Critical Role for FoxO3a-Dependent Regulation of p21CIP1/WAF1 in Response to Statin Signaling in Cardiac Myocytes

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Abstract—Statins are widely used clinical drugs that exert beneficial growth-suppressive effects in patients with cardiac hypertrophy. We investigated the role of the cell cycle inhibitor p21CIP1/WAF1 (p21) in statin-dependent inhibition of hypertrophic growth in postmitotic cardiomyocytes. We demonstrate that lovastatin fails to inhibit cardiac hypertrophy to angiotensin II in p21−/− mice and that reconstitution of p21 function by TAT.p21 protein transduction can rescue statin action in these otherwise normally developed animals. Lovastatin specifically recruits the forkhead box FoxO3a transcription factor to the p21 promoter, mediating transcriptional transactivation of the p21 gene as analyzed in isolated primary cardiomyocytes. Lovastatin also stimulates protein kinase B/Akt kinase activity, and Akt-dependent phosphorylation forces p21 in the cytoplasm, where it inhibits Rho-kinases contributing to the suppression of cardiomyocyte hypertrophy. Loss of p21 or FoxO3a by RNA interference causes a general inhibition of lovastatin signal transduction. These results suggest that p21 functions as FoxO3a downstream target to mediate an statin-derived anti-hypertrophic response. Taken together, our genetic and biochemical data delineate an essential function of p21 for statin-dependent inhibition of cardiac myocyte hypertrophy. (Circ Res. 2007;100:50-60.)

Key Words: hypertrophy □ molecular biology □ myocardium □ signal transduction □ statins

Statins are widely used in patients because they exert beneficial effects in prevention of cardiovascular diseases.1–5 Statins are cholesterol-lowering drugs that act by inhibiting 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMG-CoA), the rate-limiting enzyme in cholesterol biosynthesis.6,7 They also inhibit the isoprenoid pathway, thereby blocking the activities of the Rho family of small GTPases.8,9 Rho proteins exert many of their effects through Rho-kinases (ROCKs), and activation of the RhoA-ROCK pathway promotes cardiac hypertrophy.10–12 Inhibition of this pathway by statins leads to endothelial NO synthase activation, establishing the current perspective that statin-induced NO production is cardioprotective.13–17

FoxO-Smad proteins are crucial mediators of the cytoplastic effect of transforming growth factor (TGF)-β.18 This FoxO-Smad pathway is involved in the regulation of cardiac hypertrophy and remodeling.19–24 On phosphorylation by receptor kinases, Smad2 and Smad3 heterooligomerize with Smad4 before translocation to the nucleus, where they regulate gene expression. The FoxO3a transcription factor functions as key partner of Smad2/3–Smad4 complexes for the transcriptional induction of p21 required for cytoplastic response.25 Signaling through the phosphatidylinositol 3-kinase (PI3K)–Akt pathway is important for physiological growth and inhibition of pathological hypertrophy.14,26–29 In proliferating cells, FoxO proteins are negatively regulated by this pathway because Akt-dependent phosphorylation of FoxO factors causes their exclusion from the nucleus, eliminating their transactivation capacity.18

The cyclin-dependent kinase (cdk) inhibitor p21 is a potent inhibitor of cell growth and division. Transcriptional activation of the p21 gene is regulated through p53-dependent and -independent mechanisms. Commonly, p21 is viewed as a nuclear protein that antagonizes cell division through inhibition of cyclin E/cdk2. However, recent evidence has shown that p21 can exert cdk2- and, thus, cell cycle–independent functions.30 Heart failure is the leading major cause of mortality in all developed nations. Because of the extraordinary clinical importance of cardiovascular disease, we investigated whether the growth suppressor p21 is mechanistically implicated in statin-dependent inhibition of cardiac hypertrophy. Here, we demonstrate that lovastatin (Lova) uses the transcription factor FoxO3a as signal transducer to achieve induction of the p21 gene. Lova also stimulates Akt kinase activity, and Akt-dependent phosphorylation of p21 leads to

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inhibition of ROCK kinases and suppression of cardiac myocyte hypertrophy.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Primary Rat Cardiomyocyte Culture and p21 Knockout Mice
Spontaneously beating cardiomyocytes from heart ventricles of neonatal rats were cultured and subjected to treatment with angiotensin II (Ang II) with and without pretreatment with Lova. Animals used were in accordance with approved institutional animal care guidelines. Age-matched male wild-type (WT) C57BL/6 and p21−/− mice (5 to 8 weeks, 18 to 22 g) were used in this study. Ang II was chronically infused subcutaneously by osmotic pumps (model 2000, Alzet) at a dose of 1.4 μg/kg per minute for 14 days. Lova was injected intraperitoneally once daily for 14 days at a dose rate of 20 mg/kg. Lova and mevalonate were preactivated by opening the lactones through alkaline hydrolysis.

Statistical Analyses
Factorial design ANOVA or r-tests were used to analyze data as appropriate. Significant ANOVA values were followed by simple main effects analyses or post hoc comparisons of individual mean using the Tukey method were appropriate. The level of significance was 0.05 (in vivo studies) or 0.005 (cultured cardiomyocytes).

Results
Inactivation of p21 by RNA Interference Abrogates the Antihypertrophic Effect of Lova in Isolated Cardiomyocytes
Lova inhibited Ang II–triggered hypertrophic growth in primary cardiomyocytes, which was susceptible to mevalonate, the downstream product of the HMG-CoA reductase reaction in steroid biosynthesis (Figure 1A and 1B). To determine whether p21 is required for Lova growth arrest, we used RNA interference of p21 (sip21) to specifically reduce the amount of endogenous p21 (Figure 1C). The response to Lova in sip21-expressing cardiomyocytes cultivated in the presence of Ang II or phenylephrine (PE) was completely abolished (Figure 1A through 1D; Figure IA through IC of the online data supplement). Elimination of p21 but not of the closely related cdk-inhibitor p27KIP1 rendered cardiomyocytes refractory to Lova action, establishing that targeted loss of p21 causes a general inhibition of Lova signal transduction. These results suggest that p21 elicits an essential function during Lova action in isolated cardiomyocytes.

