SUPPLEMENTARY DATA

RNA- dependent association with myosin IIA promotes F-actin- guided trafficking of the AU-rich element binding HuR protein to polysomes

Anke Doller, Sebastian Schulz, Josef Pfeilschifter and Wolfgang Eberhardt

Supplemental Figure Legends

Figure S1*.* Quiescent HMC were stimulated for 2 h with vehicle (-) or AngII (0.1µM) without or with either latrunculin A (Lat. A) or colchicine (Colch.) as indicated. Both inhibitors were preincubated for 30 min before the addition of AngII. For Western blot analysis, 30 µg of nuclear and total cell lysates from the indicated cells were subjected to SDS-PAGE and successively immunoblotted with the indicated antibodies. HDAC1 was used as a marker of nuclear extracts.

Figure S2. *(A).* HMC were serum starved for 16 h before being treated for the indicated time points without (vehicle) or with Blebbistatin (5 µM). Total protein (50 µg) was subjected to Western blot analysis and successively probed with an anti-phospho-specific p38 antibody (phospho-p38) and with an antibody raised against total p38 (p38). *(B).* Serum starved HMC were stimulated for 16 h with vehicle (-) or Blebbistatin (5.0 µM) without or with colchicine (0.1 mg/ml) as indicated. The levels of HuR protein in cytoplasmic extracts were monitored by Western blot analysis. Loading of equal amounts of cytoplasmic protein was ascertained by reprobing the blot with an anti-β-actin antibody. *(C).* HMC were transfected for 72 h with duplex siRNA of human myosin heavy chain 9 (siRNA-MYH9) or with control siRNA (siRNA-control). After transfection, cells were serum starved for 16 h and subsequently left untreated or, stimulated for the indicated time points with Blebbistatin (5 µM), before cells were lyzed for cytoplasmic protein extracts. The levels of myosin IIA and HuR protein in cytoplasmic extracts were monitored by Western blot analysis with corresponding antibodies. Loading of equal amounts of cytoplasmic protein was ascertained by reprobing the blots with an anti-β-

actin antibody. The Western blots shown are representative for two independent experiments with similar results.

Figure S3. Indirect immunofluorescence was applied to test for possible changes in the nucleo-cytoplasmic distribution of HuR in serum-straved HMC by different microfilament inhibitors. Serum-starved cells were treated for 4 h with vehicle or with either latrunculin A (0.1 µM), or blebbistatin (5 µM). After fixation and permeabilization cells were stained with an anti-HuR antibody and subsequently with the Alexa-Fluoro 488 coupled (green) secondary antibody. F-actin was visualized with rhodamine-conjugated phalloidin. Merged pictures showing HuR plus F-actin staining. Cell nuclei were visualized by staining with DAPI. Bars, 50 µm. Data shown are from a single representative experiment out of two repeats.

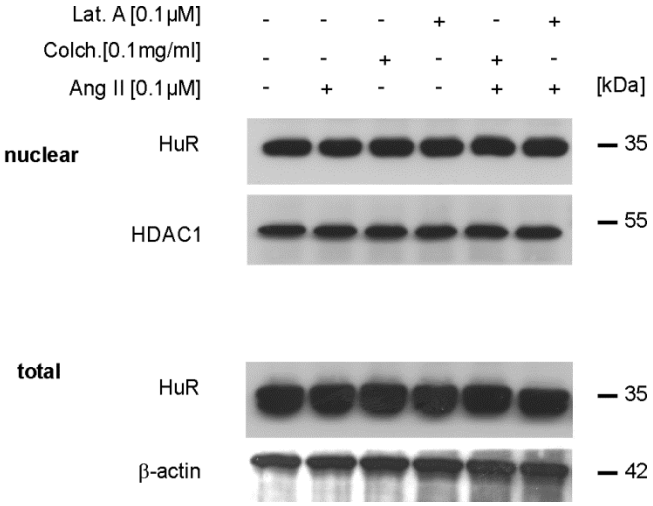
Figure S4. *(A).* Serum-starved HMC were subjected to sequential detergent and salt extraction to release free polysomes (FP), cytoskeletal bound polysomes (CBP) and membrane bound polysomes (MBP) as described in *Materials and Methods*. Equal volumes of the different fractions were assayed for LDH activity showing a highest activity in free polysomes (FP). Data are expressed as percentage of LDH activity normalized to the total protein concentration of the indicated fractions and represent means ± SD (n=3). *(B).* HMC were preincubated for 16 h with a cytokine mix consisting of IL-1β and TNFα (both at 2 nM) before addition of AngII (+ AngII) for 2 h in the absence (-) or presence of the indicated cytoskeletal inhibitors which were given 4 h prior to the administration of AngII. Subsequently, nuclear and cytosolic fractions were separated as described in *Materials and Methods* before RNA from these fractions was prepared and steady-state levels of COX-2 mRNA measured by qRT-PCR. The graph shows relative COX-2 mRNA contents in both fractions in %. *(C).* HMC were treated for 2 h with AngII (0.1 µM) and subsequently lyzed for sequential fractionation of free polysomes (FP) and cytoskeletal bound polysomes (CBP) as described in *Materials and Methods*. Prior to polysome fractionation, cell lysates were pretreated for 30 min with vehicle (-) or with MG 132 (10 µM) before either protein

