Hypertension impairs myocardin function: a novel mechanism facilitating arterial remodelling

Larissa Pfisterer, Anja Feldner, Markus Hecker, and Thomas Korff*

Institute of Physiology and Pathophysiology, Division of Cardiovascular Physiology, University of Heidelberg, Im Neuenheimer Feld 326, 69120 Heidelberg, Germany

Received 30 April 2012; revised 26 June 2012; accepted 20 July 2012; online publish-ahead-of-print 26 July 2012

Aims
Hypertension evokes detrimental changes in the arterial vessel wall that facilitate stiffening and thus lead to a further rise in mean blood pressure, eventually causing heart failure. The underlying pathophysiological remodelling process is elicited by an increase in wall stress (WS) and is strictly dependent on the activation of vascular smooth muscle cells (SMCs). However, it remains unclear as to why these cells fail to maintain their contractile and quiescent phenotype in a hypertensive environment.

Methods and results
In this context, we reveal that the knockdown of myocardin—a pivotal transcriptional determinant of the contractile SMC phenotype—is sufficient to induce SMC proliferation. In line with this observation, immunofluorescence analysis of the media of remodelling arteries from hypertensive mice demonstrated a significant decrease in the abundance of myocardin and an increase in SMC proliferation. Subsequent analyses of isolated perfused mouse arteries and human cultured SMCs exposed to cyclic stretch (i.e. mimicking one component of WS) suggested that this biomechanical force facilitates serine phosphorylation of myocardin. Furthermore, this biomechanical stimulus promotes rapid translocation of myocardin from the nucleus to the cytoplasm, inhibits its mRNA expression, and causes proteasomal degradation of the cytoplasmic protein.

Conclusions
Collectively, these findings suggest that hypertension negates the activity of myocardin in SMCs on multiple levels, hence eliminating a crucial determinant of SMC quiescence. This mechanism may control the initial switch from the contractile towards the synthetic SMC phenotype during hypertension and may offer an interesting novel approach to prevent cardiovascular disease.

Keywords
Myocardin • Wall stress • Stretch • Vascular smooth muscle cells • Hypertension

1. Introduction
Chronic increase in blood pressure is one of the most important risk factors for atherogenesis, hence causing stroke and myocardial infarction by forcing the arterial vessel wall to respond through remodelling its architecture. One hallmark of this remodelling process is the migration and proliferation of smooth muscle cells (SMCs) located in the media. Consequently, hypertension is accompanied by medial thickening, which has repeatedly been shown to be driven by an increase in wall stress (WS), a biomechanical force with major impact on SMC phenotype. In accordance with Laplace’s law, WS predominantly depends on the difference in transmural pressure and is equivalent to the degree of stretch to which the mural cells are exposed. Thus, stretching of the vessel wall caused by prolonged exposure to supra-physiological levels of transmural pressure elicits media hypertrophy and/or hyperplasia. These morphological changes are accompanied by the activation of quiescent and contractile SMCs, which acquire a synthetic phenotype allowing them to migrate and proliferate as well as to degrade or synthesize components of the extracellular matrix. As a consequence of these structural changes, arterial stiffness increases, further raising mean arterial blood pressure and thus accelerating the development of heart failure.

Mechanistically, this shift in SMC phenotype is orchestrated by the activity of a distinct set of transcription factors that control the expression of genes supporting the contractile or synthetic state of these cells. For instance, stretch-induced vascular remodelling processes are controlled in part by transcription factor activator protein-1 (AP-1), which regulates the expression of many stress-response genes, including those associated with the pro-inflammatory state of both endothelial cells and SMCs. On the other hand, there are several transcription factors for maintaining the quiescent and contractile phenotype that seem crucial for SMC differentiation during embryonic development.
In this context, serum response factor (SRF) plays a pivotal role in SMCs in controlling expression of many genes encoding cytoskeletal and contractile proteins. However, its activity is tightly regulated by transcriptional coactivators such as myocardin, which directly binds to SRF and enables the expression of SMC marker genes such as SM-α-actin or calponin, and Elk-1, which displaces myocardin from SRF and thus inhibits transcription of these genes. The preeminent role of myocardin in SMC differentiation is evidenced by the fact that myocardin knockout mice die at embryonic day 10.5 (E10.5) due to vascular defects including an insufficiently developed aorta lacking adequate SMC coverage. Although much is known about the role of myocardin in maintaining SMCs in the contractile and quiescent state, the regulation of its activity during pathophysiologic remodelling of adult arteries is still poorly understood.

