DOI: 10.1111/j.1365-2362.2011.02628.x

ORIGINAL ARTICLE

Use of atorvastatin to inhibit hypoxia-induced myocardin expression

Chiung-Zuan Chiu*,†,‡, Bao-Wei Wang‡ and Kou-Gi Shyu*,‡

*Graduate Institute of Clinical Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, †School of Medicine, Fu-Jen Catholic University, New Taipei City, Taiwan, ‡Division of Cardiology, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan

ABSTRACT

Background Hypoxia induces the formation of reactive oxygen species (ROS), myocardin expression and cardiomyocyte hypertrophy. The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) have been demonstrated to have both antioxidant and antihypertrophic effects. We evaluated the pathways of atorvastatin in repressing ROS and myocardin after hypoxia to prevent cardiomyocyte hypertrophy.

Materials and methods Cultured rat neonatal cardiomyocytes were subjected to hypoxia, and the expression of myocardin and ROS were evaluated. Different signal transduction inhibitors, atorvastatin and N-acetylcysteine (NAC) were used to identify the pathways that inhibited myocardin expression and ROS. Electrophoretic motility shift assay (EMSA) and luciferase assay were used to identify the binding of myocardin/serum response factor (SRF) and transcription to cardiomyocytes. Cardiomyocyte hypertrophy was assessed by ^{3}H-proline incorporation assay.

Results Myocardin expression after hypoxia was inhibited by atorvastatin, RhoA/Rho kinase inhibitor (Y27632), extracellular signal-regulated kinase (ERK) small interfering RNA (siRNA)/ERK pathway inhibitor (PD98059), myocardin siRNA and NAC. Bindings of myocardin/SRF, transcription of myocardin/SRF to cardiomyocytes, presence of myocardin in the nuclei of cardiomyocytes and protein synthesis after hypoxia were identified by EMSA, luciferase assay, confocal microscopy and ^{3}H-proline assay and were suppressed by atorvastatin, Y27632, PD98059 and NAC.

Conclusions Hypoxia in neonatal cardiomyocytes increases myocardin expression and ROS to cause cardiomyocyte hypertrophy, which can be prevented by atorvastatin by suppressing ROS and myocardin expression.

Keywords Cardiomyocyte hypertrophy, reactive oxygen species, statins, transcription.

Eur J Clin Invest 2012; 42 (5): 564–571

Introduction

The heart responds to different stress signals, resulting in cardiomyocyte hypertrophy to adapt through the expression of certain cardiac-restricted genes [1–4]. Sustained cardiac hypertrophy may ultimately lead to heart failure and significantly contribute to morbidity and mortality. Hypoxia elicits reactive oxygen species (ROS) and a series of functional responses in cardiomyocytes, including cell proliferation, cell hypertrophy and cell death [5,6]. Cardiomyocytes cope with hypoxia to maintain homeostasis by expressing certain cardiac-restricted genes through a variety of signalling cascades [6]. However, the detailed signalling mechanisms regulating cardiomyocytes in response to hypoxia remain unclear. Myocardin, a transcriptional cofactor, cooperates with serum response factor (SRF) and plays an important role in the genetic regulation of growth and differentiation of various organs, including heart, lung, vessel and brain. [2,7–12]. Our previous study has indicated that hypoxia induces both myocardin expression and cardiomyocyte hypertrophy with earlier expression of angiotensin II (Ang II) through the extracellular signal-regulated kinase (ERK) pathway [13], and other studies have also indicated that Ang II may increase ROS production and result in cardiomyocyte hypertrophy [5,14–16]. The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have been demonstrated to have both antioxidant and antihypertrophic effects on cardiomyocytes [17–21]. It was not known whether atorvastatin could prevent cardiomyocyte

hypertrophy induced by hypoxia. In this study, we evaluated the pathways of atorvastatin in repressing ROS and myocardin expression after hypoxia to prevent cardiomyocyte hypertrophy.

Materials and methods

Please see the supporting information online Materials and methods section for more details.

Primary culture of ventricular cardiomyocytes

Cardiomyocytes were obtained from 2- to 3-day-old Wistar rats by trypsinization, and primary culture was performed as previously described [13,22]. The enriched myocytes were then subjected to hypoxia. The rats were handled according 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). The study was approved by the Institutional Animal Care and Use Committee of Shin Kong Wu Ho-Su Memorial Hospital.

Hypoxia setting in incubator

Hypoxic conditions were achieved as previously described [13] by adding medium pre-equilibrated with nitrogen gas to cells prior to their incubation in a Plexiglas chamber purged with water-saturated nitrogen gas by an oxygen controller (PROOX model 110; BioSpherix, Ltd., Redfield, NY, USA). The hypoxia settings were (i) 10% O_2, 5% CO_2 and 85% N_2; (ii) 5% O_2, 5% CO_2 and 90% N_2; and (iii) 2·5% O_2, 5% CO_2 and 92·5% N_2. A lower oxygen concentration (1%) was attempted, but the cardiomyocytes had difficulty surviving under such severe hypoxia. The durations of hypoxia were 1, 2, 4, 6 h and longer.