Next, we generated recombinant TAT.p21 and inactive negative control TAT.p21ΔC in bacteria as C-terminal fusion proteins with the HIV-1 TAT protein transduction domain and a hexahistidine (His6) tag. TAT-fusion proteins are capable of transducing 100% of targeted cells in a concentration-dependent and receptor- and transporter-independent manner (see the online data supplement). In contrast to vast overexpression by viral gene delivery, TAT-mediated protein transduction allowed us to precisely control the amount of p21 protein reexpressed in p21-deficient cardiomyocytes. Transduction of 0.5 μmol/L TAT.p21 protein could rescue Lova growth arrest in sip21-expressing cardiomyocytes, implying that p21 has the ability to complement Lova action (Figure 1E; supplemental Figure ID through IF). This dosage of TAT.p21 was insufficient to block hypertrophy in the absence of statin demonstrating (1) that the physiological effect of TAT.p21 was specific and (2) that Lova action uses other antihypertrophic mechanisms in addition to p21 activation. Notably, p21 can also function independently from statin action, because transduction of 1.0 μmol/L TAT.p21 counteracted cardiomyocyte hypertrophy in the absence of Lova (Figure 1E, lane 5).

Lova-Activated FoxO3a Induces p21 Gene Transcription
We observed that Lova significantly enhanced p21 protein levels in an Ang II–independent manner in cardiomyocytes, as determined by ELISA technique (Figure 2A; supplemental Figure II). Based on a previous supposition,25 we reasoned that FoxO3a might be responsible for p21-induction by Lova in cardiomyocytes. To investigate this possibility, cardiomyocytes were transiently infected with siFoxO3a or transcriptionally inert FoxO3aΔN. We noticed that FoxO3aΔN inhibited p21 induction by Lova. This effect was specific, because engaging the RNA interference machinery by targeting siFoxO3a resulted in loss of p21 induction. In contrast, transduction of siFoxO3aΔN did not result in a blockade of p21 upregulation by Lova. Next, Northern blot analysis of p21 mRNA at 5 hours after Lova treatment confirmed that this drug induces p21 gene transcription (Figure 2B). Again, this rise in p21 transcript level was not observed in the presence of ectopic FoxO3aΔN or in cardiomyocytes expressing siFoxO3a. We infer from these findings that Lova engages the transcription factor FoxO3a to mediate p21 expression in cardiomyocytes.

The Smad-binding region (SBR) in the p21 gene promoter contains a Smad-binding element (SBE) and a FoxO-binding element (FHBE) that is conserved in the human, mouse, and rat p21 promoters.25,31 Thus, we more critically determined whether Lova action requires FoxO3a activation of p21 gene transcription. Mutation of the FHBE- or SBE-site abrogated the Lova response of the p21 promoter (Figure 2C). FoxO3a-dependent induction of the p21-SBR by Lova was dependent of the FHBE and SBE and further increased by FoxO3a overexpression. In contrast, ectopic FoxO3aΔN abolished the Lova response of the p21 promoter.

Because FoxO3a induction of p21-promoter activity was dependent of the FHBE and SBE, we investigated whether FoxO3a may form a Lova-regulated complex with Smads. Indeed, coimmunoprecipitation analysis revealed that endogenous FoxO3a binds to endogenous Smad2/3 and Smad4 (Figure 2D). Formation of FoxO3a-Smad and Smad2/3–Smad4 complexes was absolutely dependent on Lova action. As a synthetic oligonucleotide, the SBR region bound to endogenous Lova-activated FoxO3a but not FoxO1/4 (Figure 2E). Expression of siFoxO3a completely eliminated the induction of FoxO3a binding to the probe by Lova. Next, we used chromatin immunoprecipitation (ChIP) assays to analyze p21 gene-promoter occupancy by FoxO3a. In response to Lova, FoxO3a specifically bound to contiguous sites in the p21 promoter mediating p21 activation (Figure 2F, top). The specificity of our ChIP assay was confirmed with primers annealing to the α-myosin heavy chain promoter because
transcription of this gene is not thought to be under the control of either FoxO. Thus, significant levels of \( H9251 \)-myosin heavy chain were never amplified. We were unable to detect significant binding of FoxO3a to the p21 promoter by ChIP in the absence of Lova, confirming that this process requires statin action. We infer from these results that FoxO3a is recruited to the p21 gene promoter and that this cellular response is absolutely dependent on Lova.

Reportedly, Smad2/3 undergo constant nucleocytoplasmic shuttling. In the absence of phosphorylation, Smad2/3 are transcriptionally inactive. Phosphorylated Smad2/3 associate with and translocate Smad4 into the nucleus, resulting in transcriptional complex formation with FoxO3a. In response to Lova, Smad2, Smad3, and Smad4 specifically bound to contiguous sites in the p21 promoter (Figure 2F, bottom). Smad2/3 are phosphorylated on Lova action by as
yet uncharted pathway in cardiomyocytes, and their phosphorylated isoforms are confined to the nuclear compartment (Figure 2G). Accordingly, a significant fraction of Smad4 is concentrated in the nucleus of Lova-treated cardiomyocytes. We noticed that expression of FoxO3a is restricted to nuclear fractions because FoxO3a is not phosphorylated by Akt on T32/S253 in the presence or absence of Lova. Thus, Smad2, Smad3, and Smad4 appear to function as Lova-regulated FoxO3a partners, participating in p21 activation in cardiomyocytes.

Because induction of p21 by Lova was lost in siFoxO3a-transduced cardiomyocytes, we assessed the physiological relevance of FoxO3a in Lova-dependent inhibition of cardiomyocyte hypertrophy. Depletion of FoxO3a by siRNA rendered cardiomyocytes refractory to Lova action as these cells continued to grow in the presence of Ang II (Figure 2H). We conclude that FoxO3a mediates Lova responses including p21 activation.