In this context, we hypothesized that in the course of hypertension-induced arterial remodelling, the shift in SMC phenotype is not just initiated by mechanisms promoting the expression of synthetic genes but also by deactivation of mechanisms that facilitate the contractile state. With regard to the role of myocardin in the latter, we hypothesized that the phenotype shift is preceded by a decline in myocardin activity and/or expression, which would consequently remove the brake en route to the synthetic state. Therefore, we investigated the abundance, localization, and activity of myocardin during experimentally evoked hypertension and in human cultured SMCs exposed to cyclic stretch.

2. Methods

2.1 Antibodies

Anti-human/mouse myocardin antibodies were purchased from R&D Systems (Wiesbaden, Germany; MAB4028) and Santa Cruz Biotechnology (Heidelberg, Germany; sc-21561 and sc-33766), and monoclonal anti-mouse CD31 antibody (clone: MEC 13.3) was also obtained from the latter. Antibodies against Ki67, PCNA, phosphoserine, and calponin were obtained from Abcam (Cambridge, UK). The ERK1/2 inhibitor PD98059 was purchased from Calbiochem (Merck Biosciences, Königsmann im Taunus, Germany), bortezomib was from LC laboratories (Woburn, MA, USA), and sodium orthovanadate was obtained from Sigma–Aldrich (Munich, Germany).

2.2 Animal models

All animal studies were performed with permission of the Karlsruhe Regional Council, and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). C57BL/6 male mice (at least 12 weeks old) were anesthetized with 3% (v/v) isoflurane, and hypertension was induced by subcutaneously implanting deoxycorticosterone acetate (DOCA)-salt slow-release pellets ( Innovative Research of America, Sarasota, FL, USA) according to the manufacturer’s instructions and by providing 1% (w/v) sodium chloride in the drinking water. Adequate anesthesia was confirmed by monitoring the foot pad reflex. The resulting increase in blood pressure was monitored by applying a specialized tail-cuff blood pressure measurement method, which allowed determination of both systolic and diastolic pressure changes (NIBP, NIBPchart software; Panlab/Harvard apparatus, Holiston, MA, USA) every third day. The animals were euthanized by cervical dislocation after 10 days, and their arteries perfused with zinc fixative in situ, excised, and processed for histological examination.

2.3 Perfusion of isolated mouse arteries

After cervical dislocation, the femoral arteries were extracted and inserted into the chamber of a Culture Myograph (DMT, Copenhagen, Denmark). The chambers were placed in an incubator at 37 °C with 5% CO2, and the arteries were continuously perfused for 6 h with Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen, Darmstadt, Germany) containing 15% foetal calf serum (FCS) at a longitudinal pressure gradient of 20 mm Hg and a flow rate of ~0.07 ml/min. Perfusion was established by applying an intraluminal pressures of 70 and 50 mm Hg [control conditions, mean transmural pressure difference (ΔPtm): 60 mm Hg], and 120 and 100 mm Hg (hypertensive conditions, ΔPtm: 110 mm Hg) at the ends of the arteries. The functional and cellular integrity of the segments was routinely checked by their vasodilator response to acetylcholine (5 μM). After perfusion, vessel segments were fixed in zinc fixative (4% C, 16 h), and subsequently dehydrated in a series of ethanol and iso-propanol, embedded in paraffin (56 °C, 16 h), and processed for sectioning.