phosphatase 1 (+PPtase) or, alternatively, RNaseA/T_1 (+RNase) was added to the lysates as described in *Materials and Methods*. After isolation of the indicated fractions similar amounts of proteins were subjected to SDS-PAGE and successively immunoblotted with antibodies against HuR or α-actinin, the latter of which was used as a marker for cytoskeletal cell fractions.

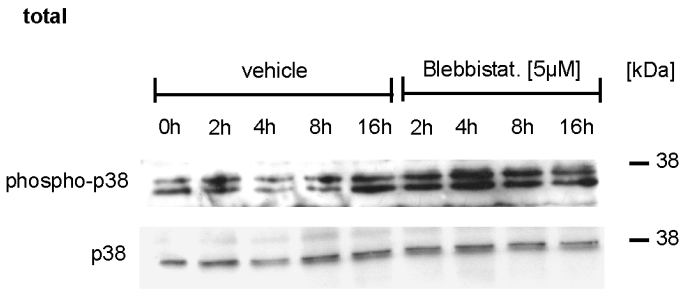
Figure S5. HMC were either treated with control siRNA (siRNA-control), or with siRNA specific for HuR (siRNA-HuR) for 72 h before cells were stimulated for 16 h with the cytokine-mix in the presence (-) or absence (+) of AngII which was added 2 h before cell lysis. Total HuR levels were determined by Western blot analysis using an anti-HuR antibody. Equal protein loading was confirmed by probing the blots additionally with an anti-β-actin antibody.

Supplemental Figures

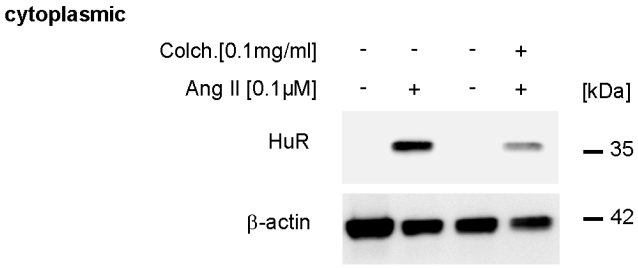
Doller et al., Fig. S1



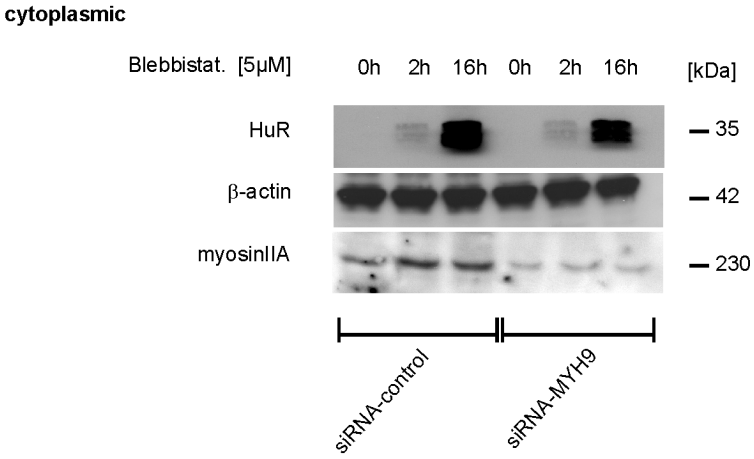
A.