**Lova Accomplishes Cytoplasmic Accumulation of p21 by Stimulating Akt-Dependent Phosphorylation of p21 at T145**

Akt is reportedly a p21 kinase in tumor cells, and statins induce Akt kinase activity in endothelial cells. Thus, we investigated whether Akt would also regulate the subcellular localization of p21 in response to Lova in cardiomyocytes. First, we determined the influence of Lova toward Akt kinase activity. Akt activation as evaluated by phosphorylation on S473 was increased within 5 hours in Lova-treated cells (Figure 3A). We noticed that Ang II exerted only a modest stimulatory effect on Akt-Pi-S473 phosphorylation, which is in good agreement with previous results. Preincubation with the PI3K inhibitor LY294002 blocked the reactivity of anti-Akt.Pi-S473 and abolished glycogen synthase kinase 3 (GSK3) substrate phosphorylation.

Next, we analyzed the subcellular compartmentalization of endogenous p21 in cardiomyocytes at an early time point (5 hours) after addition of Ang II by immunocytochemistry. At this stage, cells did not yet exhibit any morphological signs of hypertrophy. Under resting conditions, p21 protein expression was exclusively nuclear (Figure 3B). p21 was sequestered in the cytoplasm in Lova-treated cells, whereas inhibition of Akt by LY294002 evoked nuclear accumulation of p21. These results suggest that p21 is specifically retained in the cytoplasm by Lova action. To confirm this view, cardiomyocytes were preincubated with LY294002 to block the Akt pathway, or transduced with kinase-dead dn.Akt. The subcellular localization and phosphorylation of p21 was determined by immunoblotting of fractionated cellular extracts. Akt-dependent phosphorylation events of p21 on T145 were confined to cytoplasmic fractions and solely observed in the presence of Lova (Figure 3C and 3D). Pharmacological inhibition of Akt as well as ectopically delivered dn.Akt diminished T145 phosphorylation and cytoplasmic localization of p21.

**Lova Growth Arrest Is Associated With p21-Dependent Inhibition of ROCKs**

Because of their ability to block endothelial NO synthase activity, the RhoA effector kinases ROCK1 and ROCK2 are important regulators of statin action and cardiac hypertrophy. To define whether p21 can inhibit ROCK1 in cardiomyocytes, we used anti-ROCK1 kinase-assays (Figure 4A and 4B). Ang II–induced ROCK1 activation was susceptible to statin action, because Lova-activated cytoplasmic p21 forms an inhibited complex with ROCK1 as endogenous proteins in the cytoplasm, as determined by communoprecipitation analysis (Figure 4A, lane 4). We did not observe Lova-dependent inhibition of ROCK1 in sip21-transduced cardiomyocytes, whereas cotransduction of TAT:p21 (0.5 μmol/L) restored the inhibitory effect of Lova toward ROCK1 activity (Figure 4B). This dosage of TAT:p21 was insufficient to block ROCK1 in the absence of statin. We explored whether the inhibitory effects of Lova on cardiomyocyte hypertrophy occurs at the level of ROCKs and whether these effects are dependent on an intact p21 protein. When the kinase-inactive version ROCK1.K105A, which induces dominant-negative effects on RhoA was cotransduced in sip21-expressing cells exposed to Lova, hypertrophic growth was constrained (Figure 4C). Taking these findings into consideration, we surmise that Lova-mediated decreases in expression of hypertrophic markers are a consequence of ROCK inhibition caused by increased p21 expression.

**p21-Deficient Mice Are Resistant to Lova Action**

To analyze whether p21 is required for Lova action in vivo, we determined the impact of Lova on Ang II–induced cardiac hypertrophy in p21−/− mice lacking an overt phenotype. Heart/body weight ratio, myocyte cross-sectional area, and atrial natriuretic factor and brain natriuretic peptide mRNA levels were significantly increased in WT and p21−/− mice infused with Ang II, whereas they were much lower in WT mice treated with Lova/Ang II (Figure 5A through 5D). Notably, Lova did not prevent cardiac hypertrophy to Ang II in p21−/− mice.

Next, we sought evidence that p21 would also complement Lova action in vivo. Therefore, p21−/− mice were intraperitoneally injected with TAT:p21 at a dose of 4 mg/kg once daily for 14 days. This dose is below the threshold level necessary to block myocardial hypertrophy in the absence of Lova. In combination with Lova, transduction of TAT:p21 inhibited cardiac hypertrophy in p21−/− mice. Thus, reconstitution of p21 protein abundance in the myocardium of p21−/− mice was accompanied by rescue of Lova action.

Next, we tested various physiological responses of Lova action in total left ventricular heart tissue samples by Western blot analysis. The immunoreactivity with the p21.Pi-T145 antibody revealed that p21 was phosphorylated on T145 in Lova/Ang II–treated WT siblings (Figure 5E). Anti-Akt.Pi-S473 failed to react with Akt in samples from saline-injected control mice and reacted with Akt only in lysates prepared from Lova/Ang II–treated WT and p21−/− animals. All of these results are consistent with our notion that p21 is necessary for Lova action in the heart.