2.4 Cell culture

Human arterial SMCs (HUASMCs) were isolated from human umbilical cord arteries and cultured in DMEM supplemented with 15% FCS. Only cells cultured up to passage three were used throughout. The isolation of HUASMCs was approved by the local Ethics Committee (Heidelberg, Germany) and conformed to the principles outlined in the Declaration of Helsinki (1997). In order to expose HUASMCs to cyclic stretch, cells were cultured on plastic dishes or BioFlex™ Pronectin 6-well plates (Flexcell, Hillsborough, NC, USA). Stretching was performed by using an FX-5000 Tension System (Flexcell) with 13% cyclic elongation at 0.5 Hz. Cyclic elongation is needed to prevent the cells from evading the biomechanical stimulus through rearranging their focal contacts.

2.5 siRNA gene silencing

For functional studies of myocardin, an siRNA-based silencing approach was used. Customized siRNA (sense: CULUGAAGUCUUAUAAAUU; antisense: AAUUAUAAAGACUUACUAAG) was purchased from Sigma–Aldrich. HUASMCs were transfected by utilizing the MATRa system (IBA, Göttingen, Germany) according to the manufacturer’s instructions. Transfected cells were incubated for 48 h before further use. The efficiency of the siRNA-mediated myocardin knockdown was verified by western blot and immunofluorescence analysis (Supplementary material online, Figure S1).

2.6 Conventional RT–PCR

Total RNA was isolated from the cultured cells by solid-phase extraction with an RNeasy kit (Qiagen, Düsseldorf, Germany) according to the manufacturer’s instructions. Subsequently, reverse transcription (RT) and PCR for the target sequence was performed, with 605 ribosomal protein L32 (RPL32) cDNA as an internal standard. Primers with the following sequences were used for amplification: myocardin (forward: TCC AACCGGCTTTACCACTT, reverse: CACCTTCTGTTCTCCACCA), calponin (forward: GAGGAGGGAAGTGGTCGAC, reverse: GTTGG CCTCAAAATTGTCTG, and RPL32 (forward: GTTCACTCCGCGC CAGTCG, reverse: ACAGTGCACTAGCGTGCTCA). Densitometric analyses were performed by using Scion Image (Scion Corporation, Frederick, MD, USA).

2.7 Immunoprecipitation and western blot analysis

HUASMCs were lysed using sample buffer containing 1% Triton X-100 and 2 mM orthovanadate. Immunoprecipitation was performed by incubating the protein samples with anti-phosphoserine antibodies for 2 h at room temperature, which were then captured by protein A-agarose beads (Thermo Fisher, Bremen, Germany). After washing, the proteins...
were eluted from the beads by incubating in sample buffer at 95°C (10 min). Protein samples were separated by 10% SDS–PAGE, blotted onto nitrocellulose membranes and analyzed by chemiluminescence-based immunodetection according to standard procedures. Sodium orthovanadate (2 mM) was added to the buffers to inhibit the activity of phosphatases.

2.8 Immunofluorescence analysis
Deparaffinized vessel sections and methanol-fixed HUASMCs were blocked in TRIS buffer (pH 7.6, containing 3% BSA) and permeabilized with 2% saponin in PBS. Primary antibodies (sc-21561 and sc-33766) were diluted in blocking solution and incubated with the samples for 18 h at 4°C. After washing, sections or cells were incubated with secondary antibodies for 2 h, then for an additional 10 min with DAPI (Invitrogen) in PBS, and mounted using MOMIOL reagent (Calbiochem). Quantification of the staining intensity and/or nuclear localization of target proteins in cultured cells was performed by using the morphological analysis software Cell^R (Olympus, Hamburg, Germany). Three to five randomly selected microscopic fields of view containing up to 50 cells were evaluated in each experiment. When analyzing cytoplasmic fluorescence intensities, size-matched areas were compared. Nuclei containing myocardin or Ki67 were detected as a purple colour upon merging the DAPI fluorescence (blue) with the fluorescence indicating the target proteins (red). When analyzing the fluorescence intensity of medial smooth muscle cells in arteries, three to five size-matched areas per section of artery were quantified by using Cell^R.

2.9 Statistical analysis
All results are expressed as means ± SD. Differences between two matched experimental groups were analyzed by unpaired Student’s t-test, with P < 0.05 considered statistically significant. Differences among three or more experimental groups were analyzed by ANOVA, followed by a Bonferroni post hoc test for selected pairs of groups, with P < 0.05 considered statistically significant. Correlation analyses were performed by calculating the Pearson’s correlation coefficient (r).

