0026-895X/04/6506-1440–1451$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 65:1440–1451, 2004

Vol. 65, No. 6
2896/1151865
Printed in U.S.A.

Down-Regulation of Soluble Guanylyl Cyclase Expression by Cyclic AMP Is Mediated by mRNA-Stabilizing Protein HuR

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Received August 13, 2003; accepted February 27, 2004

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

We analyzed whether the cyclic AMP induced down-regulation of the nitric oxide (NO) receptor soluble guanylyl cyclase (sGC) is mediated by the mRNA-protecting protein HuR. Exposure (up to 24 h) of isolated rat aortic segments to the activator of adenylyl cyclase, forskolin (10 μM), and to both activators of cAMP-stimulated protein kinase (PKA), 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′- cyclic monophosphorothioate, Sp- isomer (Sp-5,6-DCl-cBIMPS; 400 nM), and N^6-phenyl-cAMP (10 μM), strongly reduced sGC$\alpha_1\beta_1$ and HuR protein and mRNA expression in a time-dependent and actinomycin D (10 μM)-sensitive fashion. In vitro degradation of sGCα_1 and β_1 poly(A)$^+$ mRNA by native rat aortic protein was markedly increased by pretreatment of intact aortas with forskolin. Native protein extract from rat aorta shifted the electrophoretic mobility of biotin-labeled riboprobes from the 3′-untranslated region of sGCα_1 and β_1 mRNA, and these bands was supershifted by a monoclonal antibody directed against the mRNA-stabilizing protein HuR. Forskolin decreased the HuR-sGCα_1 and β_1 mRNA interaction and HuR protein expression in rat aorta, and this was prevented by the PKA inhibitory cAMP analog 3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPS). In cultured smooth muscle cells from rat aorta, forskolin induced a rapid increase in Fos/p-Fos protein levels and activator protein 1 (AP-1) binding activity. Inhibition of this transcription factor by an AP-1 decoy prevented the forskolin-induced down-regulation of HuR. We conclude that forskolin/cAMP decrease the expression of heterodimeric sGC in rat aortic smooth muscle cells via activation of Fos/AP-1, which decreases the expression of HuR and thus destabilizes the sGCα_1 and β_1 mRNA.

Soluble guanylyl cyclase (sGC) is a key signaling component of the L-arginine-NO-cyclic GMP pathway (Denninger and Marletta, 1999; Bellamy et al., 2002). The propagation of NO signaling via cyclic GMP formation is influenced at different time scales, most rapidly by allosteric activation of heterodimeric sGC upon binding of NO to its heme-iron. In addition, NO signaling may be affected by altered expression of sGC subunits, as has been shown in different pathological conditions (Li et al., 1999; Kloss et al., 2000; Marques et al., 2001; Mülsch et al., 2001; Takata et al., 2001; Telfer et al., 2001; Tzao et al., 2001) and developmental stages (Giuili et al., 1994; Bloch et al., 1997; White et al., 2000; Behrends et al., 2001a,b; Ibarra et al., 2001). The molecular mechanisms underlying altered sGC expression in these and other conditions have not yet been revealed. Post-transcriptional events may play a prominent role in regulation of sGC expression. Indeed, NO donors (Filippov et al., 1997), cGMP (Ujiie et al., 1994), nerve growth factor (Liu et al., 1997), and cAMP (Shimouchi et al., 1993; Papapetropoulos et al., 1995) have been shown to reduce sGC expression by destabilization of sGC mRNAs. The 3′-untranslated regions (UTR) of the sGCα_1 and β_1 mRNA bear AU-rich elements (ARE), which are targeted by *trans*-acting factors for regulation of mRNA stability. One of these factors is the *elav*-like ubiquitous 34-kDa protein HuR, which binds to AREs, thereby protecting the respective mRNA from degradation (Ma et al., 1996; Fan and Steitz, 1998). Recently, HuR and other ARE-binding proteins were also found to regulate translation of target mRNAs, by recruitment to mammalian stress granules formed in response to metabolic stress (Kedersha and Anderson 2002). We recently analyzed the mechanism whereby cGMP and cGMP-eliciting agents, such as YC-1, decrease the expression of sGCα_1 mRNA. We found that HuR interacts with the 3′UTR of sGCα_1 mRNA, and that YC-1 reduced the stability of sGCα_1 mRNA via down-regulation of HuR (Kloss

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 553, TP C10 to A.M.).

ABBREVIATIONS: sGC, soluble guanylyl cyclase; UTR, untranslated region; ARE, AU-rich element; RASMC, rat aortic smooth muscle cells; RT-PCR, reverse transcriptase-polymerase chain reaction; Sp-5,6-DCl-cBIMPS, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′- cyclic monophosphorothioate, Sp- isomer; Rp-cAMPS, 3′,5′-cyclic monophosphorothioate, Rp-isomer; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; AP-1, activator protein 1; DsDNA, double-string DNA; TAE, Tris-acetate/EDTA; EMSA, electrophoretic mobility shift assay; ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; PKA, protein kinase A; ef II, elongation factor II; kb, kilobase(s); act.D, actinomycin D; p-Fos, phosphorylated Fos; ODN, oligodesoxynucleotide; HuR, *elav*-like protein.

et al., 2003). In accordance with these findings, knock-down of HuR by siRNA approach reduced the expression of sGCα_1 subunit (Kloss et al., 2003). We now assessed whether or not down-regulation of HuR accounts for cAMP-induced depression of sGCα_1 and also β_1 in rat aorta and rat aortic smooth muscle cells (RASMC).

Materials and Methods

Materials. The polyclonal chicken antibody directed against the α_1- and β_1-subunit of the rat lung sGC was from Alexis GmbH (Grünstadt, Germany), and the rabbit-anti-chicken antibody was from Biogenes (Berlin, Germany). The monoclonal HuR antibody (19F12) was kindly provided by Dr. Furneaux (Memorial Sloan-Kettering Cancer Center, New York, NY). The anti-Fos antibody (rabbit) was from Santa Cruz Biotechnology (Heidelberg, Germany). The oligonucleotides for RT-PCR, in vitro transcription, and gel-shift analysis were synthesized by BioSpring GmbH (Frankfurt, Germany) and MWG Biotech (Ebersberg, Germany), respectively. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-cyclic monophosphorothioate, Sp-isomer (Sp-5,6-DCl-cBIMPS), adenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPS), and N^6-phenyl-cAMP were from Biolog (Bremen, Germany). Forskolin and the anti-α-actin antibody (murine) were from Sigma (Dreieich, Germany).

Cell Culture. To assess the effect of actinomycin D and forskolin on intracellular localization and expression of HuR and to perform transcription factor decoy experiments, we used cultured RASMC. The cells were isolated from the thoracic aorta of male Wistar rats and cultured in minimum essential medium containing 2 mM L-glutamine, 5 mM TES, 5 mM HEPES (both at pH 7.3), 100 U/ml penicillin, 50 μg/ml streptomycin, and 10% FCS as described previously (Schini-Kerth et al., 1997). Confluent cells (passage 10–12) were placed for 24 h in serum-free medium containing 0.1% fatty acid-free bovine serum albumin before incubations with forskolin (10 μM) or other treatments started.

Isolation and Organ Culture of Rat Aortic Rings. The thoracic aorta was isolated from young (2-month-old) male Wistar rats (Möllegard, Skensved, Denmark) and the endothelium mechanically removed as described previously (Kloss et al., 2003). Rings of 3-mm length were placed in culture dishes in minimum essential medium supplemented by N^G-nitro-L-arginine (30 μM, to block residual NO synthase activity), under a carbogen atmosphere (4.5% CO_2) at 37°C. After 2 h, the rings were exposed to forskolin (10 μM), or appropriate solvent (0.1% DMSO) for up to 16 h. Some rings were pre-exposed to actinomycin D (10 μM) for 30 min before addition of forskolin. Thereafter, the rings were snap-frozen in liquid nitrogen and stored at −70°C.

Western Blot. Western blotting of HuR, α-actin, and sGC subunits was performed as described previously (Kloss et al., 2003). For immunodetection of c-Fos, the blots were incubated with a 1:1000 dilution (in blocking buffer) of anti-c-Fos antibody for 12 h at 4°C. After repeated washes, the blots were exposed to a peroxidase-linked anti-rabbit-IgG antibody (1:10,000) and further processed for chemiluminescence-detection of immunoreactive protein as described previously (Kloss et al., 2003).

mRNA Degradation Assay (Northern Blot). Poly(A)$^+$ mRNA was purified from rat lung total RNA by means of the Messagemaker kit (Invitrogen, Karlsruhe, Germany) (Kloss et al., 2003). The denatured poly(A)$^+$ RNA sample was fractionated in a 1.2% agarose-formaldehyde gel and blotted overnight onto nylon membrane. The mRNA was fixed by UV–cross-linking, baked at 80°C for 2 h, and then prehybridized for 2 h at 42°C. Hybridization occurred at 42°C overnight with biotinylated DNA probes specific for elongation factor II and sGC α_1 and β_1 mRNA, which had been synthesized by using published primers (Kloss et al., 2000) and the Bioprime DNA labeling system (Invitrogen). Blots were then washed twice at 65°C, blocked for 1 h at 65°C, and incubated with a streptavidin-alkaline phosphatase conjugate (1:1000) for 10 min at RT. The blots were washed twice and immunoreactive mRNA bands were visualized by chemiluminescence and exposure to X-ray film.