**The Inhibitory Effects of Lova on ROCK Kinases Are Abolished in p21-Deficient Mice**

Having shown that Lova cannot constrain cardiac hypertrophy to Ang II in p21−/− mice, we surmised that unrestrained
Figure 2. FoxO3a mediates p21 induction by Lova. A, Cardiomyocytes were transiently cotransfected with lentiviral vectors encoding siFoxO3a or siFoxO3a-mis for 36 hours before transduction with transcriptionally inert FoxO3a-N for 36 hours. Cells were then pre-treated with Lova for 1 hour and incubated with Ang II for 5 hours before lysis. Aliquots of total cardiomyocyte extracts (0.2 to 6 mg) were analyzed by sandwich ELISA using anti-p21. Mean±SEM, n=4. B, Cardiomyocytes lentivirally transduced as indicated were pre-treated with Lova for 1 hour and then incubated with Ang II for 5 hours before lysis. Total RNA was subjected to Northern blotting with probes to p21 and GAPDH. Mean±SEM, n=3. C, Cardiomyocytes transfected with p21 promoter constructs driving luciferase reporter and FoxO3a variants as indicated were preincubated with Lova for 1 hour and then incubated with Ang II for 24 hours before lysis, and luciferase activity was determined. The mt.FHBE and mt.SBE p21 gene promoter constructs carry mutations in the SBR (FoxO3a/Smad}
ROCK activity attributable to lack of p21 may account for this effect. Therefore, we determined whether p21 can also form inhibited complexes with ROCKs in vivo. ROCK1/2 activities were much lower in Lova/Ang II–treated WT mice when compared with the same p21/H11002/H11002/H11002 grouping (Figure 6A and 6B). The association of p21 with ROCKs was restricted to Lova/Ang II–infused WT mice, whereas it was absent in the same p21/H11002/H11002/H11002 grouping, as inferred from coimmunoprecipitation analysis. Moreover, the inhibitory impact of Lova on ROCKs was reconstituted in p21/H11002/H11002/H11002 mice transduced with TAT.p21 but not TAT.p21ΔC. These results argue for the direct involvement of Lova-activated p21 in ROCK inhibition in the myocardium.

ROCK1/2 are downstream effector molecules of the small GTPase RhoA. Thus, we examined the effect of Lova on RhoA activity. The amount of GTP-loaded RhoA was substantially the same in extracts derived from WT and p21/H11002/H11002/H11002 mice infused with Ang II (Figure 6C). Ang II–dependent formation of activated RhoA-GTP was inhibited in Lova-treated WT siblings and in their p21/H11002/H11002/H11002 counterparts.

Figure 3. Lova induces Akt-dependent phosphorylation of p21 on T145, leading to its cytoplasmic retention. A, Quantitative analysis of Akt-kinase activity (left). Cardiomyocytes were pretreated with the PI3K-specific inhibitor LY294002 (20 μmol/L) for 30 minutes, before treatment with Lova for 1 hour, and then incubated with Ang II for 5 hours before lysis. Total cellular extracts (1 to 2 mg) were incubated with anti-Akt, and immunocomplex kinase assays were performed using paramyosin-GSK3β-/β-croetidide fusion protein as substrate. The extent of GSK3 phosphorylation was determined by Western blot analysis using phosphorylation site–specific anti-GSK3.Pi-S21/S9, and densitometry using TINA software. Mean±SEM, n=3. Equal amounts of total cellular extracts (50 μg) were subjected to immunoblotting with anti-Akt, or anti-Akt.Pi-S473 specifically recognizing the activated Akt protein variant (right). B, Subcellular localization of p21. Cardiomyocytes were processed for immunostaining with anti-p21 (fluorescein isothiocyanate; green) 5 hours after addition of Ang II. Scale bar=10 μm. C, Lova induces cytoplasmic accumulation of p21 by Akt-dependent phosphorylation of p21 on T145. Fractionated cell extracts were immunoblotted using anti-p21 or anti-p21.Pi-T145. dn.Akt, kinase-dead Akt.K179M. D, Immunoblot analysis for subcellular localization of Akt. CP indicates cytoplasmic fraction; β-Gal, β-galactosidase; MHC, myosin heavy chain; NC, nuclear fraction.

Figure 2 (cont). binding region). Mean±SEM, n=4. D, Lova-dependent formation of a FoxO3a-Smad transcription factor complex in cardiomyocytes. Cells were pretreated with Lova for 1 hour and incubated with Ang II for 5 hours before lysis (D through G). Total cellular cardiomyocyte extracts (500 μg) were immunoprecipitated (IP) with specific antibodies, as indicated on the top of each panel, and immunoblotted with antibodies indicated on the left. WB indicates Western blot. E, FoxO3a binds specifically to biotinylated double-stranded SBR oligonucleotide probes derived from the p21 promoter, which is diminished by FHBE (FoxO3a oligonucleotide-binding sequence) and SBE (Smad-binding element) mutations. Determination of the presence of FoxO3α in the precipitate was analyzed by immunoblotting with antibodies to FoxO family members. F, Specific recruitment of FoxO3α and not other FoxO family members to the p21 promoter by Lova. ChIPs were performed with the indicated antibodies (top). PCR was performed with primers specific for the p21 promoter region harboring the SBR and primers specific for the α-myosin heavy chain (α-MHC) promoter as Lova-unresponsive control. G, Lova induces phosphorylation-dependent nuclear accumulation of Smad2/3. Equal amounts (50 μg) of biochemically fractionated cell extracts were subjected to immunoblotting using antibodies, as indicated on the left of each panel. CP indicates cytoplasmic fraction; NC, nuclear fraction. H, Expression of siFoxO3α abrogates the inhibitory effect of Lova on cardiomyocyte hypertrophy to Ang II. Mean±SEM, n=4. *P<0.005 vs unstimulated cells, **P<0.005 vs Ang II, #P<0.005 vs Lova/Ang II.
parts. In both groupings, p21 and TAT.p21 were not associated with RhoA, as analyzed by coimmunoprecipitation analysis, indicating that p21 does not inhibit formation of RhoA-GTP. In addition, Lova also stimulated FoxO3a binding to biotinylated double-stranded SBR oligonucleotide probes in vivo, which was diminished by FHBE and SBE mutations (Figure 6D). Lova-dependent recruitment of FoxO3a to the p21 gene promoter was further corroborated by ChIP analysis (Figure 6E). These results provide evidence for the importance of FoxO3a in the p21 response to Lova in the myocardium.