3. Results
3.1 Silencing of myocardin expression facilitates SMC proliferation and down-regulates calponin gene expression
To assess the relevance of myocardin expression for the SMC phenotype, its expression was knocked down in cultured HUASMCs by an siRNA-based approach (Figure 1A, Supplementary material online, Figure S1). Therefore, the cells were cultured as three-dimensional spheroids, to more closely mimic the architecture of the arterial vessel wall and to support organotypic behaviour of the SMCs. Immunofluorescence analysis confirmed that the calcium-binding protein calponin was also associated with a decline in the con- tractile phenotype and attenuating proliferation. Thus, we hypothesized that myocardin must be depleted before arterial remodelling starts, as this is usually associated with, and dependent upon, the activation of SMCs that migrate and proliferate to reorganize the architecture and structural integrity of the media. A prototypic example of such an arterial remodelling is initiated by hypertension, which entails the thickening of the arterial vessel wall. This process was experimentally evoked by treating mice with DOCA-salt (Figure 2A). Subsequent immunofluorescence analysis of femoral arteries showed an almost uniform distribution of the proliferation marker PCNA in the nuclei of medial SMCs 10 days after the onset of hypertension (Figure 2B). Hypertension also resulted in a decrease in the abundance of the calcium-binding protein calponin in the media of femoral arteries (Figure 2C). While myocardin was located in the cytoplasm as well as in the nuclei of SMCs under control conditions (Figure 2D–G), hypertension markedly diminished its abundance throughout the cells (Figure 2D, H–J).

3.2 Myocardin disappears from the nucleus and cytoplasm of arterial SMCs upon hypertension
The above results suggested that myocardin acts as an inhibitor of SMC activity by facilitating the expression of genes directing their contractile phenotype and attenuating proliferation. Thus, we hypothesized that myocardin must be depleted before arterial remodelling starts, as this is usually associated with, and dependent upon, the activation of SMCs. Consequently, we explored potential regulatory mechanisms which may underlie such an arterial remodelling is initiated by hypertension, which entails the thickening of the arterial vessel wall. This process was experimentally evoked by treating mice with DOCA-salt (Figure 2A). Subsequent immunofluorescence analysis of femoral arteries showed an almost uniform distribution of the proliferation marker PCNA in the nuclei (Figure 2B). Hypertension also resulted in a decrease in the abundance of the calcium-binding protein calponin in the media of femoral arteries (Figure 2C). While myocardin was located in the cytoplasm as well as in the nuclei of SMCs under control conditions (Figure 2D–G), hypertension markedly diminished its abundance throughout the cells (Figure 2D, H–J).

3.3 Exposure of isolated arteries to supra-physiological transmural pressure inhibits SMC expression of myocardin
As elevation of WS is a hallmark of arterial hypertension, we next hypothesized that this is a biomechanical force that is responsible for triggering the decrease in myocardin abundance in SMCs in vivo. To mimic the rise in WS and to exclude any effects of DOCA-salt treatment on myocardin expression, isolated perfused mouse femoral artery segments were exposed to a supra-physiological longitudinal pressure gradient of 120 mm Hg (inflow) to 100 mm Hg (outflow) or to physiological pressure gradient (70 to 50 mm Hg). Both conditions produced a constant axial pressure drop of 20 mm Hg (and thus the same flow) but different ΔPtm: ~110 and 60 mm Hg, respectively. Immunofluorescence analysis confirmed that elevated ΔPtm markedly diminished the abundance of myocardin in the nuclei and in the cytoplasm of the medial SMCs (Figure 3G, cf. Figure A, C, E, arrows, and B, D, F). This WS-induced decline in myocardin abundance was accompanied by a significant drop in myocardin mRNA expression (Figure 3H). Expression of the myocardin target gene calponin was also strongly reduced under these conditions (Figure 3I).