DNA-Electrophoretic Mobility Shift Assays (DNA-EMSA). Nuclear protein was prepared from RASMC (10-cm culture dishes) in a modification (Schini-Kerth et al., 1997) of the method by (Schreiber et al., 1989) and stored at −70°C. The binding reaction proceeded in 100 mM NaCl, 1.5 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 1 μg of poly(dI/dC), and 5 mM HEPES-NaOH, pH 7.9, for 30 min at RT with 20 μg of nuclear protein and 6 μg of biotin-labeled AP-1–specific 19-mer dsDNA (5′-AGCTTG*TGAGTCA*GAAGCT-3′) (Kitabayashi et al., 1991), or an AP-1 mismatch 19-mer (5′-AGCTTG*AATC*TCAGAAGCT-3′). The mixture was loaded on a nondenaturing 2% TAE-buffered agarose gel and electrophoresed for 2 h at 90 V. Thereafter the gel was wet-blotted overnight on a nylon membrane, the membrane was incubated with phosphatase-labeled streptavidin, and the free and shifted probes detected by chemiluminescence, as described for Northern blots.

RNA-EMSA. Electrophoretic mobility shift assays (EMSA) were carried out as described recently (Kloss et al., 2003). The biotin-labeled oligoribonucleotides DRα_1GC3UTR2 (5′-UAUCUGUGAUAAAACAUUUUAAUUAAUAGUAACAAUGUAC-3′), comprising bases 3256 to 3295 of the 3′-UTR from the sGCα1 mRNA, and β_1GC3UTR1 (5′-AAACUGCUUUUCUGUAAAAAUGUUUGUCUUUCAUUUAGUA-3′), comprising bases 2929 to 2968 of the 3′-UTR from the sGCβ_1 mRNA, were from MWG Biotech. The oligoribonucleotides (150 ng) were incubated with 20 μg of total native extract (nuclear and cytosolic) from endothelium-denuded rat aorta, and a reaction mix [10× reaction buffer (100 mM Tris, pH 7.5, 500 mM KCl, 10 mM dithiothreitol; LightShift chemiluminescent EMSA Kit; Pierce Perbio, Rockford, IL; 1.5% glycerol, 5 mM $MgCl_2$, 0.05% Nonidet P-40, 2 units/μl RNase inhibitors (40 units/μl, RNaseOUT; Invitrogen), 200 ng/ml total tRNA, and rRNA] for 30 min at 4°C. Complexes were resolved by native 8% polyacrylamide gel electrophoresis for 2 to 3 h at 4°C and electroblotted onto nitrocellulose filters (Protrans; Schleicher and Schuell). Blocking and detection of biotin-labeled bands was performed as described previously (Kloss et al., 2003). For supershifts, 4 μg of the monoclonal HuR-antibody was incubated with the native protein extract for 1 h on ice before the specific riboprobe was added; all subsequent steps were performed as described for native gels.

AP-1 Decoy. A modification of a published method (Morishita et al., 1996) was used. Cultured RASMC (200,000 cells/well; 9–11 passages) were starved for 24 h, washed with balanced salt solution (137 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.6) and then transfected with either the match or the mismatch AP-1 dsDNA (19-mer used for EMSA; 10 μM) for 4 h by the effectene method according to the supplier's manual (QIAGEN, Hilden, Germany). The salt solution was replaced by serum-free medium, and the cells were incubated for a further 6 h in the absence and presence of forskolin (10 μM), harvested, and processed for AP-1 binding activity (EMSA) and expression of HuR.

Determination of sGC Activity. The enzymatic activity of sGC in native protein extracts (nanomoles of cGMP formed per minute per milligram of protein) was determined by assessing the conversion of [α-^{32}P]GTP to [^{32}P]cGMP exactly as described previously (Brandes et al., 2000), using 15 μg of aortic protein per sample (100 μl).

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

Results

Influence of Forskolin and Stimulatory cAMP Analogs on sGC and HuR Expression. To analyze the influence of cAMP elevation on the expression of sGC and HuR in

rat aorta, freshly isolated aortic tissue was incubated in organ culture for up to 24 h in the absence ("untreated") and presence of solvent (0.1% DMSO), forskolin (10 μM), and adenylyl cyclase activator, or a mixture of Sp-5,6-DCl-cBIMPS (400 nM) and N^6-phenyl-cAMP (10 μM), protein kinase A (PKA)-stimulating cAMP analogs. The tissue was homogenized and RNA and protein extracted (see *Materials and Methods*). The expression of HuR protein was assessed by Western blot, and the blot was stripped and subsequently probed for sGC subunits α_1, β_1, and α-actin. As illustrated in Fig. 1, after a small transient increase at 2 and 4 h, the expression of HuR and sGC subunits slowly and concomitantly decreased in untreated and solvent-treated (0.1% DMSO) rat aorta within 24 h of organ culture. This decrease was markedly accelerated by either forskolin or cAMP analogs, such that HuR was barely detectable at 8 h and sGCα_1 and β_1 at 12 h. The expression of α-actin was stable for this period of time and was not affected by either treatment (Fig. 1).

The changes in protein expression were accompanied by similar changes in mRNA abundance of HuR and sGCα_1 and β_1 subunits assessed by RT-PCR, as illustrated in Fig. 2. Under control conditions, mRNA levels of HuR and sGC subunits transiently increased at 2 and 4 h, respectively and remained constant for at least 8 h. Forskolin and cAMP analogs accelerated the time-dependent decrease of all three mRNAs. Expression of elongation factor II mRNA (ef II) somewhat increased under control conditions but remained constant for up to 12 h with solvent (0.1% DMSO), forskolin, or stimulatory cAMP analogs (Fig. 2).

Forskolin Decreases the Interaction of HuR with the 3′UTR of sGCα_1 mRNA. To investigate whether the cAMP-induced down-regulation of HuR is reflected by a corresponding loss in HuR function, we assessed HuR sGCα_1 and β_1 mRNA-binding activity in forskolin-pretreated rat aorta by electrophoretic RNA gel-shift analysis (RNA-EMSA). Endothelium-denuded rat aorta was treated for 0 and 8 h under organ culture conditions (see *Materials and Methods*), in the