**Discussion**

We demonstrate that Lova activates a FoxO3a transcription factor complex, which turns on the growth-inhibitory p21 gene. p21 is phosphorylated by Akt, which sequesters p21 in the cytoplasm, where it inhibits ROCK kinases. Figure 6F illustrates the relationship between the Lova-
Figure 5. p21 is important for Lova action in vivo. Lova inhibits cardiac hypertrophy to Ang II in WT mice but not in p21⁻/⁻. Transduction of TAT.p21 protein complements Lova action in p21⁻/⁻ mice. A, Heart weight corrected for body weight, cross-sectional cardiomyocyte area, and determination of atria natriuretic factor (ANF) and brain natriuretic peptide (BNP) mRNA expression by Northern blotting. Mean±SEM, n=6 to 8. *P<0.05 vs sham saline, **P<0.05 vs Ang II, #P<0.05 vs WT Lova/Ang II, ##P<0.05 vs p21⁻/⁻ Lova/Ang II. B, Gieson stain and fluorescein isothiocyanate–conjugated wheat germ agglutinin stain of myocardial cross-sections. Scale bars: 2 mm (Gieson); 200 μm (cross area). C, Visualization of TAT.p21 by immunofluorescence microscopy using anti-His₆ (anti-his) tag antibody (fluorescein isothiocyanate; green). Scale bar=200 μm. MHC indicates myosin heavy chain. D, Northern blot analysis of hypertrophic markers in left ventricles. E, Physiological responses of Lova action in total left ventricular heart tissue samples (60 μg), as analyzed by immunoblotting.
Figure 6. Lova-mediated inhibition of ROCKs by p21 contributes to inhibition of myocardial hypertrophy. Lova inhibits ROCK activities in WT mice but not in p21^{-/-} animals. Transduction of TAT.p21 protein complements Lova action on ROCK kinase activities. A and B, Left ventricular extracts (2 to 3 mg) were subjected to anti-ROCK1/2 immunocomplex kinase assays and aliquots of the reactions were immunoblotted with anti-ROCK1/2, or anti-p21/anti-His6 tag to detect ROCK-bound p21. C, p21 does not prevent formation of RhoA-GTP in the mouse myocardium. Lysates were incubated with GST-Rhotekin and immunoblots were probed with anti-RhoA to detect RhoA-GTP, or anti-p21 for analysis of p21 associated with RhoA. Experiments in A through C were performed twice with similar results. D and E, Lova recruits FoxO3a to the p21 gene promoter in vivo. D, FoxO3a binds specifically to biotinylated double-stranded p21-SBR oligonucleotide probes, which is diminished by FHBE/SBE mutations. Presence of FoxO3a in the precipitate was determined by immunoblotting. E, ChIP analysis of FoxO3a binding to the p21 promoter in vivo. F, Model for Lova-mediated inhibition of cardiomyocyte hypertrophy through induction of p21. Statins activate FoxO3a transcription factor complexes to target the p21 promoter for activation. Statin signaling events also activate the PI3K/Akt pathway, and Akt-dependent phosphorylation of p21 leads to nuclear exclusion of p21, enabling cytoplasmic p21 to inhibit ROCKs. The RhoA-independent activation of ROCKs has been discussed elsewhere. 

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generated FoxO3a p21-activation complex and Lova-directed engagement of Akt to accomplish cytoplasmic p21 expression, contributing to suppression of cardiomyocyte hypertrophy.

The conceptually important points that emerge from our study are (1) that Lova fails to inhibit cardiac hypertrophy in p21−/− mice (Figure 5); (2) that Lova action is lost when p21 is disabled by siRNA-mediated knockdown in cultured cardiomyocytes (Figure 1); and (3) that both inactivation of endogenous FoxO3a by RNA interference and by dominant-negative FoxO3aAN eliminate induction of p21, allowing hypertrophy to proceed in the presence of Lova (Figure 2). The interaction of FoxO3a with the p21 promoter and its transactivation is stringently dependent on Lova stimulation (Figures 2 and 6). We observed no differences in Lova-dependent formation of FoxO3a complexes in p21−/− mice when compared with their WT counterparts, indicating that these Lova downstream signaling events are not compromised. All of our findings suggest that FoxO3a is functionally integrated in p21 activation by Lova. We concede that statins may also signal through FoxO-independent as well as transcription-independent mechanisms. However, our observations from genetic deletions and transducing proteins strongly argue that FoxO3a is specifically engaged by Lova to activate p21.

FoxO3a function is inhibited by the PI3K/Akt pathway in proliferating cells where Akt-dependent phosphorylation can induce nuclear exclusion of FoxO3a. Here, we describe a different scenario for Lova-FoxO3a−dependent induction of p21 in nondividing cardiomyocytes. In these cells, FoxO3a complexes are still recruited to the p21 promoter, albeit Akt kinases are catalytically active (Figures 2 and 3). One possible explanation is that FoxO3a is simply not amenable to Akt-dependent inactivation because of their contrasting subcellular localization (compare Figure 2G with Figure 3D).

Ang II induces NADPH oxidase−dependent superoxide anion production, and it is widely accepted that reactive oxygen species (ROS) have a critical function for the development of cardiac hypertrophy. Statins can decrease ROS production through inhibition of Rac1 and this reduction in the levels of intracellular oxidative cell stress is an important clinically as aspect of their cholesterol-independent effects. Strong experimental evidence has described a primary role of FoxO3a in the detoxification defense against ROS, eg, by inducing the antioxidative mitochondrial gene manganese superoxide dismutase. However, because intracellular ROS can activate numerous signaling pathways downstream of growth factor receptors, the precise molecular mechanism underlying the ability of Lova to induce the transactivation activity of FoxO3a is at present unknown.

Lova growth arrest was rescued by ectopic TAT.p21 in p21−/− mice and sip21-expressing cardiomyocytes, whereas mutant TAT.p21ΔC failed to functionally complement Lova action (Figures 1 and 5). Because the amino terminus of p21 was not sufficient to inhibit cardiac hypertrophy, the most plausible explanation is that, for p21 to function in this context, it may not have to bind to cdks and to other partners (such as SAPks) with which this region interacts. The present findings indicate that, besides playing a crucial role in mediating statin action, p21 can be involved in the progression of cardiomyocytes through differentiation-associated processes. In this model, the carboxy terminus of p21 may function as a specific bridge between signaling complexes (such as ROCKs) and the transcriptional machinery involved in the hypertrophic program.