Antibodies and reagents

Antibodies and reagents used are described in the supplemental section. The roles of c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), ERK, phosphatidylinositol 3-kinase (PI3K), Rac1 and RhoA in hypoxia-induced myocardin expression were determined by pretreatment of the myocytes with 25 μM SP-600125, 3 μM SB-203580, 50 μM PD-98059, 50 μM Rac1 inhibitor and 1 μM Y27632 for 30 min before hypoxia. About 30 nM and 500 μM of Dp44mT and N-acetyl-L-cysteine (NAC) were added, respectively. The dose of mevalonate was 200 μM. SP-600125 is a selective and reversible inhibitor of JNK. SB-203580 is a specific inhibitor of p38 MAPK. PD-98059 is a specific inhibitor of the ERK pathway. Dp44mT is a ROS generator. NAC is a free radical scavenger. Y27632 is a selective inhibitor of RhoA/Rho-associated kinase (ROCK). HMG-CoA is reduced to mevalonate by HMG-CoA reductase, which is a rate-limiting step in cholesterol synthesis and can be inhibited by statins.

RNA isolation and real-time quantitative PCR

RNA isolation and real-time quantitative PCR were performed as described [13]. Primers were designed for the detection of myocardin gene expression (forward: 5′-GGACTGCTCTGG-CAACCCAGTGC-3′; reverse: 5′-CATCTGCTG ACT-CCGGGTCATTTGC-3′). GAPDH gene expression was used as internal controls (forward: 5′-GAGAGGCTCTCTGTCGAC-TAC-3′; reverse: 5′-TAGTGT AGGTTGGGCGCTCAA-3′).

Western blot analysis

Western blot analysis for cardiomyocytes was performed as previously described [13].

RNA interference

Small interfering RNA (siRNA) was prepared as described [13]. Neonatal cardiomyocytes were transfected with 800 ng of ERK- and myocardin-annealed siRNA (Dharmacon, Lafayette, CO, USA). ERK siRNAs are target-specific 20- to 25-nt siRNAs designed to knock down gene expression. Myocardin siRNAs are also designed to knock down gene expression.

Electrophoretic motility shift assay

Nuclear protein concentrations from cultured myocytes were determined by the Bradford method as commercialized by Santa Cruz Biotechnology, and Electrophoretic motility shift assay (EMSA) was performed as described [13]. Consensus and control oligonucleotides were labelled by polynucleotide kinase incorporation of [γ-^{32}P] ATP. In each case, mutant or cold oligonucleotide was used as a control to compete with the labelled sequences.

Promoter activity assay

A −968 to +44 bp rat myocardin promoter construct was generated as previously described [13]. The myocardin promoter contains myocardin-binding sites for SRF (sequences: CGGTTTAGGG) located at −514 to −505 bp of the promoter region. We used the binding sites to detect the transcriptional activity of myocardin. We also changed the mutant sequences located at −507 to −506 bp from CGGTTTAGGG to CGGTTTATTG. For the mutant, the myocardin–SRF-binding sites were mutated using a mutagenesis kit (Stratagene, La Jolla, CA, USA). Site-specific mutations were confirmed by DNA sequencing. Plasmids were transfected into cardiomyocytes using a low-pressure accelerated gene gun (Bioware, Taipei, Taiwan).

Measurement of intracellular ROS

Intracellular ROS generation was measured using 5-(and-6)-chloromethyl-2′, 7′-dichlorofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Invitrogen, Carlsbad, CA, USA), a

cell-permeant indicator for ROS. Confluent cells were subjected to serum starvation for 24 h and then used for experiments. Starved cells were then treated with hypoxia, atorvastatin, Y27632, NAC and Dp44mT for 5 min at 37 °C and imaged by inverted fluorescence microscopy.

Determination of protein synthesis

Protein synthesis was examined by measuring 3H-proline incorporation into the cells as previously described [13].

Statistical analysis

All results were expressed as mean ± SD. Statistical significance was evaluated using ANOVA test followed by Tukey–Kramer multiple comparisons test (GRAPHPAD Software Inc., San Diego, CA, USA). A value of $P < 0{\cdot}05$ was considered to denote statistical significance.

Results

Hypoxia-induced myocardin expression is suppressed by atorvastatin

Myocardin expression was noted as having the most evident effect in 2·5% O_2 hypoxia for 4 h, so we selected this concentration to conduct further analysis (Figure S1A,B). Myocardin protein and mRNA levels increased and reached peaks after 4 and 1 h, respectively (Figure S1C–E).

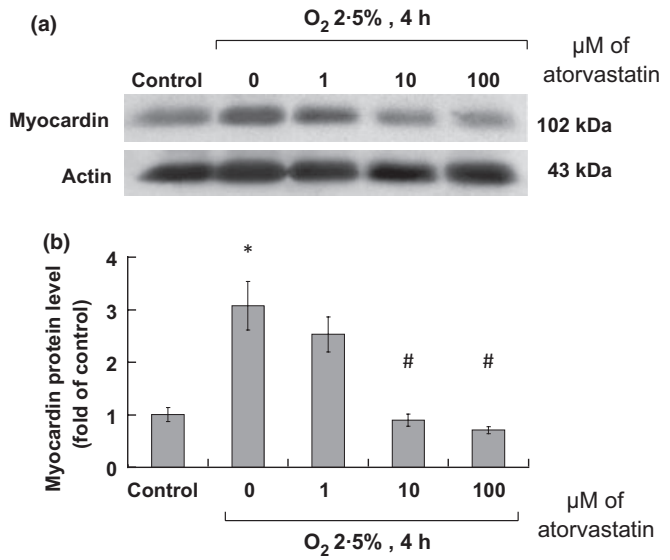

Figure 1 Effect of atorvastatin on hypoxia-induced myocardin expression. (a,b) Atorvastatin inhibited myocardin expression under hypoxia and had significant effect if its concentration reached more than 10 μM. *$P < 0{\cdot}05$ vs. normoxia control. #$P < 0{\cdot}05$ vs. 4 h. ($n = 3$ per group).