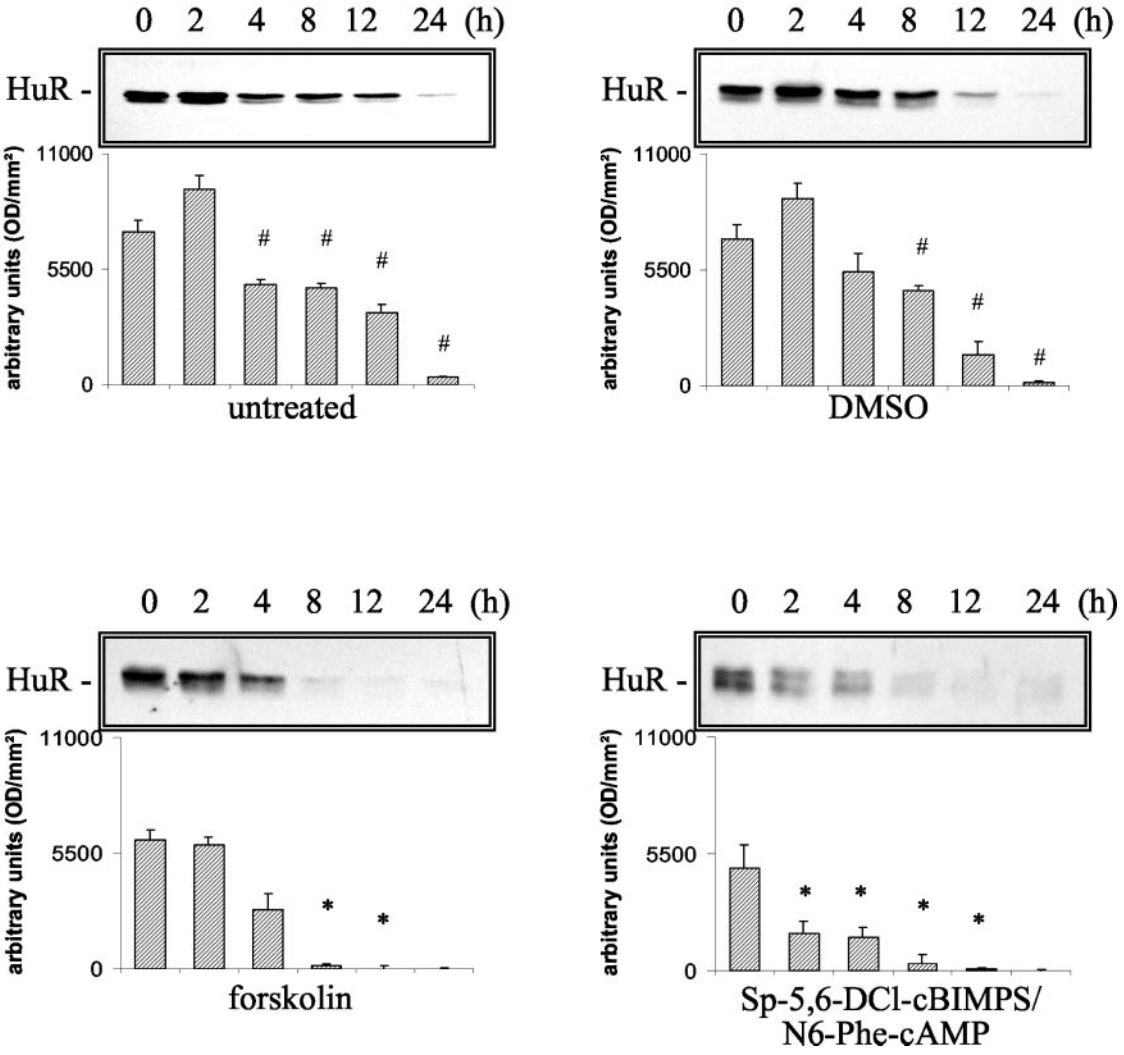

Fig. 1. Forskolin and stimulatory cAMP-analogs decrease HuR and sGC protein. Rat aortic rings were incubated without any additions (untreated), with solvent (0.1% DMSO), with 10 μM forskolin, or PKA agonists (400 nM Sp-5,6-DCl-cBIMPS/10 μM N^6-phenyl-cAMP) for 0, 2, 4, 8, 12, and 24 h. Total protein extracts (20 μg) were probed for HuR (34 kDa; this page), sGCα_1 (81.5 kDa; facing page), and β_1 (70 kDa; facing page) subunit using specific antibodies. Equal protein loading was verified by immunostaining of smooth muscle α-actin (47 kDa; B). The bar graph below each blot combines densitometric data (arbitrary units, optical density per square millimeter) from three different experiments (mean ± S.D.). #, significant difference ($P < 0.05$; ANOVA) versus 0 h within the same treatment group. *, significant difference ($P < 0.05$; ANOVA) versus the same time period in the solvent (DMSO) group.

presence of either solvent control (0.1% DMSO), forskolin (10 μM), or forskolin after pretreatment (45 min) with the PKA inhibitor Rp-cAMPs (50 μM). Total native protein extracts were prepared from the vascular tissue, and the HuR-like ARE binding activity was assessed by RNA-EMSA with a biotinylated ARE-containing oligoribonucleotide from the 3′-UTR of sGCα_1 and β_1 (DRα_1GC3UTR2 and β_1GC3UTR1; see *Materials and Methods*). In the presence of 20 μg of protein from DMSO-treated control aortas, a band-shift was observed [Fig. 3, A (sGCα1) and B (sGC β1), lanes 2 and 3]. In contrast, with protein from forskolin-exposed aorta, the shifted band markedly decreased after 8 h (Fig. 3, A and B, lane 5). This effect of forskolin was prevented by pretreatment of the aortas with Rp-cAMPs (Fig. 3, A and B, lane 7). A very similar pattern of bands was observed in three further experiments. Addition of a monoclonal HuR antibody (4 μg, 1-h pretreatment at 4°C) to control aortic protein induced a strong supershift (Fig. 3, A, lane 8, and B, lane 9). Addition of an unlabeled competitor probe $(AUUUA)_4$ prevented the shift (Fig. 3B, lane 8). These results show that forskolin induces a decrease of the HuR binding activity for conserved AREs in the 3′-UTR of GCα_1 mRNA in a PKA-activation-dependent fashion.

Forskolin Pretreatment of Rat Aorta Induces Destabilization of sGC α_1 and β_1 mRNA in Vitro. It was then important to see whether the cAMP-induced decrease in HuR expression and sGC mRNA-binding activity translates into decreased sGC mRNA stability in vitro. Therefore, poly(A)$^+$-enriched RNA from rat lung was incubated for 10 to 50 min at 37°C with a total native protein extract from either control (0.1% DMSO) or forskolin-treated (10 μM, 16 h) rat aorta, the reaction was stopped, and the amount of sGC α_1 (mRNA size, 5.5 kb) and β_1 (3.4 kb) as well as elongation factor II mRNA (2.6 kb) was assessed by Northern blot analysis, using spe-

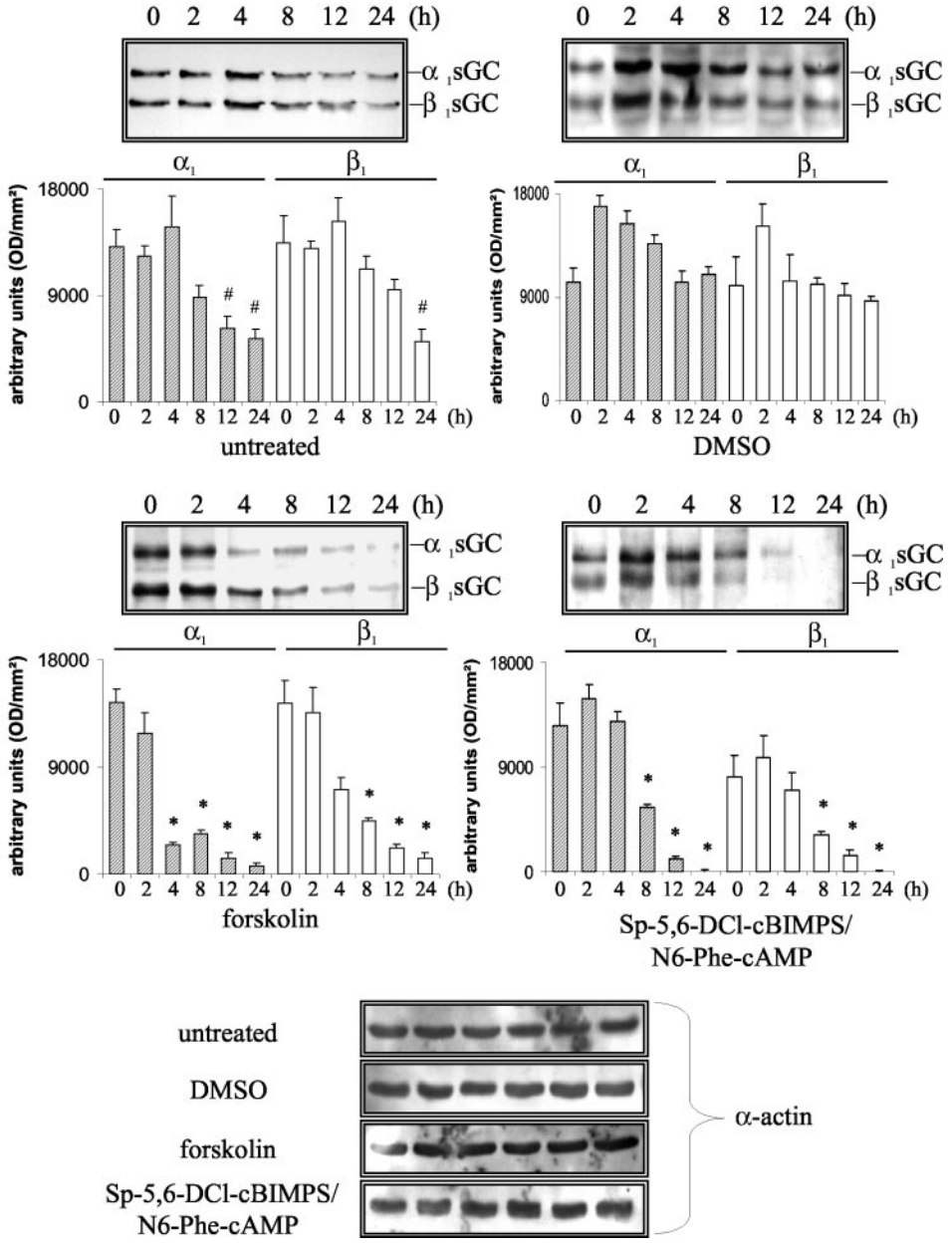

Fig. 1. (*continued*)

cific biotinylated probes (see *Materials and Methods*). When incubated in the absence of aortic protein for up to 45 min, the sGC and ef II mRNAs were not degraded at all (Fig. 4, "no protein"). After exposure to protein (20 μg) from control aorta, the sGCα_1 and β_1 mRNA content of the poly(A)$^+$-RNA mixture decreased, with a half-time of about 30 min (Fig. 4, "solvent-treated aorta"). The ef II mRNA signal did not decrease within 45 min. When the mRNA was incubated with protein from forskolin-treated rat aorta, the decay of both sGC subunit mRNAs was markedly accelerated compared with incubation with control aortic protein, whereas ef II mRNA stability was not different from control (Fig. 4, "forskolin"). Addition of actinomycin D (10 μM) during exposure of rat aorta to forskolin prevented the aortic protein-induced