total
vehicle
Blebbistat. [5μM]
[kDa]
0h 2h 4h 8h 16h 2h 4h 8h 16h
phospho-p38
38
p38
38

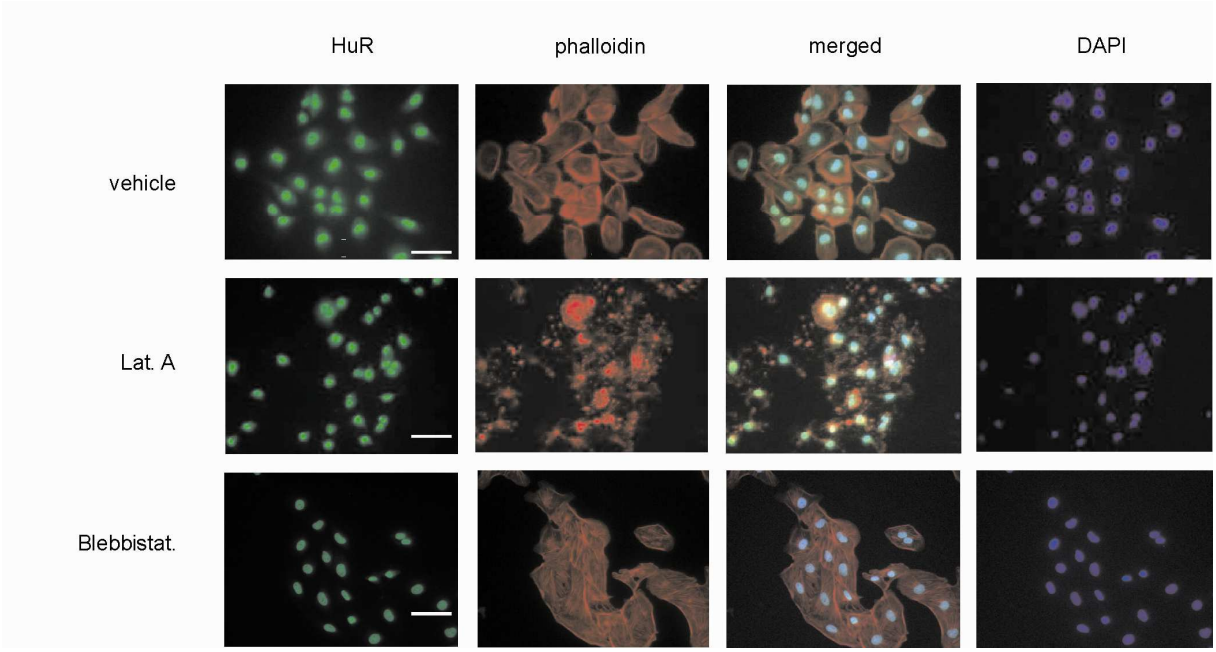
B.


cytoplasmic
Colch.[0.1mg/ml] - - - +
Ang II [0.1μM] - + - +
[kDa]
HuR
35
β-actin
42

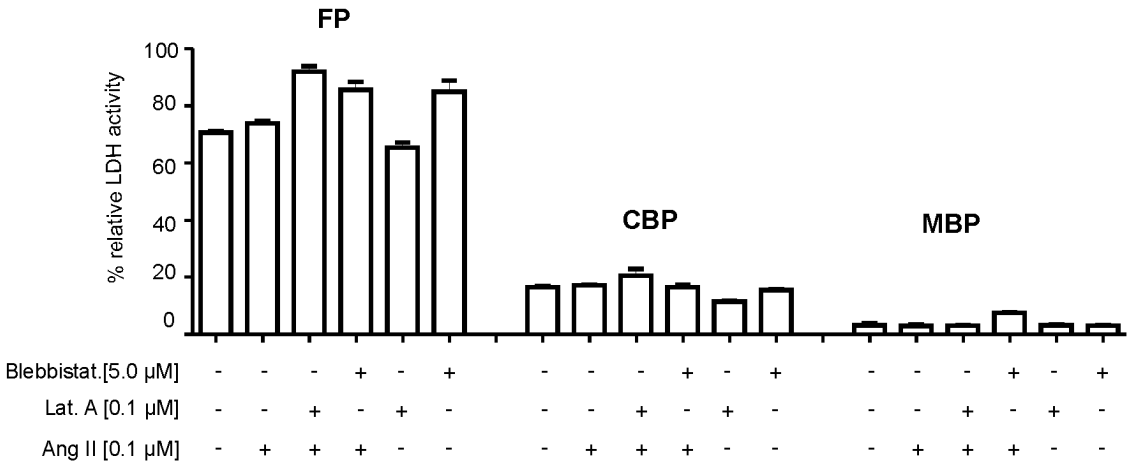
C.