Hypoxia-induced myocardin expression could be suppressed by different concentrations of atorvastatin (Fig. 1a,b). Based upon the dose–response effect of atorvastatin on myocardin, we selected 10 μM atorvastatin in the following experiments. With regard to the influence of mevalonate on atorvastatin in the suppression of myocardin, we found that neither mevalonate nor atorvastatin with mevalonate was able to change hypoxia-induced myocardin expression (Figure S2). This finding indicated that atorvastatin inhibited hypoxia-induced myocardin expression through a HMG-CoA reductase–dependent pathway. In addition, atorvastatin did not inhibit myocardin expression under normoxia (Figure S2).

Hypoxia-induced myocardin expression in cardiomyocyte is through ERK and RhoA/Rock pathway

Hypoxia may stimulate genetic expressions through different pathways [6]. Other studies have indicated that stress-induced ROS generation may be conducted through the Rho pathway and be suppressed by statins [17,19,20,23]. We used different signal pathway inhibitors to detect the pathways of signal transduction. Myocardin expression was evidently inhibited by PD98059, Y27632, Rac1 inhibitor, ERK and myocardin siRNA, atorvastatin and NAC (Fig. 2, S3 and S4). Exogenous addition of Dp44mT without hypoxia also increased myocardin expression (Fig. 2).

Hypoxia increases binding activity between myocardin and SRF and myocardin promoter activity

Under hypoxia or Dp44mT with normoxia, binding activity between myocardin and SRF increased. Atorvastatin, NAC, PD98058 and Y27632 decreased the binding activity between myocardin and SRF induced by hypoxia (Fig. 3a). In Fig. 3a, there are two shifted bands in EMSA. Both upper and lower bands are indicated as SRF–DNA complex. As both bands are super-shifted in the presence of SRF antibody, both shifted bands should include SRF–DNA complex. This finding indicates that hypoxia in cardiomyocytes may increase the cooperation between myocardin and SRF, and hypoxia may increase myocardin expression and myocardin/SRF-binding activity.

We used a luciferase reporter assay to identify the genetic transcription activity of myocardin to cardiomyocytes under hypoxia (Fig. 3b). We found that hypoxia increased myocardin promoter activity in cardiomyocytes. Mutant myocardin promoter failed to have the same effect as wild type under hypoxia. This finding indicates that the SRF-binding site in myocardin promoter is essential for transcriptional regulation in cardiomyocytes under hypoxia. Exogenous addition of Dp44mT also increased the transcriptional activity of

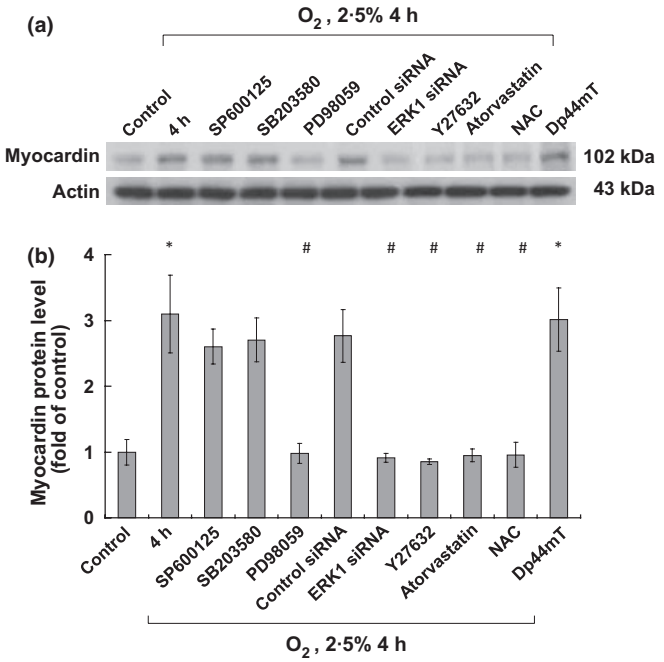

Figure 2 Myocardin expression after hypoxia is inhibited by extracellular signal-regulated kinase (ERK) pathway inhibitor, ERK siRNA, RhoA inhibitor, atorvastatin, N-acetyl-L-cysteine (NAC) and myocardin siRNA. (a,b) Myocardin expression 4 h after hypoxia was inhibited by ERK pathway inhibitor (PD98059), RhoA inhibitor (Y27632), ERK siRNA, atorvastatin and NAC. Exogenous addition of ROS generator (Dp44mT) under normoxia also increased the expression of myocardin. The result was normalized to actin. $^{*}P < 0{\cdot}05$ vs. normoxia control. $^{\#}P < 0{\cdot}05$ vs. 4 h. ($n = 3$ per group).

myocardin. Atorvastatin, PD98508, Y27632 and NAC suppressed the transcriptional activity of myocardin.