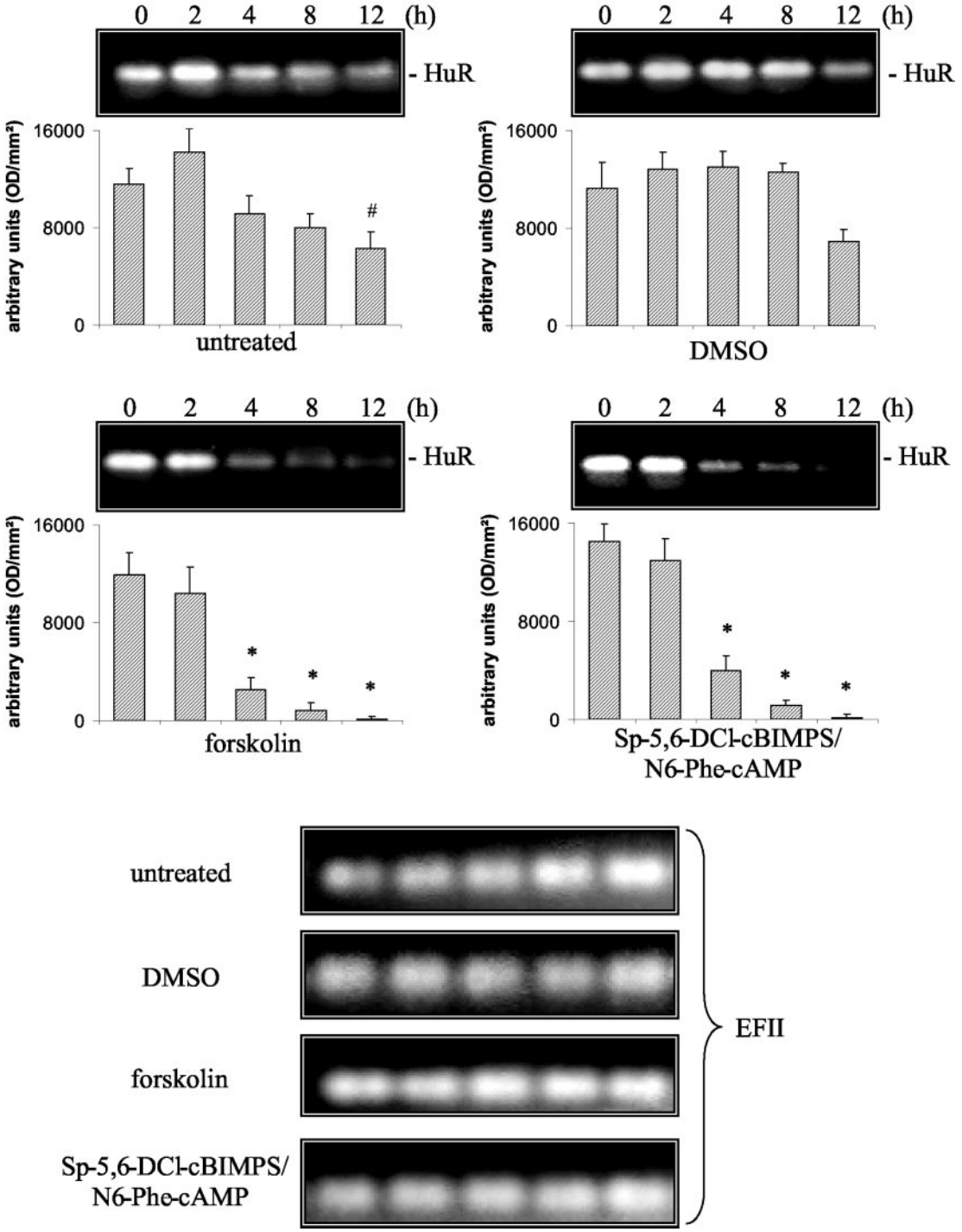

Fig. 2. Forskolin and stimulatory cAMP-analogs decrease HuR and sGC mRNA levels. RT-PCR analysis of mRNA isolated from rat aortic rings (treatment as described in Fig. 1). The graph shows ethidium bromide-stained agarose gels (1%) containing RT-PCR products of the HuR (this page), sGCα_1 (facing page), sGCβ_1 (facing page), and ef II (this page) mRNA amplified from 2 μg of total RNA. The bar graphs below combine densitometric data (arbitrary units, arbitrary units, optical density per square millimeter) from four different experiments (mean ± S.D.). #, significant difference versus 0 h ($P < 0.05$; ANOVA) within the same treatment group. *, significant difference ($P < 0.05$; ANOVA) versus a corresponding time interval of the solvent (DMSO)group.

degradation of sGC mRNA (Fig. 4, "forskolin/act.D"). This experiment shows that forskolin specifically induces a destabilization of both sGC subunit mRNAs, not of ef II mRNA, presumably because of decreased HuR-dependent protection.

Effect of Actinomycin D on Forskolin-Induced Depression of HuR and sGC. To start an analysis of intracellular signaling pathways mediating down-regulation of HuR, we then assessed the influence of actinomycin D, an inhibitor of gene transcription, on the depressing effect of forskolin/cAMP on sGC. As described in the first section under *Results*, isolated rat aorta was exposed for 6 and 16 h to forskolin (10 μM), now either in the absence or presence of act.D (10 μM),

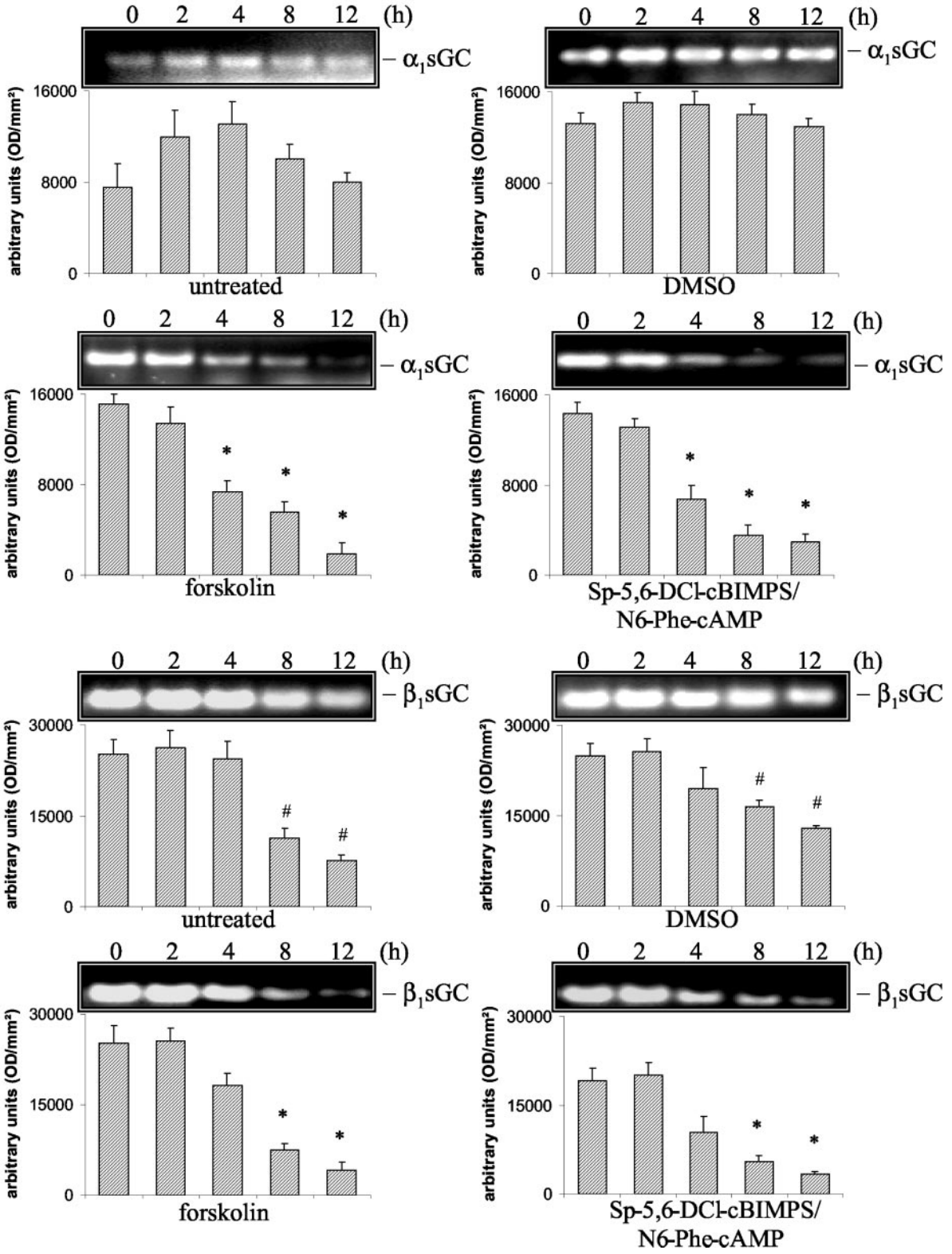

Fig. 2. (*continued*)