3.4 Cyclic stretch facilitates the redistribution of myocardin to the cytoplasm and inhibits myocardin mRNA expression
Given the above results, increased stretch (as a result of the rise in WS) seemed to control the overall level and distribution of myocardin in the SMCs. Consequently, we explored potential regulatory mechanisms which may underlie such an arterial remodelling is initiated by hypertension, which entails the thickening of the arterial vessel wall. This process was experimentally evoked by treating mice with DOCA-salt (Figure 2A). Subsequent immunofluorescence analysis of femoral arteries showed an almost uniform distribution of the proliferation marker PCNA in the nuclei (Figure 2B). Hypertension also resulted in a decrease in the abundance of the calcium-binding protein calponin in the media of femoral arteries (Figure 2C). While myocardin was located in the cytoplasm as well as in the nuclei of SMCs under control conditions (Figure 2D–G), hypertension markedly diminished its abundance throughout the cells (Figure 2D, H–J).
mechanisms in human umbilical artery SMCs that were cultured on flexible elastomers and subjected to cyclic stretch for 24 h. As this experimental setup served as a biomechanical surrogate for an increase in WS, longer stimulation times were needed to affect the expression of myocardin. A prominent relocation of myocardin from the nucleus to the cytoplasm of the cultured SMCs occurred (Figure 4A–F), and this was confirmed by analyzing cells over-expressing GFP-tagged myocardin (Supplementary material online, Figure S6); it was clearly dependent on the level of stretch applied (Supplementary material online, Figure S7). Subsequent western blot analysis revealed that the overall amount of myocardin protein did not change over 24 h (Figure 4G), but was strongly diminished after 48 h of stretching (Figure 4G). Myocardin mRNA expression also dropped significantly in SMCs after 24 h exposure to stretching (Figure 4H), and was usually accompanied by a similar decrease in calponin gene expression (Supplementary material online, Figure S3–5).

3.5 Blocking of ERK1/2 counteracts the stretch-induced export of myocardin from the nucleus and inhibits its stretch-induced phosphorylation

Cyclic stretch or an increase in WS has frequently been shown to stimulate extracellular signal-regulated kinase (ERK1/2) signaling in vascular SMCs. Consequently, we hypothesized that myocardin is a target of these kinases in stretch-stimulated SMCs. Immunoprecipitation-based analysis of protein lysates suggested that this biomechanical stimulation increases the phosphorylation of...
serine residues in myocardin (Figure 5A). Given the change in stretch-dependent serine-phosphorylation, we assumed that stretch stimulation of ERK1/2 affects the export of myocardin from the nuclei of the cells. Consequently, the activity of these kinases was blocked by treating cultured HUASMCs with PD98059, a specific inhibitor of MAPK/ERK kinase 1 and 2 (MEK1/2), which are rate-limiting for the phosphorylation and thus activation of ERK1/2. While cyclic stretching of the cultured SMCs usually caused phosphorylation of myocardin (Figure 5A), its loss from the nucleus (Figure 5B), and its accumulation in the cytoplasm (Figure 5D, F, and I), all of these effects were virtually abolished upon pre-treatment with PD98059 (Figure 5A, C, E, G, and J). Consequently, the stretch-induced decline in calponin expression was fully abrogated upon blocking of ERK1/2 activity (Supplementary material online, Figure S8). We further examined whether phosphatases also affect the nuclear localization of myocardin in stretched SMCs by employing the broad phosphatase inhibitor orthovanadate. As shown in Figure 5C and E, loss of phosphatase activity in the cultured SMCs resulted in a pronounced loss of myocardin from the nucleus and its accumulation in the cytoplasm (Figure 5E, H and K).

3.6 Inhibition of the proteasome results in accumulation of myocardin in the cytoplasm

Although the export of myocardin from the nucleus seemed to occur within the first 24 h following the onset of stretch, we also noted an
overall decrease in protein abundance both in the media of native isolated perfused femoral artery segments and in cultured SMCs upon prolonged exposure to cyclic stretch. It would appear therefore that a rise in WS or stretch facilitates the degradation of myocardin in the cytoplasm, presumably via the proteasome. When treated with the proteasome inhibitor bortezomib (also known as Velcade®) there was in fact a prominent rise in cytoplasmic myocardin in stretched HUASMCs (Figure 6A, cf. Figures D and E, arrows). Interestingly, under static conditions bortezomib also significantly increased the amount of myocardin in the cytoplasm of the SMCs, pointing towards a significant basal turnover of the protein via the proteasome.