Atorvastatin inhibits hypoxia-induced myocardin expression through the ERK pathway

Western blot analysis revealed that both atorvastatin and PD98059 inhibited the phosphorylation of ERK (Fig. 4a,b). These findings revealed that hypoxia-induced ROS generation and myocardin expression may be conducted through the ERK pathway and can be inhibited by atorvastatin.

Atorvastatin attenuates hypoxia and ROS-induced intracellular ROS formation

Intracellular ROS increased after hypoxia for 1 h and was inhibited by atorvastatin, NAC and RhoA inhibitor. Intracellular ROS generated by Dp44mT under normoxia was also inhibited by atorvastatin, NAC and Y27632 (Fig. 5).

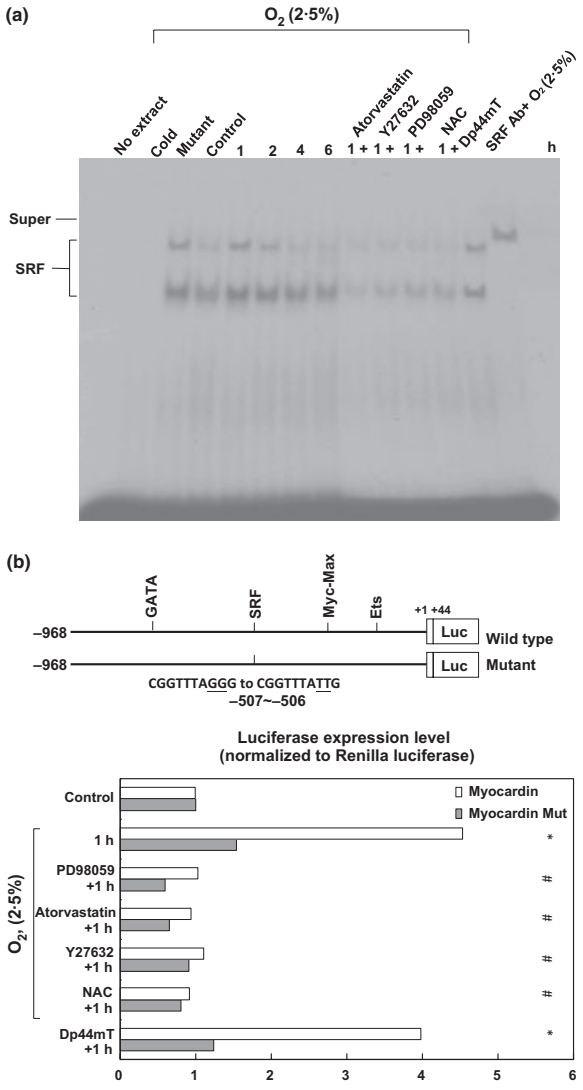

Figure 3 Binding activity between myocardin and SRF and genetic transcription activity at SRF-binding site of myocardin promoter increase in neonatal cardiomyocytes under hypoxia. (a) EMSA showed increased binding between myocardin, SRF and SRF antibody (Ab; super-shift) under 2·5% O_2 hypoxia, which was suppressed by atorvastatin, Y27632, PD98059 and N-acetyl-L-cysteine (NAC). Exogenous addition of Dp44mT under normoxia also increased the binding between myocardin and SRF. (b) Luciferase reporter assay revealed that 2·5% O_2 hypoxia increased transcriptional activity at SRF-binding sites of myocardin promoter when compared with the myocardin mutant, which was suppressed by atorvastatin, Y27632, PD98059 and NAC. Exogenous addition of Dp44mT under normoxia also increased the transcriptional activity in neonatal cardiomyocytes. $^{*}P < 0{\cdot}05$ vs. normoxia control. $^{\#}P < 0{\cdot}05$ vs. 6 h ($n = 3$ per group).

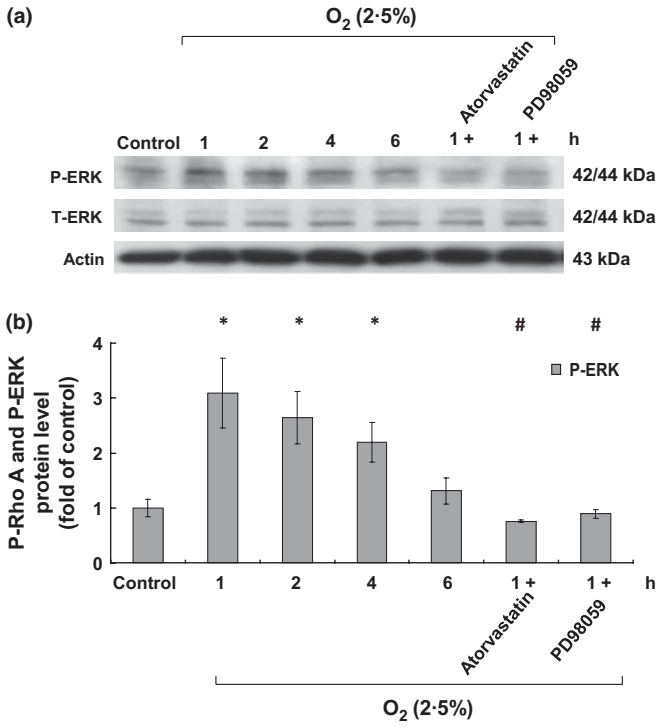

Figure 4 Atorvastatin inhibits hypoxia-induced myocardin expression through the extracellular signal-regulated kinase (ERK) pathway. (a,b) Phosphorylation of ERK increased after hypoxia and was inhibited by atorvastatin and PD98059. $^{*}P < 0{\cdot}05$ vs. normoxia control. $^{\#}P < 0{\cdot}05$ vs. hypoxia ($n = 3$ per group).