and sGC expression (taken as readout of altered HuR activity) was assessed by Western blotting, using a polyclonal chicken antibody that detects both sGC subunits simultaneously (Kloss et al., 2003). Compared with controls exposed only to solvent (0.1% DMSO). Forskolin treatment induced a down-regulation of both sGC subunits after 16 h (Fig. 5, upper blots), which was completely prevented by act.D in case of sGCα_1 and to a large extent also in case of β_1. By densitometric evaluation, we verified that this decrease was significant ($p < 0.05$; ANOVA) (Fig. 5, bar graph). This finding translated into similar changes in NO-stimulated sGC activity measured in extracts from forskolin and act.D exposed (12 h) aortae. In the presence of a maximally activating concentration of sodium nitroprusside (100 μM SNP), the specific sGC activity (nanomoles per milligram per minute) amounted to 0.53 $\pm$ 0.02 in untreated controls, 0.77 $\pm$ 0.05 after 12-h solvent exposure, 0.16 $\pm$ 0.01 after forskolin exposure (significantly different from control, solvent, and act.D; $p < 0.05$; ANOVA), and 0.49 $\pm$ 0.02 in aorta treated with both forskolin and act.D ($n = 3$).

Because act.D reportedly can change the intracellular localization of HuR (Peng et al., 1998), we sought to determine whether this mechanism could account for the observed inhibition of the forskolin-effect. Therefore, cultured RASMC were exposed for 1 and 6 h to either solvent (0.1% DMSO) or forskolin (10 μM), with or without act.D (10 μM). Cells were lysed, and cytosolic- and nuclear-protein-enriched fractions prepared by centrifugation (see *Materials and Methods*). HuR was assessed in both fractions by Western blotting. As shown in Fig. 6 HuR primarily localized to the nuclear frac-

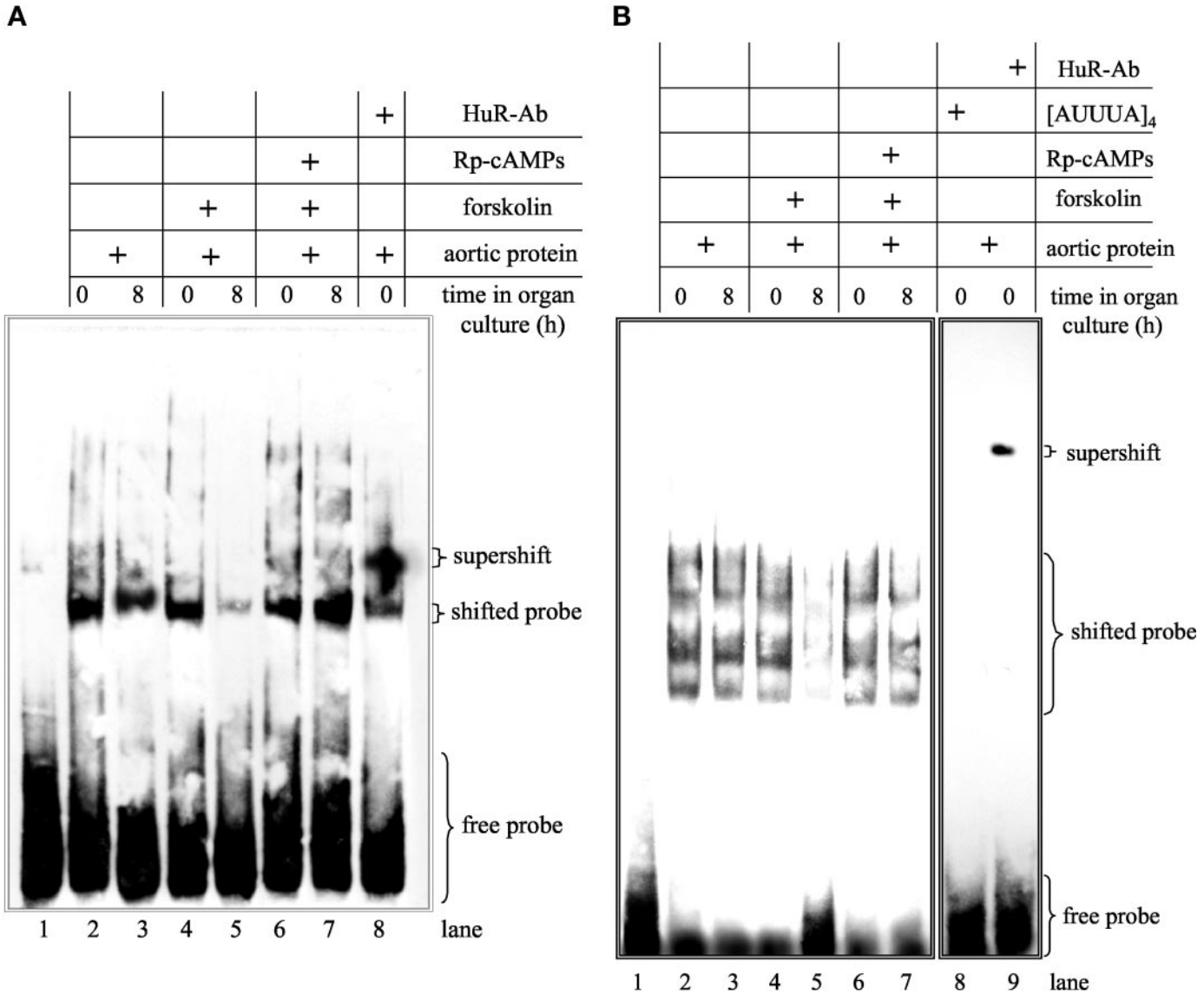

Fig. 3. sGC-mRNA-binding activity of HuR is decreased by forskolin in a PKA-inhibitor sensitive manner. Rings of endothelium-denuded rat aorta were incubated for 0 and 8 h in organ culture with solvent control (0.1% DMSO; lanes 2, 3, 8, and 9), with forskolin (10 μM; lanes 4 and 5), or with forskolin (10 μM) and Rp-cAMPs (50 μM; lanes 6 and 7), then frozen and homogenized. A, biotinylated oligoribonucleotide from the 3′-UTR of GCα_1 mRNA (DRα_1GC3UTR2; 7.5 ng) or B, sGCβ1 mRNA (β_1GC3UTR1; 7.5 ng) was incubated for 30 min at 4°C with aortic protein (20 μg), and the EMSA was performed using an 8% TAE-polyacrylamide gel. Lane 1, free probe; lane 8 (A) and lane 9 (B), supershift induced by addition (1 h at 4°C) of 4 μg of HuR antibody to aortic protein from untreated aortas. Lane 8B, shows the effect of addition of unlabeled competitor riboprobe $(AUUUA)_4$ (375 ng). Representative data from four independent experiments performed with aortas from four rats.

tion. After 6 h, forskolin strongly decreased HuR protein expression in both fractions, and this effect was completely prevented by act.D. The distribution of HuR between nuclear and cytosolic protein as seen in controls was not affected by act.D. These findings show that cAMP induces a down-regulation of HuR via a transcriptional event.