Depletion of p21 by sip21 and genetic deletion of p21 in mice specifically prevent Lova-dependent inhibition of ROCKs (Figures 4 and 6). The formation of inhibited p21-ROCK complexes and suppression of the hypertrophic phenotype also correlate: both processes occur to a large extent in WT cardiomyocytes and are undetectable under conditions in which p21 function has been eliminated. These results are consistent with our concept that p21 is essential and sufficient to restrain cardiac hypertrophy by blocking ROCK kinases.

Our findings lend support to the idea that p21 is of potential therapeutic importance for the prevention of heart failure. Important issues that remain to be examined are potential immune responses of the TAT-domain and side effects associated with long-term administration of TAT.p21. In conclusion, the results from our study indicate that the induction of p21 by statins is an important aspect of their cardioprotective properties.

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Disclosures
None.

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Critical role for FoxO3a-dependent regulation of p21\textsuperscript{CIP1/WAF1} in response to statin signaling in cardiac myocytes

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Short title: p21 mediates statin dependent cardiac growth arrest

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Supplemental Methods

Primary rat neonatal ventricular cardiomyocyte culture and p21 knockout mice

Hearts from 3-day postnatal Wistar rats were dissected, minced, and enzymatically isolated with collagenase II (0.5 mg/mL, Invitrogen) and pancreatin (1 mg/mL, Sigma). To selectively enrich for cardiomyocytes, the resultant cell suspension was plated onto 10 cm-collagen I (Gibco)–coated dishes in culture medium DMEM/F12 containing 3 mmol/L Na-pyruvate, 2 mmol/L glutamine, routine antibiotics (GIBCO), 0.2% BSA, 0.1 mmol/L ascorbic acid, and 0.5% insulin-transferrin-selenium (Sigma) for 60 minutes. After preplating, myocytes were held for 36 hours in the presence of 25 mmol/L araC and 5% horse serum (Sigma) to inhibit noncardiomyocyte proliferation. Cardiomyocytes were stimulated in culture medium without BSA.

Lova and Meva were pre-activated by opening the lactones through alkaline hydrolysis using 0.15 mL of 0.1 N NaOH for 4 mg of drug dissolved in 0.1 mL of ethanol (99%) at 50°C for 2 hours. The resulting solution was neutralized with HCl to a pH of 7.4 and was brought up to a volume of 1 mL with cell-culture grade water.

Animals use were in accordance with approved animal care guidelines of the Max-Delbrueck-Center. Aged matched male wt-C57BL/6 and p21−/− mice (5-8 weeks, 18-22 g) were used in this study. Ang was chronically infused subcutaneously by osmotic pumps (model 2000, Alzet) at a dose of 1.4 µg/kg per minute for 14 days. Lova was injected intraperitoneally once daily for 14 days at a dose rate of 20 mg/kg. Mice were anesthetized with 1% isofluorane in 70% N2O and 30% O2 using a vaporizer. Body temperature was maintained at 37°C using a thermally controlled surgical table. In randomly selected animals, the right femoral artery was cannulated with a PE10 catheter for blood pressure and heart rate. Application of Ang did not elevate systolic blood pressure. After euthanasia, the heart was excised, the atria and aorta were removed, and the heart weight was assessed. After fixation in formalin, a transverse midsection (3 µm thickness) was stained with Gieson (HT25A, Sigma) for cardiac muscle or FITC-
conjugated wheat-germ agglutinin (L4895, Sigma) for cross sectional measurements. Lova, LY294002, Y27632 were obtained from Calbiochem. Ang, PE, Meva, were purchased from Sigma.

**Lentiviral vectors**

Design of siRNA was performed using the internet applications of Ambion/Dharmacon. The following target oligonucleotide sequences were used to generate shRNA-lentiviruses in pLenti6/BLOCK-iT-DEST (Invitrogen). sip21 sense (GenBank U24174) 5'-AGTATGCGTCGCTGTGGT-3'. sip21mis 5'-AGTATGAGTCTGTGTTG-3'. sip27 (D86924) 5'-ACCGAGCACCACCAAGCCTT-3'. sip27mis 5'-ACCGAGCACCTCAAGCCTT-3'. sipFoxO3 (XM_215421) 5'-GATTCGGCCAGGCTGCTGT-3'. sipFoxO3amis 5'-GATTCGGAAAGGCTGCTGT-3'. Cells were transduced with the indicated lentiviral vectors at 10 pfu/cell. In siRNA mis, two base pairs of the target sequences have been mutated.

Kinase-dead Akt.K179M,1 transactivation-deficient FoxO3aΔN (Δ1-256),2 and kinase-dead ROCK1.K105A,3 were in lentivirus pLenti6-DEST (Invitrogen). Mutations were introduced employing the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene). Each individually cDNA was sequenced using the big dye terminator cycle sequencing kit according to the manufacturer’s specifications (Abiprism, Perkin Elmer).