4. Discussion
The adult arterial system is exposed to a plethora of physiological influences that have the capacity to initiate remodelling of the vessel wall if specific thresholds are exceeded. As such, hypertension has been widely accepted as an important determinant of maladaptive changes in the structure of the vessel wall. Likewise, a supra-physiological...
increase in WS due to hypertension is a potent driver for arterial remodelling\textsuperscript{7,21–23} and—as a consequence of the hypertensive state—causes thickening of the arterial vessel wall and eventually its stiffening and malfunction.\textsuperscript{2,24} In our study we observed that medial SMCs in mouse arteries proliferate in response to hypertension, and this was paralleled by a decline in expression of the calcium-binding protein calponin (a prototypic transcriptional target of myocardin). In this context, our loss-of-function experiments indicate that a significant drop in the abundance of myocardin is sufficient to decrease calponin expression and promote SMC proliferation—both of which are indicative of a switch towards the synthetic phenotype. These results are corroborated by findings that myocardin controls cellular proliferation through inhibiting the expression of cyclin D1\textsuperscript{25} and NF-κB(p65)-dependent cell-cycle progression.\textsuperscript{26}

As a new finding, we noted that experimental hypertension led to a decline in the abundance of myocardin (a transcriptional coactivator in vascular SMCs that helps to maintain the contractile phenotype). Based on further analysis aimed at delineating the biomechanical force primarily responsible for this effect, we could exclude the possibility that indirect shear-stress-mediated effects (such as an increase in the production of nitric oxide) affect myocardin gene expression in SMCs (Supplementary material online, Figure S9). Rather, by utilizing isolated pressure-perfused mouse arteries, we verified that increased transmural pressure (and thus WS) accounts for this decline in myocardin gene expression. Remarkably, perfusing isolated arteries under conditions mimicking hypertension resulted in the loss of myocardin from the nuclei of the medial SMCs. This effect was similarly observed in cultured SMCs subjected to cyclic stretch—albeit this stimulation represents only one component of circumferential wall stress in vivo. Nevertheless, in both setups the absence of myocardin in the nuclei was paralleled by a decline in calponin abundance. Since the promotor of the calponin gene contains at least 4 CArG-box elements, its expression is tightly associated with the activity of SRF\textsuperscript{27,28} and thus also controlled by myocardin.\textsuperscript{29,30}

Considering the putative impact of myocardin on SMC phenotype, its stretch-induced export from the nucleus seems to govern SMC differentiation during hypertension. Whereas further experiments indicated that myocardin translocation is independent of exportin CRM1, Rho kinase, phospholipase C, or changes in intracellular calcium (data not shown), the activity of ERK1/2 seems pivotal for this effect to occur. At the functional level, myocardin phosphorylation may impair the activity of SRF, as suggested by over-expressing...
phosphomimetic mutants of myocardin in vascular SMCs. Correspondingly, we observed that serine phosphorylation of myocardin is associated with its loss from the nuclei of both native and cultured SMCs subjected to a supra-physiological increase in WS or stretching. Both stimuli are well-known determinants of ERK1/2 activity, both in vivo and in vitro,\textsuperscript{19,20,32} by activating MAP kinases, for instance by the Src family, or integrin-dependent phosphorylation of focal adhesion kinases,\textsuperscript{33} or through stretch-induced association of caveolin with β1-integrins and the signaling proteins Fyn and Shc.\textsuperscript{34} Interestingly, stretching also promotes the translocation of ERK1/2 from the cytoplasm to the nucleus,\textsuperscript{35} which might result in the phosphorylation of nuclear rather than cytoplasmic myocardin. In fact, blockade of ERK1/2 through inhibiting its upstream kinases MEK1/2 abolished the stretch-induced translocation of myocardin from the nucleus to the