Induction of the Immediate Early Gene *c-fos* and Activation of the Transcription Factor AP-1 Accounts for down-Regulation of HuR by Forskolin. To identify transcription factors that might mediate the cAMP-induced depression of HuR, we assessed the expression of Fos, the protein coded by the immediate early gene *c-fos*, which is known to be activated by cyclic AMP (Angel and Karin, 1991; Seternes et al., 1998) and is one constituent of the heterodimeric transcription factor AP-1 (Fos/Jun). Confluent RASMC were stimulated with either forskolin (10 μM) or solvent control (0.1% DMSO) for up to 4 h, then harvested; expression of Fos (62 kDa) and phosphorylated Fos (p-Fos; 64 kDa) in the nuclear extract was assessed by Western blotting. Fos and p-Fos immunoreactive bands were markedly and stably enhanced in forskolin-stimulated cells compared with control cells (Fig. 7A). An increase in Fos/p-Fos was already seen in "0 min" forskolin-exposed cells, which were harvested immediately after forskolin addition. Although the harvesting process takes a few minutes, this is obviously sufficient time to allow Fos to increase in the nuclear fraction.

To determine whether forskolin-induced increased Fos/p-Fos expression is associated with increased Fos activity, we assessed AP-1 binding activity in nuclear extracts by DNA-gel-shift analysis, using a biotin-labeled AP-1–specific oligodesoxynucleotide as probe. As shown in Fig. 7B, AP-1 binding activity was low in solvent control cells and increased slightly after 4 h (lanes 2–5). In contrast, forskolin induced a strong and transient increase in AP-1 activity, which peaked between 20 min and 1 h of forskolin exposure (Fig. 7B, lanes 6–9). The AP-1 mismatch probe did not yield a signal with nuclear extract from cells exposed to forskolin for 1 h (Fig. 7B, lane 1). These results show that forskolin increases Fos/p-Fos and the activity of the transcription factor AP-1.

To finally prove that activation of AP-1 accounts for cAMP-induced down-regulation of HuR, we used a transcription factor decoy approach (Morishita et al., 1996). RASMC starved for 24 h were pretreated (4 h) or not with either an AP-1–cognate oligodesoxynucleotide (match ODN) or a mutated ODN (mismatch), and then incubated for 6 h with solvent (0.1% DMSO) or forskolin (10 μM). The cells were harvested, and HuR expression was assessed by Western blotting. As shown in Fig. 8, the down-regulation of HuR by

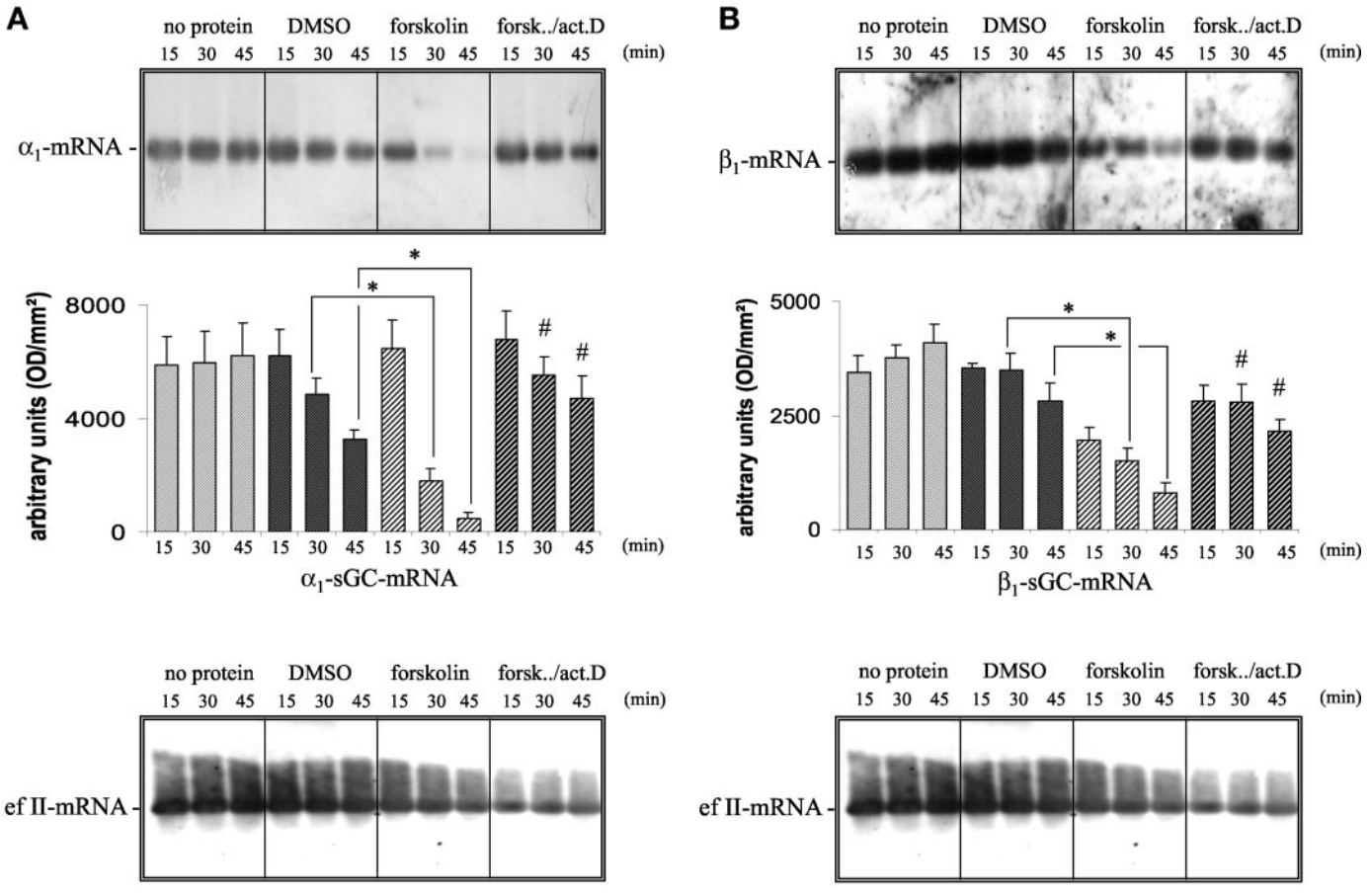

Fig. 4. Forskolin-induced factors in rat aorta accelerate degradation of sGC α_1 and β_1 mRNA in vitro. Native protein (20 μg) isolated from rat aortas that had been exposed to solvent (0.1% DMSO), forskolin (10 μM), or forskolin and act.D (10 μM) for 16 h was incubated at 37°C with 1 μg of poly(A)$^+$ RNA isolated from rat lung, and the amount of sGCα_1 (5.5 kb; A), sGCβ_1 (3.4 kb; B) and ef II (2.6 kb) mRNA remaining after different periods of time (15, 30, and 45 min) was assessed by Northern blotting. The mRNAs were rapidly degraded in the presence of aortic protein from forskolin-treated aorta, and this effect was blocked by act.D. The bar graph below each blot combines densitometric data (arbitrary units, optical density per square millimeter) from three different experiments (mean ± S.D.). *, significant differences ($P < 0.05$; ANOVA) of forskolin versus DMSO. #, significant difference between the forskolin- and the actinomycin D/forskolin-treated tissue ($P < 0.05$; ANOVA).

forskolin was prevented by the match AP-1 ODN, not by the mismatch ODN.

Discussion

Cyclic nucleotides such as cAMP and cGMP play important biological roles as second messenger molecules to transmit extracellular signals to intracellular effectors, thereby eliciting the proper cellular response. Cyclic nucleotide-dependent gene expression is an important component of this response and has primarily been regarded as a consequence of altered gene transcription (Eigenthaler et al., 1999) because of activation of specific transcription factors. Recently, however, we and others could show that cGMP also regulates gene expression by a post-transcriptional mechanism that affects mRNA stability. So-called AU-rich elements present in the 5′- and 3′-UTRs of many mRNAs target these mRNAs for degradation by endonucleases. HuR, a protein of the *elav* family, protects mRNAs from degradation by binding to AREs (Fan and Steitz, 1998). HuR is down-regulated in rat vascular smooth muscle cells (Kloss et al., 2003) and mesangial cells