cytoplasmic
Blebbistat. [5μM]
0h 2h 16h 0h 2h 16h
[kDa]
HuR
35
β-actin
42
myosinIIA
230
siRNA-control
siRNA-MYH9

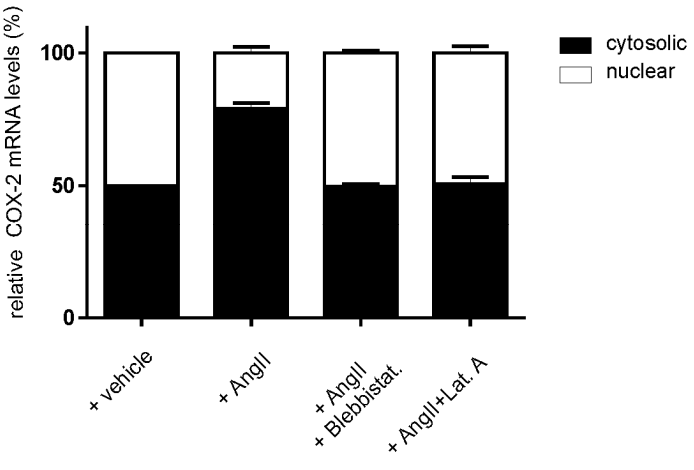
Doller et al., Fig. S3



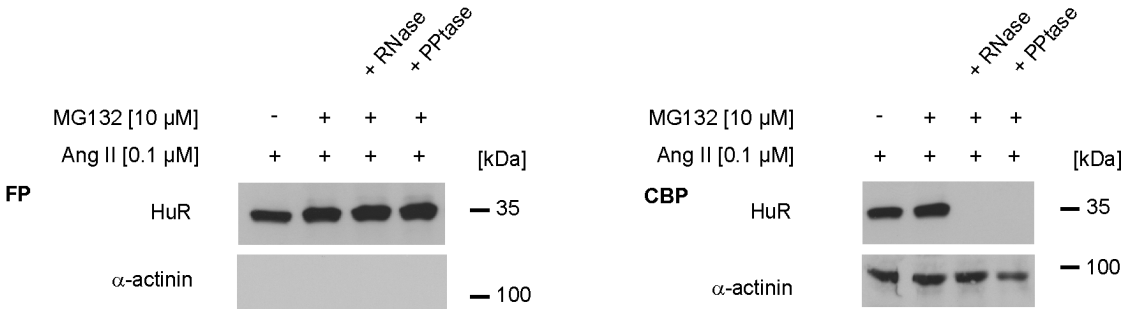
A.


FP
CBP
MBP
% relative LDH activity
100
80
60
40
20
0
Blebbistat.[5.0 µM] - - - + - + - - - + - + - - - + - +
Lat. A [0.1 µM] - - + - + - - - + - + - - - + - + -
Ang II [0.1 µM] - + + + - - - + + + - - - + + + - -

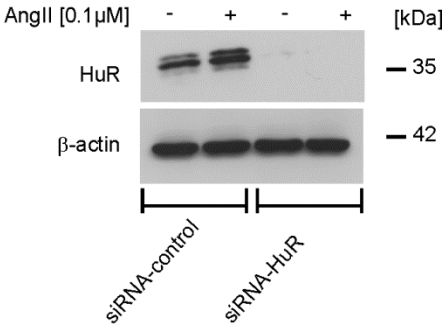
B.


relative COX-2 mRNA levels (%)
100
50
0
cytosolic
nuclear
+ vehicle
+ AngII
+ AngII + Blebbistat.
+ AngII+Lat. A

C.