**Figure 5** Analysis of myocardin-specific fluorescence intensity and myocardin phosphorylation after inhibiting ERK1/2. Immunoprecipitation analysis revealed increased serine phosphorylation of myocardin (A, *P* < 0.05 vs. control, *n* = 3) after 24 h of stretching, which was not detectable (n.d.) upon inhibition of ERK1/2 activity by PD98059 (ERK1/2 inhibitor). The number of myocardin-positive nuclei decreased in stretch-stimulated (24 h) HUASMCs (B, *P* < 0.05 vs. control, *n* = 3). Treatment with PD98059 (50 μM) abolished this export of myocardin from the nucleus (C, *P* < 0.05 vs. stretch, *n* = 3, cf. F, I and G, J, arrows) whereas blockade of phosphatase activity by sodium orthovanadate (2 mM) augmented this process (C, **P** < 0.05 vs. stretch, *n* = 3, cf. F, I and H, K, arrows; scale bar: 100 μm). Conversely, cytoplasmic myocardin abundance was increased in stretch-stimulated (24 h) HUASMCs (D, **P** < 0.01 vs. control, *n* = 3, and treatment with PD98059 decreased its concentration in the cytoplasm (E, **P** < 0.01 vs. stretch, *n* = 3, cf. F, I and G, J, arrowheads). This effect was reversed upon phosphatase inhibition (cf. F, I and H, K, arrowheads).
cytoplasm. Although further details about the mechanism of this export are presently unknown, the import of myocardin into the nucleus might be mediated by importin α/β1 as a corresponding nuclear localization sequence is present in its NH2-terminal basic domain.15,36

Prolonged stretching, on the other hand, facilitates the degradation of myocardin by the proteasome, which may serve as a versatile instrument to prohibit its re-import into the nucleus. Accordingly, ubiquitination as well as proteasomal processing of myocardin has been shown to control its transcriptional activity, by stabilizing the protein or repressing its function.37–39 In this context, we observed that bortezomib (Velcade®: the first therapeutic inhibitor of the 26S proteasome, used to treat multiple myeloma)40 prevents the stretch-induced degradation of myocardin. Thus, it is tempting to speculate that drugs supporting the nuclear localization of myocardin and/or inhibiting its depletion may serve as interesting therapeutic tools to stabilize the contractile SMC phenotype and prevent hypertension and/or inhibiting its depletion may serve as interesting therapeutic tools to stabilize the contractile SMC phenotype and prevent hyper tension and/or arteriosclerosis-mediated detrimental changes in the architecture of the vessel wall.

In summary, our findings indicate that: (i) hypertension-induced remodelling of the arterial vessel wall is associated with a decline in myocardin abundance in arterial SMCs, and (ii) the hypertension-mediated increase in WS (hence stretching) leads to removal of myocardin from the nucleus through ERK1/2-dependent phosphorylation and its subsequent proteasomal degradation in the cytoplasm (summarized in Supplementary material online, Figure S10). Collectively, these mechanisms render myocardin incapable of maintaining its pivotal function as a transcriptional coactivator and determinant of the contractile and quiescent SMC phenotype. They thus appear to be prerequisites for the phenotype shift of arterial SMCs to the synthetic state by disabling the expression of genes supporting the contractile phenotype while enabling SMC proliferation.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

---

**Figure 6** Determination of the proteasome-dependent degradation of myocardin in stretched SMCs. Inhibition of the proteasomal degradation of myocardin by treating the cultured HUASMCs with bortezomib (2.5 μmol/L) led to an increase in myocardin staining intensity in the cytoplas (A, ***P < 0.001 vs. control; #P < 0.05 vs. stretch, n = 3; arrows in the representative images B–E; scale bar: 100 μm).

---

**Acknowledgements**

The authors would like to acknowledge the excellent technical assistance of Gudrun Scheib, Maria Harlacher, Ender Serbest and Tim Scholta.

**Conflict of interest:** none declared.

**Funding**

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB TR23, projects C5 and C6) and by a grant from the Deutsche Forschungsgemeinschaft and the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (International Research Training Group 880, project 7).

**References**

Hypertension controls myocardin activity