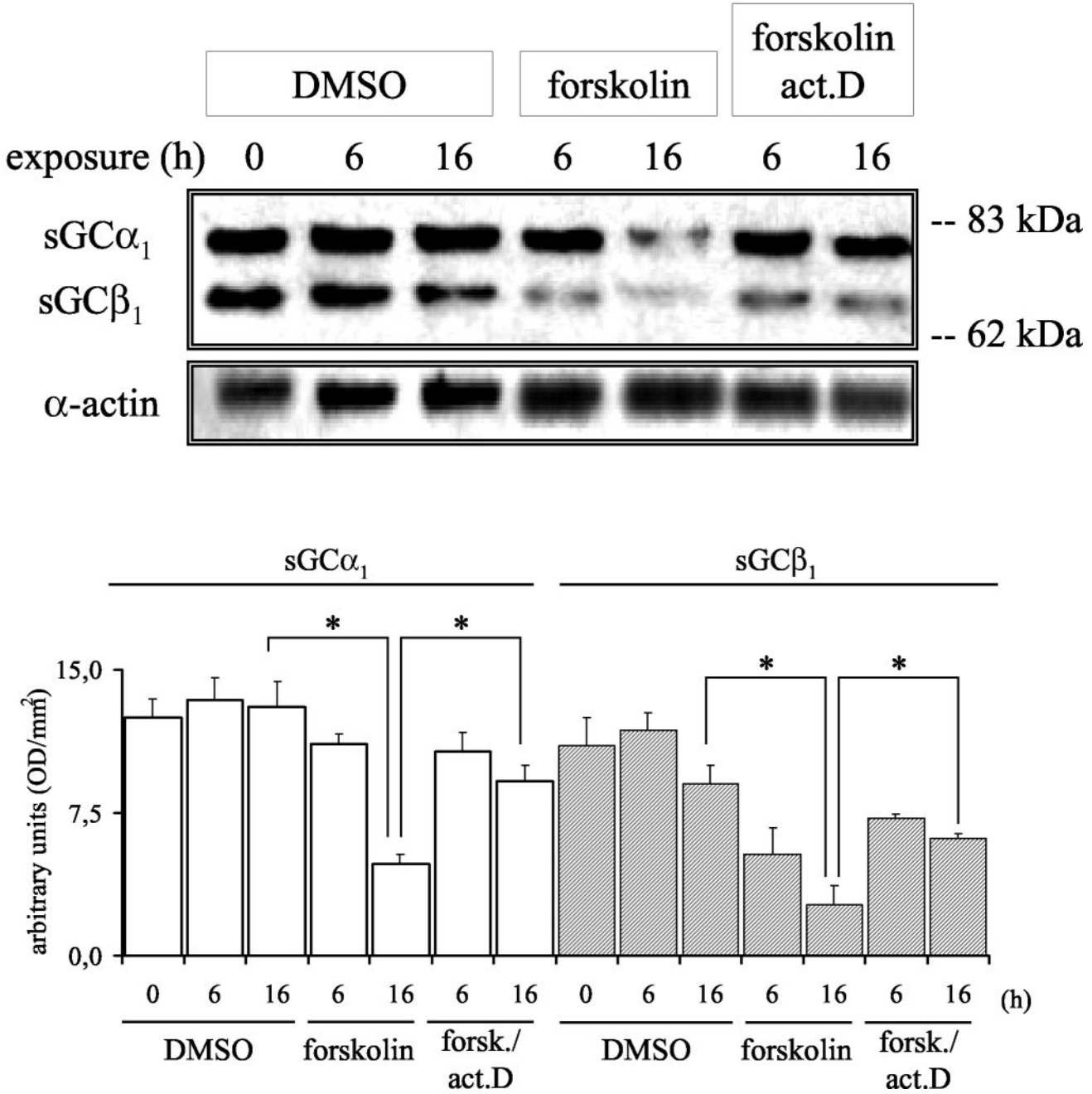

Fig. 5. Influence of actinomycin D on forskolin-induced depression of sGC. Isolated rat aorta was incubated for 6 and 16 h with either solvent (0.1% DMSO), forskolin (10 μM), or forskolin and act.D (10 μM). Total native protein (40 μg) from the aortic tissue was separated by SDS-polyacrylamide gel electrophoresis and assessed for sGC α_1 and β_1 protein by Western blotting (upper graph), using a polyclonal chicken antibody. To verify equal protein loading, the blots were also probed for α-actin. Forskolin-induced a depression of sGC subunits after 16 h that was prevented by act.D. Representative blot from one of three experiments. Summarized data from densitometric evaluations of three different Western blots are shown in the bar diagram below. *, significant difference ($P < 0.05$; ANOVA).

(Akool et al., 2003) by cGMP-eliciting agonists. Consequently, mRNAs specifically protected by HuR, such as soluble guanylyl cyclase α_1 subunit (sGCα_1) and matrix metalloproteinase 9 are rapidly degraded. Because expression and mRNA stability of sGC subunits is also decreased by cAMP-eliciting agonists (Shimouchi et al., 1993; Papapetropoulos et al., 1995), we then investigated whether cAMP also controled ARE-dependent mRNA stability by depression of HuR.

We show herein that forskolin, a cAMP-eliciting direct activator of adenylyl cyclase, and PKA-activating cAMP analogs decrease the expression of both HuR and sGC in isolated rat aorta, at the protein and mRNA levels in a time-dependent fashion (Figs. 1 and 2). In the absence of cAMP-eliciting conditions, we noticed a markedly slower decrease in HuR and sGC expression in rat aortic tissue (12 h; Fig. 1). The underlying mechanism was not further analyzed here. The interaction of endogenous HuR with ARE-containing oligoribonucleotides from the 3′-UTR of sGCα_1 and β_1 (EMSA) was strongly decreased in protein extracts from forskolin-exposed aorta, and this effect was blocked by Rp-cAMPS, an PKA-inhibitory cAMP analog (Fig. 3). The decrease in HuR interaction with the sGC3′UTR probes as shown in Fig. 3, A and B, could in principle be caused by either decreased expression of HuR, reduced binding activity of HuR, or poor integrity of the protein extract. However, as shown in Fig. 1, a decreased expression of HuR at the protein and mRNA levels is the most likely explanation for the reduced HuR sGC mRNA interaction, which was consistently observed in four independent experiments (Fig. 3). As a consequence of decreased HuR expression and mRNA binding activity, the stability of sGCα_1 and β_1 mRNA in in vitro degradation assays was considerably reduced by native protein extracted from forskolin-exposed aortic tissue, compared with protein from control aorta (Fig. 4). These findings clearly show that HuR expression and activity is decreased by the cAMP/PKA signaling pathway. This mechanism probably accounts for decreased expression of sGC subunits, because HuR-dependent protection of sGC mRNA is lost. The decisive role of HuR for sGC mRNA stability regulation was highlighted by our previous finding that knock-down of HuR by siRNA decreases sGC expression (Kloss et al., 2003). It is conceivable that the expression of many other HuR-regulated genes is similarly affected by cAMP and that decreased expression of HuR accounts for the previously observed cAMP-induced destabilization of several mRNAs (for example, the human β1-adrenergic receptor) (Dunigan et al., 2002). We cannot exclude, at this point, the possibility that additional mechanisms could contribute to the cAMP-elicited decrease of sGC mRNA stability [e.g., induction of mRNA-destabilizing factors such as AUF 1 (Pende et al., 1996)], but HuR seems to play a pre-eminent role in sGC mRNA stability regulation.

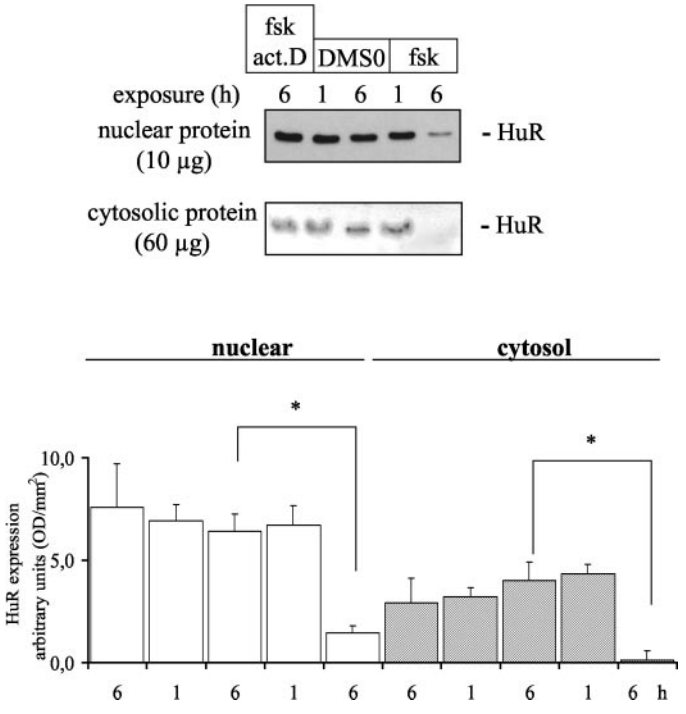

Fig. 6. Influence of actinomycin D on forskolin-induced depression of HuR. Cultured RASMC were exposed to DMSO, forskolin, and forskolin/act.D for 1 and 6 h, then harvested and probed for HuR abundance in cytosolic (60 μg/lane) and nuclear protein (10 μg/lane) fractions by Western blotting. The forskolin-induced decrease of HuR after 6 h was prevented by act.D. Summarized data from densitometric evaluations of three different Western blots are shown in the bar diagram below. *, significant differences ($P < 0.05$; ANOVA).