Atorvastatin decreases the presence of myocardin induced by hypoxia or ROS stimulation in nuclei of neonatal cardiomyocytes

We used confocal microscopy to identify the presence of myocardin on cardiomyocytes, which showed an increased presence of myocardin in the nuclei from 2 to 4 h after hypoxia. This was suppressed by atorvastatin, Y27632, PD98059 and NAC. Dp44mT further increased the presence of myocardin in the nuclei of cardiomyocytes (Figure S5).

Hypoxia or ROS stimulation induces cardiomyocyte hypertrophy

We also evaluated protein synthesis in cardiomyocytes by measuring ^{3}H-proline incorporation into the cells. The results showed increased protein synthesis after hypoxia for 4–8 h, which represented a hypertrophic change of the cardiomyocytes (Figure S6). Pretreatment with PD98059, atorvastatin, NAC and myocardin siRNA inhibited the protein synthesis induced by hypoxia. Exogenous addition of Dp44mT also increased ^{3}H-proline incorporation similar to the effect of hypoxia (Figure S6).

Discussion

Hypoxic injury is a pathological phenomenon in cardiovascular diseases and causes a stress to cardiomyocytes with subsequent expression of foetal cardiac-restricted genes [5,6,13]. Cardiomyocyte hypertrophy is the first adaptive response with enlargement of cells and organelles and may finally result in heart failure and even cardiac death [14,16,24]. Myocardin is a recently discovered potent transcriptional cofactor by Wang *et al.* [2], which cooperates with SRF to enhance growth and differentiation in cardiomyocytes by binding to the SRF-binding sites of its promoters [7–13]. Upregulated expressions of myocardin in failing ageing human hearts and porcine myocardium as compared with healthy individuals have recently been identified [25]. Fibroblasts from postmyocardial infarction scars acquiring properties of cardiomyocytes after transduction with recombinant myocardin gene has been another promising finding [26,27]. Other studies have indicated that pressure overload in cardiomyocytes increases the expression of myocardin and results in cardiomyocyte hypertrophy [28].

In our study, we identified that hypoxia in neonatal cardiomyocytes increased the expression of myocardin to cause cardiomyocyte hypertrophy [13]. Previous studies have indicated that hypoxia may stimulate ROS formation with subsequent cardiomyocyte hypertrophy [5,6]. Our study also identified that hypoxia and exogenous addition of Dp44mT increased myocardin expression, binding between myocardin and SRF, and transcriptional activities to the SRF-binding sites of myocardin promoter to cause cardiomyocyte hypertrophy. The hypertrophic response could be inhibited by myocardin siRNA and NAC.

Statins have been demonstrated to have antioxidant and antihypertrophic effects on cardiomyocytes [17–21]. In addition to inhibiting cholesterol synthesis, statins also block the formation of mevalonate and synthesis of isoprenoid intermediate metabolites in the biosynthetic pathway, such as farnesyl pyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP). FPP and GGPP are important lipid attachments for the post-translational modification of a variety of cellular proteins, including Rac1 and RhoA [29–31]. Rac1 is a component of the NADPH (nicotinamide adenine dinucleotide phosphate) oxidative complex that drives superoxide generation and plays a pivotal role in cardiac hypertrophy. The inhibition of Rac1 by statins leads to the reduction of oxidative stress and subsequently prevents cardiac hypertrophy [17,20,21,23]. Our study revealed the same findings that hypoxia-induced myocardin expression, Rac1 expression and myocardial hypertrophy can be inhibited by atorvastatin in neonatal rat cardiomyocytes. In addition to Rac1, RhoA/ROCK activity is one of the best-characterized downstream mediators of action of Rho pathways. Recent studies suggested that the RhoA/ROCK pathway plays