The rat p21 FHBE/SBE promoter reporter constructs were derived from pGL3-basic (Promega) and carried for copies of the FoxO/Smad binding elements.2,4 Luciferase assays were carried out using the Promega assay kit, Lipofectamine 2000 reagent (Invitrogen), and a Berthold luminometer as described previously.5 Nucleotide sequence of Smad-binding element in the rat p21 promoter (Genbank U24172) located -2715 to -2677 upstream of p21 coding sequences: wt 5'-CTCCAAAGTAAACAGACAGACAAATGTCTCTTCTATCT-3' FBHE SBE, mt.FBHE 5'-CTCCAGGATCCACAGACAGACAAATGTCTCTTCTATCT-3', mt.SBE 5'-CTCCAAAGTAAACAGAGATCCAAATGTCTCTTCTATCT-3'.
**Cellular extracts and cell fractionation**

In an attempt to rule out any in vitro artifacts associated with the preparation of lysates, extractions buffers contained broad phosphatase inhibitors (1 mmol/L Na$_3$VO$_4$, 20 mmol/L NaF, 10 mmol/L β-glycerophosphate, 1 mmol/L NaP$_2$O$_7$). NP40-buffer was used for whole cellular extracts: 50 mmol/L Tris-HCl pH 7.5, 250 mmol/L NaCl, 0.5% NP-40, 5 mmol/L EDTA pH 8.0, 1 mmol/L DTT, protease inhibitor cocktail (Roche). For subcellular cell fractions, 2.5x10$^6$ cardiomyocytes in 10 cm-dishes were trypsinized and lysed in 500 µl harvest buffer (10 mmol/L HEPES pH 7.9, 50 mmol/L NaCl, 500 mmol/L sucrose, 0.1 mmol/L EDTA, 1 mmol/L DTT, protease inhibitors). After centrifugation the supernatant was designated as cytoplasmic extract. The remaining pellet was washed in buffer A (10 mmol/L HEPES pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, protease inhibitors) and nuclei were lysed using 500 µl buffer C (10 mmol/L HEPES pH 7.9, 500 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.1% NP-40, 1 mmol/L DTT, protease inhibitors). After centrifugation (14,000 x g; 1 hour; 4°C) the supernatant was designated as nuclear fraction.

**RhoA-p21 binding assay**

Samples were lysed in 50 mmol/L Tris-HCl (pH 8.0), 2 mmol/L MgCl$_2$, 0.2 mmol/L Na$_2$S$_2$O, 10% glycerol, 20% sucrose, 2 mmol/L DTT, phosphatase/protease inhibitors. The supernatant was incubated with GST-tagged rhotekin (17-294, Upstate). Immunoblotting was done with anti-RhoA to detect RhoA-GTP, or anti-p21 for RhoA associated p21.

**ELISA, immunoprecipitation, oligonucleotide precipitation, ChIP assays, and antibodies**

For sandwich ELISA technique, microtiterplates were coated with anti-p21 (sc-397), and captured proteins were detected with anti-p21 (sc-6246) and alkaline phosphatase-secondary antibody (715-056-150, Jackson) and pNPP substrate (PS613, Sigma). Samples were reacted for 45 minutes at room
temperature and absorption at 405 nm was determined in a microtiter plate reader (Dynatech). The linear range of the standard curve was employed for determination of p21 protein contents.

Cellular extracts were incubated with 50 µL protein G-agarose beads (Roche) preblocked with 1.0% (w/v) BSA in lysis buffer, and incubated with antibodies (5-12.5 µg/mL) overnight at 4°C on a rotating wheel. For coimmunoprecipitation studies, immunocomplexes were dissociated in 25 µL lysis buffer containing 1% (w/v) SDS. Lysis buffer was added to give a final concentration of 0.1% SDS, and the supernatant was incubated with 50 µL protein G-agarose beads for 2-3 hours at 4°C. The supernatant was transferred to fresh protein G-agarose beads, normal rabbit or mouse antibodies as negative controls were added, and incubated for 2 hours at 4°C. Immune complexes were then washed three times with lysis buffer and eluted in 50 µL SDS sample buffer. Boiled samples (25 µL) were electrophoretically separated, transferred to PVDF membranes, blocked, and primary antibodies (0.5 to 5 µg/mL) were incubated overnight at 4°C on a rotary platform with gentle agitation. They were subsequently probed with secondary HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (diluted 1:2000, GE Healthcare). Immune complex kinase assays were performed according to manufacturers' instructions.

ROCK1, 14-601, Upstate. Akt, 9840, Cell Signaling. Visualization of recombinant LS6SP (27 kDa) and GSK3 (30 kDa; 200 ng) was done by standard Coomassie blue staining of SDS-PAGE gels.

For oligonucleotide-precipitation lysates in 10 mmol/L HEPES (pH 7.9), 100 mmol/L KCl, 2 mmol/L MgCl₂, 10% glycerol, 1 mmol/L DTT, 0.5% NP40 were precleared with ImmunoPure streptavidin-agarose beads (Pierce) for 2 hours and incubated with 5 µg of biotinylated double stranded oligonucleotides and 10 µg poly(dI-dC) for 24 hours. After collection of DNA-bound proteins with streptavidin-agarose beads for 2 hours, samples were immunoblotted with antibodies to FoxO proteins. 6 ChIP assays were carried out essentially as described2,7 using the following PCR primers: p21 forward 5’-CCTGAGGAGAGCCAACTGCCTGC-3’, reverse 5’-GGAGGACTCCTCTCCTGTTGGG-3’. α-MHC forward 5’-CCAATGAGTACCGCGTGAA-3’, reverse 5’- ACAGTCATGCGGCTGATGAT-3’.
Antibodies: Penta-His (34670, Qiagen), Smad3 (AB3817, Chemicon), ROCK2 (07-443), RhoA (05-778, Upstate), actin (sc-7210), lamin B (sc-20682), β-tubulin (sc-5546), p21 (sc-397), p21 (sc-6246), p21.Pi-T145 (sc-20220-R), ROCK1 (sc-5560), tropomyosin (sc-28543), normal rabbit IgG (sc-2027, SantaCruz), Akt (9272), Akt.Pi-S473 (4051), Smad2 (3103), Smad2.Pi-S465/467 (3101), Smad3.Pi-S423/425 (9514), Smad4 (9515), FoxO1 (9462), FoxO3a (9467), FoxO3a.Pi-T32 (9464), FoxO3a.Pi-S253 (9466), FoxO4 (9472), eNOS (9572), eNOS.Pi-S1177 (9571), GSK3.Pi-S21/S9 (9331), p27 (2552, Cell Signaling).

