; *second lane* from *left*: siRNA from S. Sengupta; *fourth lane*: siRNA as described under "Materials and Methods") and cultured for 48 h at 37 °C. As a control RASMCs were incubated without siRNA (*C*) or with the transfection reagent only (*TR*). *A*, Western blot. Total native protein (10 μg) was probed for HuR (34 kDa) and $sGC\alpha_1$ 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, $sGC\alpha_1$, and elongation factor II (*ef II*) mRNA amplified from 2 μg of total RNA. Representative data out of two experiments.

from the control and Me_2SO-treated aortas showed a marked HuR-positive band at 34 kDa (Fig. 7*A*, *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* and *6*, and densitometric analysis shown below in Fig. 7*B*). The sGC inhibitor NS2028 prevented the down-regulation of HuR expression by YC-1 (*lanes 7* and *8*).

HuR Knockdown by RNA Interference Decreases Expression of $sGC\alpha_1$—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 knockdown of HuR in cultured rat aortic smooth muscle cells (RASMC) affects expression of sGC. Incubation of RASMC for 24 h with two different HuR siRNA oligonucleotides decreased HuR expression at the protein (Fig. 8*A*, "*siRNA*") and mRNA level (Fig. 8*B*, "*siRNA*"). In the same cell extracts the expression of $sGC\alpha_1$ protein and mRNA was decreased as well (Fig. 8, *A* and *B*), compared with controls. Expression of actin protein (Fig. 8*A*) and elongation factor II mRNA (Fig. 8*B*) was not affected by HuR siRNA. This finding clearly shows that specific knockdown of HuR decreases $sGC\alpha_1$ 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 $sGC\alpha_1$ and β_1 mRNA (17).

We set out to reveal the mechanism accounting for the down-regulation of the $sGC\alpha_1$ subunit expression in response to sGC activation. Our rationale for studying $sGC\alpha_1$ was that in preliminary studies we found that this subunit was less expressed in rat vascular tissues than the β_1 subunit and, therefore, formation of the NO-sensitive $\alpha_1\beta_1$ holoenzyme would be limited by the α_1 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 $sGC\alpha_1$ mRNA bears several AUUUA-motifs (AU-rich elements, AREs) that 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 pro-

vide evidence by *in vitro* mRNA degradation assay and RNA-EMSA that HuR protects the rat sGCα_1 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 sGCα_1 mRNA. Consequently, the expression of the sGCα_1 subunit was decreased at the mRNA and protein level. All these effects could be blocked by an inhibitor of YC-1-stimulated sGC activity, NS2028 (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. Although 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 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 21-mer double-stranded RNA, homologous to base position 163–183 relative to the start codon of the HuR message (HuR siRNA), we observed a strong decrease in HuR as well as in sGCα_1 expression at the mRNA and protein level (Fig. 8). This experiment proves that down-regulation of sGC mRNA is a consequence of decreased HuR expression.

The signaling cascade accounting for cGMP-dependent down-regulation of HuR could not be revealed in this study. Because concomitant application of Act D during exposure of the rat aorta to YC-1 prevented the down-regulation of HuR expression and binding activity, it is likely that cGMP induces the transcriptional activation of (unknown) factors that decrease HuR expression. Our preliminary data indicate that a cGMP-activated protein kinase and the transcription factor AP-1 are involved in down-regulation of HuR by sGC activators,[2] but an in-depth study is required. AP-1 sites are present in the mouse HuR promoter region (24). Interestingly, CREB sites were also found in this promoter region (24). Agents that 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,[2] 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 underscores the increasing importance of mRNA stability regulation for gene expression (25), as compared with 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 down-regulated 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 be needed to reveal the relative importance of HuR-regulated mRNA stability *versus* transcriptional processes for NO/cGMP-dependent gene expression.

[2] S. Kloess and A. Mülsch, unpublished.

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