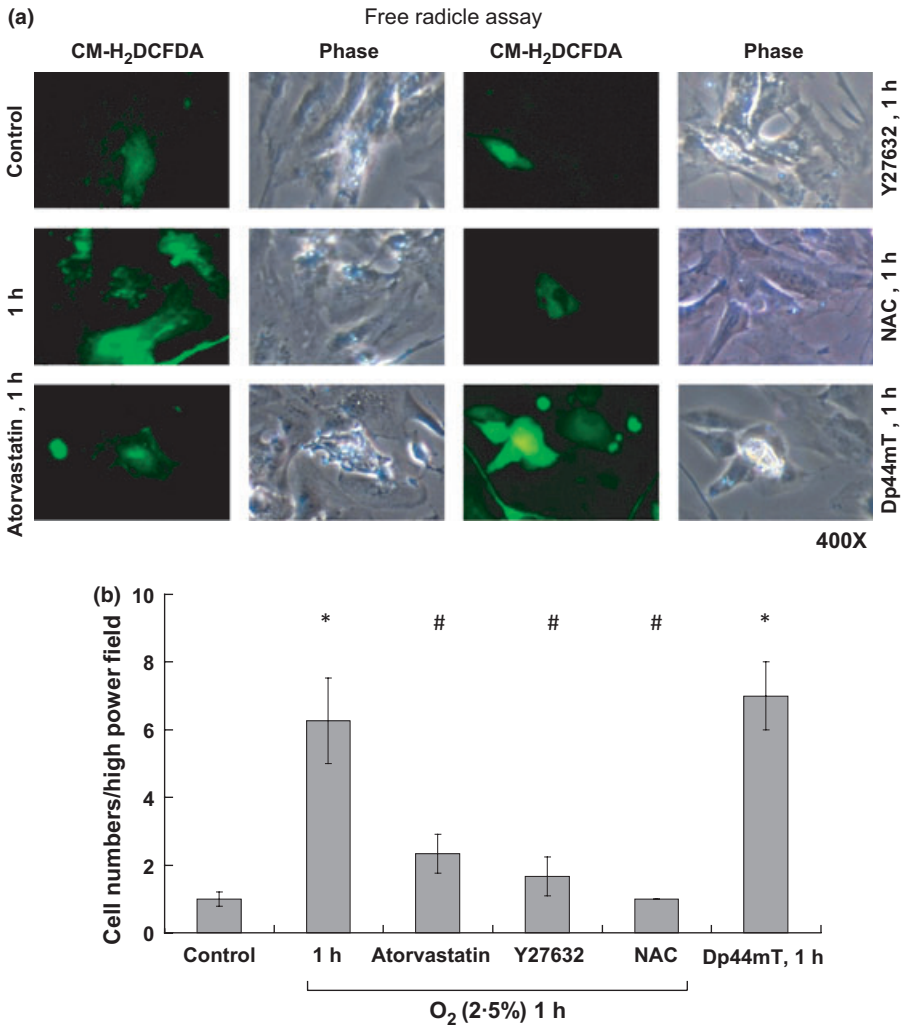

Figure 5 Atorvastatin inhibits post-hypoxia ROS formation, which, in turn, is inhibited by atorvastatin, N-acetyl-L-cysteine (NAC) and Y27632. (a,b) Intracellular ROS increased after hypoxia for 1 h and exogenous addition of ROS generator (Dp44mT) under normoxia and was inhibited by atorvastatin, NAC and RhoA inhibitor. $^{*}P < 0{\cdot}05$ vs. normoxia control. $^{\#}P < 0{\cdot}05$ vs. 6 h ($n = 3$ per group). High-power field (HPF) represents a 400 × magnification level of microscopy.

an important role in hypertension, coronary artery diseases and atherosclerosis [32–34]. Indeed, the inhibition of the RhoA/Rock signalling pathway by statins effectively improves these pathological conditions. In contrast to the clear role of RhoA/ROCK pathways in vascular control, the involvement of RhoA/ROCK pathways in cardiac hypertrophy and remodelling is relatively unclear [23]. In addition, previous studies have demonstrated only minor or fewer effects of statins in suppressing the Rho/Rock pathway than those of the Rac1 pathway to prevent cardiac hypertrophy in animal or human models [23,32–34]. Our study found that both atorvastatin and Rac1/RhoA inhibitors suppress myocardin expression, ROS generation and cardiomyocyte hypertrophy in neonatal rats under hypoxia. In the vascular smooth muscle cell (VSMC) model, the earliest described effect of RhoA was its ability to elicit serum response element–mediated transcriptional activation through a SRF-mediated pathway [35]. RhoA can regulate muscle-specific gene expression through the activation of SRF [36,37]. Reports from Treisman and others document the involvement of Rho family proteins in SRF-dependent gene transcription in a variety of cell types. However, the relationship between RhoA and SRF in genetic transcription to cardiomyocytes has not been reported before. In our study, we identified genetic transcription to cardiomyocytes under hypoxia through the RhoA pathway and the cooperation between SRF and myocardin. It has been recently shown that myocardin-related transcription

factor-A (MRTF-A), which is translocated from cytosol into nucleus and then activates SRF following Rho activation, plays an important role in cardiac hypertrophy [12]. In our study, we also found that the MRTF-A was involved in hypoxia-induced, Rho-dependent hypertrophic responses (Figure S7). Our study showed that MRTF-A expression reached a peak 4 h after 2·5% hypoxia in neonatal cardiomyocytes and was inhibited by the Y27632.

Our previous study indicated that hypoxia in neonatal cardiomyocytes caused cardiomyocyte hypertrophy and could be blocked by Ang II receptor blocker [13]. Other studies also indicated that Ang II expression may stimulate ROS and result in cardiomyocyte hypertrophy [5,32,33]. Statins have been shown to inhibit cardiomyocyte hypertrophy through the following pathways: (i) inhibition of the Rac1 pathway to prevent ROS-related cardiomyocyte hypertrophy, (ii) inhibition of Ang II expression by suppressing the activity of Ang II type 1 receptors and (iii) minor inhibition of the Rho/ROCK pathway to prevent cardiomyocyte hypertrophy [23]. In the VSMC model, the Rho/ROCK-ERK pathway has been demonstrated through the action of SRF and target genes in VSMC [35–37].

In our study, western blot analysis revealed that both atorvastatin and PD98059 inhibited the phosphorylation of ERK. This finding reveal that the inhibitory effects of atorvastatin on hypoxia-induced ROS generation, myocardin expression and cardiomyocyte hypertrophy are through the ERK pathway.