**TAT fusion protein generation**

The pRSET-based pTAT-vector was obtained from S. Dowdy (Howard Hughes Medical Institute, La Jolla, USA). Recombinant TAT.p21 and inactive TAT.p21ΔC in which the carboxy-terminal PCNA-, cyclin-binding site, and the nuclear localization signal have been deleted were generated in bacteria as C-terminal fusion proteins with the HIV-1 TAT protein transduction domain and a hexahistidine (his-) tag. To induce cytoplasmic localization, mutations were sequentially introduced into TAT.p21 to generate TAT.p21.K141A/K154A (designated TAT.p21) with the following primers. K141A 5'-CAGGGTCGA(G/A)(C/A)ACGGCGGCGACCAGC-3'. K154A 5'-CCACTCC(G/A)(C/A)ACGCACCACTCC-3'.

TAT.p21 (Genbank L25610) and TAT.p21ΔC (Δ91-164) were isolated under denaturing conditions as described. Briefly, *E.coli* BL21(DE3)pLysS (Promega) pellets (30 g wet weight) from 5.0 L overnight cultures (terrific broth) induced with 500 or 1000 μmol/L IPTG (Sigma) were lyzed in 8 mol/L urea-buffer (100 mL), 1.0 mmol/L DTT, 10 mmol/L PMSF, 15 mmol/L imidazole (Sigma), 100 mmol/L NaCl, 20 mmol/L Hepes, pH 8.0 (Calbiochem) and sonified six-times for 30 sec on ice. Cleared supernatant was subjected to Ni-NTA column (12 mL, GE Healthcare) connected to a FPLC (ÄKTA, GE Healthcare) at room temperature. TAT-fusion protein was eluted in Z-buffer containing 500 mM imidiazole and subjected to ionic exchanger chromatography (Mono Q 5/10 column, GE Healthcare).
TAT.p21 was eluted with a single 2 mol/L NaCl step and desalted in PBS (G-25 column, GE Healthcare). Purified TAT.p21 in cell culture medium (for in vitro studies) or saline (for animal experiments) was adjusted to 10% (v/v) glycerol, aliquotted, and stored at -80°C. TAT.p21 was purified to near homogeneity as judged by Coomassie-gel staining. We routinely obtain 4 to 5 mL recombinant TAT-p21 protein at 1 mg/mL from a single 5.0 L batch culture.

**Hypertrophy assays**

Mouse cDNA probes were PCR-amplified employing specific primers.\(^{11}\) ANF forward 5'-CATCACCCTGGGTCTTCTTCT-3', reverse 5'-TGGGCTCAATCCTGTCAATC-3'; BNP forward 5'-GCAGGCATGGATCTCCTGAAGG-3', reverse 5'-CCCAGGCAGAGTCAGAAACTG-3'; GAPDH forward 5' ATGTTCCAGTGACTCCACTCG-3', reverse 5'-GAAGACACCAGTAGACTCCACGCA-3'. Total RNA (20 µg) was resolved by denaturing 1.5% agarose-formaldehyde-gel electrophoresis, electrotransferred to nitrocellulose membranes (OPTITRAN BA-S85, Schleicher& Schuell, Germany), and crosslinked (UV-Stratalinker, Stratagene). PCR-amplified cDNAs (100 to 200 ng) were nick-labeled with [\(\alpha-32\)P]dCTP (111 MBq/mmol, NEN) and the multiprime DNA labeling system (Pharmacia). Unbound radioactivity was removed applying Microspin G-25 columns according to the manufacturer's instructions (Pharmacia Biotech). The membranes were reprobed to assess equivalent loading with a GAPDH-cDNA probe. Membranes were autoradiographed and the incorporated radioactive label was detected with a PhosphorImager and TINA software (Raytest). For de novo protein synthesis, cardiomyocytes were incubated with 250 µCi/mL \([^{35}\text{S}]\)methionine and Ang II for 24 hours. Aliquots of total cellular extracts (3x10^5 cells) were resolved by SDS-PAGE, electrotransferred, autoradiographed, and the amount of incorporated radioactive label per lane was quantified with a PhosphorImager. Determination of cell surface area was done by indirect immunofluorescence staining of cardiomyocytes with anti-tropomyosin and NIH image. Cells grown on coverslips were fixed in 3.7% PBS-buffered formalin and permeabilized in 1% Triton X-100 for 12
minutes. Anti-p21 positive cells were recognized with FITC-, and tropomyosin positive cells were visualized with TRITC-conjugated secondary antibodies (Jackson). Digital images were overlaid using Adobe Photoshop CS2.

References


Supplemental Figures

Figure 1S

p21 exerts essential functions in Lova-dependent inhibition of cardiomyocyte hypertrophic growth. (A) Cardiomyocytes were transduced with p21 or p27 lentiviral siRNA expression vectors for 72 hours. Cells were then incubated for 48 hours with Ang (100 nmol/L) to induce hypertrophic growth. Mean±SEM, n=4. *P<0.005 vs unstimulated cells. (B) Northern blot analysis of ANF/BNP mRNA levels. (C) Western blot analysis of endogenous p21 and p27 protein expression. (D) Transduction of TAT.p21 proteins in cardiomyocytes. Cells were transduced with the indicated recombinant proteins for 48 hours. Mean±SEM, n=4. (E) Northern blot analysis of ANF/BNP mRNA levels. (F) Immunoblot analysis of endogenous p21 and ectopic TAT.p21 proteins.

Figure 2S

FoxO3a mediates p21 induction by Lova. Cardiomyocytes were transiently cotransfected with lentiviral vectors encoding siFoxO3a or siFoxO3a\textsuperscript{mis} for 36 hours prior to transduction with transcriptionally inert FoxO3a\textsuperscript{ΔN} for 36 hours. Cells were then pre-treated with Lova for 1 hour, and incubated with Ang for 5 hours prior to lysis. Aliquots of total cardiomyocyte extracts (50 µg) were subjected to immunoblot analysis employing anti-p21.
Supplemental Figures

Figure 1S
Supplemental Figures

Figure 2S

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