+ RNase
+ PPtase
MG132 [10 µM] - + + +
Ang II [0.1 µM] + + + +
[kDa]
FP
HuR
α-actinin
35
100
CBP
HuR
α-actinin
35
100

total



Supplemental Methods

Immunoprecipitation

For immunoprecipitaiton, 400 µl of protein G sepharose beads (GE Healthcare) equilibrated with binding/wash buffer (140mM NaCl, 8mM Na3PO4, 2mM K2HPO4, 10 mM KCl, pH 7.4) were captured to 100 µg of myosin II antibody or mouse normal IgG for 15 min at 4°C. After several washing steps with binding/wash buffer, the antibody-linked Protein G sepharose was resuspended in 400 µl binding/wash buffer and subsequently covalently linked by addition of 25µl of 25 mg/ml DSS (in DMSO) for 1 h at 4°C. The excess of DSS was removed by several washing steps with binding/wash buffer and elution buffer (Thermo Scientific) and beads were finally resuspended in 400 µl of binding/wash buffer. Afterwards, 50 µl of the anti-myosin-coupled sepharose beads were incubated with 300 µg of total cell lysates overnight at 4°C. After centrifugation for 5 min at 3000 x g, the precipitated complexes were several times washed with binding/wash buffer before the bound proteins were released by elution buffer. Finally, equal volumes of eluated proteins were subjected to SDS-PAGE and analyzed by immunoblotting.

IP-qRT-PCR analysis (pull-down assay)

Briefly, cells were lyzed by adding a buffer containing 10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% NP-40, 50 mM NaF, 10 mM Na_3VO_4, 10 mM sodium pyrophosphate, 50 mM disodium glycerol phosphate and 100 U/ml RNasin. Subsequently, cell lysates were applied to immunoprecipitation by adding 2 µg of a monoclonal HuR antibody or, alternatively, the same amount of mouse IgG from mouse overnight at 4°C. Afterwards, protein G Sepharose CL-4B beads were added and incubated for further 2h. After a short centrifugation (3000 × g) beads were successively washed with low and high salt buffer before cellular RNA was collected by using the Tri-reagent (Sigma-Aldrich). The COX-2 mRNA which was bound to HuR was subjected to reverse transcription by using SuperScript reverse transcriptase (Invitrogen, Karlsruhe, Germany) and subjected to

quantitative PCR (qRT-PCR). The relative HuR binding to COX-2 mRNA was assessed by RT reaction using similar amounts of cell homogenates as used for the IP reaction and assessment of COX-2 mRNA levels.

Primers used for RT-PCR Analysis

mRNA	Primer	Tm [°C]	length [bp]
COX-2	5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′	60	304
	5′-AGATCATCTCTGCCTGAGTATCTT-3		
cyclin A	5′-ATTAGTTTACCTGGACCCAG-3′	60	443
	5′-CACAAACTCTGCTACTTCTG-3′		
cyclin D	5′-GCTGCTCCTGGTGAACAAGC-3′	60	63
	5′-TTCAATGAAATCGTGCGGG-3′		
β-actin	5′-TTGCCGACAGGATGCAGAAGGA-3′	60	128
	5′-AGGTGGACAGCGAGGCCAGGAT-3′		
AchR	5′-TGGGCTCCGAACATGAGACC-3′	60	206
	5′-TGGGGCGTGGCAGATCTACCA-3′		

Generation of c-myc-tagged HuR deletion mutants

A series of HuR deletion mutant cDNA fragments were generated by using the plasmid pTet-Myc-HuR as a template (32) and included truncations of RRM1 (ΔRRM1; amino acids 20 to 104), RRM2 (ΔRRM2; amino acids 105 to 185), the hinge region (ΔHinge; amino acids 186 to 242) or RRM3 (ΔRRM3; amino acids 243 to 326) were generated by PCR splicing by using sense primers from the corresponding nucleic acids sequence with a HindIII- site (AAGCTT) as 5´overhang and antisense primers with a 5´overhang bearing a EcoRI -restriction site (GAATTC), respectively. Subsequently, the amplificates were subcloned into HindIII/EcoRI cut pcDNA3.1 vector to generate the plasmids pcDNA3-c-myc-HuRΔ1, pcDNA3-c-myc-HuRΔ2, pcDNA3-c-myc-HuRΔHinge and pcDNA3-c-myc-HuRΔ3, respectively. Deletions and correct insertation were proven by DNA sequencing.