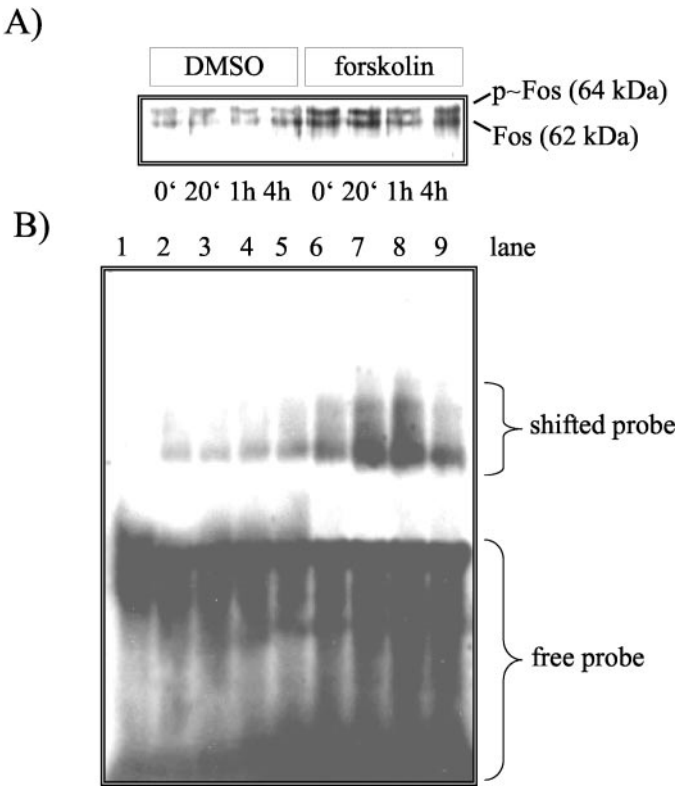

Fig. 7. Influence of forskolin on Fos/p-Fos expression and AP-1 binding activity in RASMC. RASMC were exposed for 0, 20 min, 1 h, and 4 h to either solvent (0.1% DMSO) or forskolin (10 μM), then harvested for preparation of nuclear protein. A, Western blot performed with 10 μg of nuclear protein/lane showing Fos (62 kDa) and pFos (64 kDa) immunoreactive peptides. Forskolin markedly increased both signals. B, AP-1 gelshift analysis performed on a 2% TAE-agarose gel with 20 μg of nuclear protein from the same RASMC as shown in A and an AP-1–specific, biotin-labeled dsDNA probe (6 μg). Forskolin treatment (0–4 h) induced a transient activation of AP-1 (lanes 6–9). Incubation of the AP-1 mismatch probe (lane 1) with nuclear protein from cells exposed to forskolin for 1 h (lane 8) did not yield a probe-shift. Likewise, nuclear protein from solvent (DMSO)-exposed cells induced only a weak gel-shift signal (lanes 2–5). Representative results from two independent experiments are shown.

To further elucidate the signaling pathways accounting for cAMP-induced depression of HuR, we used cultured RASMC. We obtained evidence that active transcription is a prerequisite, because the forskolin effect on HuR and sGC expression was prevented by the transcriptional inhibitor act.D (Fig. 5). Although act.D reportedly can affect the intracellular distribution of HuR (Peng et al., 1998), our results showed that the ratio of nuclear versus cytosolic HuR was not appreciably altered in vascular smooth muscle cells by act.D (Fig. 6).

In general, cAMP signaling to the nucleus is accomplished by translocation of the catalytic subunit of PKA into the nucleus, where it phosphorylates and activates activating (cyclic nucleotide responsive element-binding protein) and silencing (inducible cAMP early repressor) transcription factors (Eigenthaler et al., 1999), both of which can target cyclic nucleotide responsive element sites, such as in the *c-fos* promoter. Indeed, we could show that forskolin induced a very rapid increase in Fos and activated p-Fos (Fig. 6A) that was accompanied by a transient activation of AP-1, the heterodimeric transcription factor constituted by Fos and Jun. A causal relationship between AP-1 activation and HuR depression was established by an AP-1 decoy approach (Morishita et al., 1996). Competition of an exogenously provided AP-1 cognate dsDNA oligonucleotide with endogenous AP-1 sites inhibited forskolin-induced depression of HuR (Fig. 8).

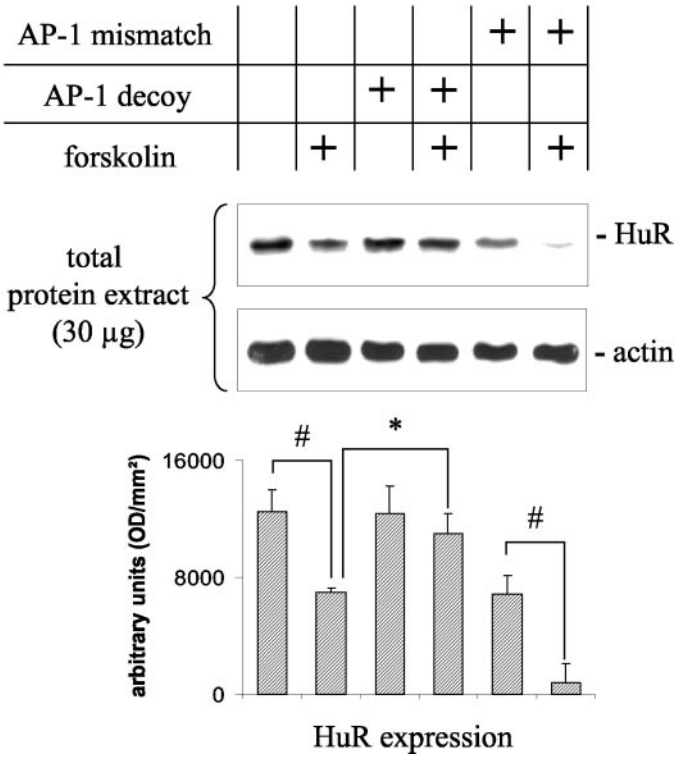

Fig. 8. AP-1 decoy prevents forskolin-induced down-regulation of HuR in RASMC. Serum-starved (24 h) RASMC were exposed for 4 h to an AP-1-cognate oligodesoxynucleotide (match ODN, 10 μM) or a mutated ODN (mismatch, 10 μM), and then incubated for 6 h with solvent (0.1% DMSO) or forskolin (10 μM). The cells were harvested, and HuR expression was assessed by Western blotting. The forskolin-induced decrease of HuR was prevented by AP-1 decoy, not by the mismatch ODN. Representative result from three experiments. The bar graph below combines densitometric data (arbitrary units, optical density per square millimeter) from three different Western blots (mean ± S.D.). * and #, significant differences ($P < 0.05$; ANOVA).

However, the complete sequence of signaling events leading to HuR depression by AP-1 activation remains to be elucidated. The mouse HuR promotor bears several AP1 and a conserved CREB site(s) (King et al., 2000), which could also mediate a direct silencing effect of cAMP on HuR promotor activity. It is noteworthy that quite similar signaling seems to account for NO/cGMP-induced down-regulation of HuR (Kloss et al., 2003). We observed that the sGC activators YC-1 and sodium nitroprusside rapidly and transiently increased Fos and activated AP-1 in RASMC (S. Kloess, A. Muelsch, unpublished observations), in accordance with the present paradigm of cGMP-dependent gene expression (Eigenthaler et al., 1999).

What might be the biological significance of cAMP- and cGMP-induced HuR-depression? cAMP inhibits growth factor-stimulated VSMC proliferation by counteracting Ras/Rho-induced degradation of $p27^{Kip1}$ via the proteasome pathway (Ii et al., 2001). HuR inhibits $p27^{Kip1}$ translation by binding to an internal ribosomal entry site (IRES) in the 5′-UTR of $p27^{Kip1}$ mRNA (Kullmann et al., 2002). Consequently, depression of HuR by cAMP and cGMP will relieve this blockade and allow efficient translation of $p27^{Kip1}$, thus inhibiting cyclin-dependent kinases and arresting cell cycle in G_1. Arrest in G_1 is also supported by cyclic nucleotide-induced decrease in cyclin D1 and A expression. This synergistic action provides a powerful mechanism to prevent injury-induced vascular intimal hyperplasia and neointima formation, for example. Furthermore, cyclic nucleotide-induced down-regulation of HuR decreases matrix metalloproteinase-9 levels (Akool et al., 2003), thereby reducing vascular remodelling.

In conclusion, we have shown that stimulation of the cAMP/PKA pathway in rat aorta and rat aortic smooth muscle cells decreases the expression of heterodimeric sGC by destabilizing sGC subunit mRNA as a result of loss of protection by HuR. Total cellular HuR is down-regulated by a cAMP-triggered signaling cascade requiring active transcription, an increase in Fos expression, and activation of the transcription factor AP-1. It is conceivable that this mechanism contributes to the antiproliferative action of cAMP, for instance.

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