According to our previous [13] and current findings, we can conclude that the signal transduction pathway of cardiomyocyte hypertrophy under hypoxia is mediated by myocardin/SRF transcription in neonatal cardiomyocytes. Hypoxia in neonatal cardiomyocytes stimulated the expression of Ang II and ROS [13] and further increased the expression of myocardin to increase transcriptional activity to neonatal cardiomyocytes, resulting in cardiac hypertrophy.

The roles of statins in suppressing hypoxia-induced cardiomyocyte hypertrophy and myocardin expression have also not been reported before. In our study, atorvastatin was an effective inhibitor of ERK, ROS, myocardin and cardiac hypertrophy. Exogenous additions of NAC, a ROS scavenger, could also suppress ROS and myocardin expression in the downstream sequence. Our study found that hypoxia induced cardiomyocyte hypertrophy and this hypertrophy could be inhibited by atorvastatin.

In conclusion, hypoxia in neonatal cardiomyocytes increases myocardin expression and ROS to cause cardiomyocyte hypertrophy. Myocardial hypertrophy can be prevented by atorvastatin by suppressing ROS and myocardin expression.

Acknowledgements

This study was sponsored in part by Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan.

Address

Graduate Institute of Clinical Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan (C-Z. Chiu, K-G. Shyu); School of Medicine, Fu-Jen Catholic University, New Taipei City, Taiwan (C-Z. Chiu); Division of Cardiology, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan (C-Z. Chiu, K-G. Shyu).

Correspondence to: Dr Kou-Gi Shyu, Division of Cardiology, Shin Kong Wu Ho-Su Memorial Hospital, 95 Wen-Chang Road, Taipei 111, Taiwan. Tel.: 886-2-28332211; fax: 886-2-28365775; e-mail: shyukg@ms12.hinet.net

Received 5 September 2010; accepted 1 November 2011

References

1 Miano JM. Serum response factor: toggling between disparate programs of gene expression. *J Mol Cell Cardiol* 2003;**35**:577–93.
2 Wang D, Chang PS, Wang Z, Sutherland L, Richardson JA, Small E *et al*. Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. *Cell* 2001;**105**:851–62.
3 Zhang X, Azhar G, Zhong Y, Wei JY. Identification of a novel serum response factor cofactor in cardiac gene regulation. *J Biol Chem* 2004;**279**:55626–32.
4 Deindl E. Arteriogenesis: focus on signal transduction cascades and transcription factors. *Thromb Haemost* 2007;**98**:940–3.
5 Inamoto S, Hayashi T, Tazawa N, Mori T, Yamashita C, Nakano D *et al*. Angiotensin-II receptor blocker exerts cardioprotection in diabetic rats exposed to hypoxia. *Circ J* 2006;**70**:787–92.
6 Seta KA, Millhorn DE. Functional genomics approach to hypoxia signaling. *J Appl Physiol* 2004;**96**:765–73.
7 Wen JK, Han M. Myocardin. A novel potentiator of SRF-mediated transcription in cardiac muscle. *Mol Cell* 2002; **8**: 1–2.
8 Firulli AB. Another hat for myocardin. *J Mol Cell Cardiol* 2002;**34**:1293–6.
9 Parmacek MS. Myocardin-related transcription factors: critical coactivators regulating cardiovascular development and adaptation. *Circ Res* 2007;**100**:633–44.
10 Miano JM. Channeling to myocardin. *Circ Res* 2004;**95**:340–2.
11 Reynolds PR, Mucenski ML, Le Cras TD, Nichols WC, Whitsett JA. Midkine is regulated by hypoxia and causes pulmonary vascular remodeling. *J Biol Chem*. 2004;**279**:37124–32.
12 Kuwahara K, Kinoshita H, Kuwabara Y, Nakagawa Y, Usami S, Minami T *et al*. Myocardin-related transcription factor A is a common mediator of mechanical stress- and neurohumoral stimulation-induced cardiac hypertrophic signaling leading to activation of brain natriuretic peptide gene expression. *Mol Cell Biol* 2010;**30**:4134–48.
13 Chiu CZ, Wang BW, Chung TH, Shyu KG. Angiotensin II and ERK pathway mediate the induction of myocardin by hypoxia in cultured rat neonatal cardiomyocytes. *Clin Sci* 2010;**109**:273–82.
14 Yamashita C, Hayashi T, Mori T, Tazawa N, Kwak CJ, Nakano D *et al*. Angiotensin II receptor blocker reduces oxidative stress and attenuates hypoxia-induced left ventricular remodeling in apolipoprotein E-knockout mice. *Hypertens Res* 2007;**30**:1219–30.
15 Yoshida T, Hoofnagle MH, Owens GK. Myocardin and Prx1 contribute to angiotensin II-induced expression of smooth muscle α-actin. *Circ Res* 2004;**94**:1075–82.

16 Sadoshima J, Malhotra R, Izumo S. The role of the cardiac renin-angiotensin system in load-induced cardiac hypertrophy. *J Card Fail* 1996;**2**:S1–6.
17 Rikitake Y, Hirata K. Inhibition of RhoA or Rac1? Mechanisms of cholesterol-independent beneficial effects of statins *Circ J* 2009;**73**:231–2.
18 Ye Y, Hu SJ, Li L. Inhibition of farnesyl pyrophosphate synthase prevents angiotensin II-induced hypertrophic responses in rat neonatal cardiomyocytes: involvement of the RhoA/Rho kinase pathway. *FEBS Lett* 2009;**583**:2997–3003.
19 Saka M, Obata K, Ichihara S, Cheng XW, Kimata H, Noda A *et al*. Attenuation of ventricular hypertrophy and fibrosis in rats by pitavastatin: potential role of the RhoA-extracellular signal regulated kinase-serum response factor signaling pathway. *Clin Exp Pharmacol Physiol* 2006;**33**:1164–71.
20 Senthil V, Chen SN, Tsybouleva N, Halder T, Nagueh SF, Willerson JT *et al*. Prevention of cardiac hypertrophy by atorvastatin in a transgenic rabbit model of human hypertrophic cardiomyopathy. *Circ Res* 2005;**97**:285–92.
21 Adam O, Laufs U. Antioxidative effects of statins. *Arch Toxicol* 2008;**82**:885–92.
22 Shyu KG, Chen CC, Wang BW, Kuan P. Angiotensin II receptor antagonist blocks the expression of connexin 43 induced by cyclical mechanical stretch in cultured neonatal rat cardiac myocytes. *J Mol Cell Cardiol* 2001;**33**:691–8.
23 Brown JH, Del Re DP, Sussman MA. The Rac and Rho hall of fame: a decade of hypertrophic signaling hits. *Circ Res* 2006;**98**:730–42.
24 Baker KM, Chernin MI, Schreiber T, Sanghi S, Haiderzaidi S, Booz GW *et al*. Evidence of a novel intracrine mechanism in angiotensin II-induced cardiac hypertrophy. *Regul Pept* 2004;**120**:5–13.
25 Torrado M, Lopez E, Centeno A, Medrano C, Castro-Beiras A, Mikhailov AT. Myocardin mRNA is augmented in the failing myocardium: expression profiling in the porcine model and human dilated cardiomyopathy. *J Mol Med* 2008;**81**:566–77.
26 Pijnappels DA, Tuyn J, Vries AF, Grauss RW, Laarse A, Ypey DL *et al*. Resynchronization of separated rat cardiomyocyte fields with genetically modified human ventricular scar fibroblasts. *Circulation* 2007;**116**:2018–28.
27 Tuyn J, Pijnappels DA, Vries AF, Vries I, Dijke IV, Knan-Shanzer S *et al*. Fibroblasts from human postmyocardial infarction scars acquire properties of cardiomyocytes after transduction with a recombinant myocardin gene. *FASEB J* 2007;**21**:3369–79.
28 Xing W, Zhang TC, Cao D, Wang Z, Antos CL, Li S *et al*. Myocardin induces cardiomyocyte hypertrophy. *Circ Res* 2006;**98**:1089–97.
29 Hakoshima T, Shimizu T, Maesaki R. Structural basis of the Rho GTPase signaling. *J Biochem J (Tokyo)* 2003;**134**:327–31.
30 Hofer F. Activated Ras interacts with the Rac1 guanine nucleotide dissociation stimulator. *Proc Natl Acad Sci U S A* 1994;**91**:11089–93.
31 Schiller MR. Coupling receptor tyrosine kinases to Rho GTPases-GEFs: what's the link. *Cell Signal* 2006;**18**:1834–43.
32 Sagara Y, Hirooka Y, Nozoe M, Ito K, Kimura Y, Sunagawa K. Pressor response induced by central angiotensin II is mediated by activation of Rho/Rho-kinase pathway via AT1 receptors. *J Hypertens* 2007;**25**:399–406.
33 Kimura K, Eguchi S. Angiotensin II type-1 receptor regulates RhoA and Rho-kinase/ROCK activation via multiple mechanisms. *Am J Physiol Cell Physiol* 2009;**297**:C1059–61.
34 Nohria A, Prsic A, Liu PY, Okamoto R, Creager MA, Selwyn A *et al*. Statins inhibit Rho kinase activity in patients with atherosclerosis. *Atherosclerosis* 2009;**205**:517–21.
35 Hill CS, Wynne J, Treisman R. The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* 1995;**81**:1159–70.
36 Gineitis D, Treisman R. Differential usage of signal transduction pathways defines two types of serum response factor target gene. *J Biol Chem* 2001;**276**:24531–9.
37 Lui HW, Halayko AJ, Fernandes DJ, Harmon GS, McCauley JA, Kocieniewski P *et al*. The RhoA Rho kinase pathway regulates nuclear localization of serum response factor. *Am J Respir Cell Mol Biol* 2003;**29**:39–47.

Supporting Information

Additional Supporting information can be found in the online version of this article:

Data S1. Materials and methods.

Figure S1. Hypoxia increases the expression of myocardin protein and mRNA in cultured rat neonatal cardiomyocytes.

Figure S2. Atorvastatin inhibits hypoxia-induced myocardin expression through a HMG-CoA reductase dependent pathway.

Figure S3. Myocardin expression under hypoxia is supressed by Rac1 inhibitor.

Figure S4. Effects of myocardin and ERK siRNAs expression in cardiomyocytes under 2·5% O_2.

Figure S5. Confocal microscopy identifies the presence of myocardin (yellow; white arrow) in the nuclei (blue) of neonatal cardiomyocytes after hypoxia and Dp44mT, which is inhibited by atorvastatin, Y27632, PD98059, and NAC. (n = 3 per group).

Figure S6. Protein synthesis in neonatal cardiomyocytes increases after hypoxia.

Figure S7. Myocardin-related transcriptional factor-A (MRTF-A) increases expression after hypoxia in neonatal cardiomyocytes.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.