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Statins Inhibit Rho Kinase Activity in Patients with Atherosclerosis

Anju Nohria^a, Adnan Prsic^a, Ping-Yen Liu^b, Ryuji Okamoto^a, Mark A. Creager^a, Andrew Selwyn^a, James K. Liao^a, and Peter Ganz^c

^a Cardiovascular Division, Brigham and Women's Hospital, Boston, MA, USA

^b Cardiovascular Division, National Cheng Kung University Hospital/Cardiovascular Research Center, National Cheng Kung University, Tainan, Taiwan

^c Cardiovascular Division, San Francisco General Hospital, San Francisco, CA, USA

Abstract

Background—In addition to inhibiting cholesterol synthesis, statins (HMG-CoA reductase inhibitors) decrease the formation of isoprenoid intermediates required for the activation of key signaling pathways, including Rho/Rho kinase (ROCK). In experimental settings, statins inhibit ROCK and reverse vascular dysfunctions in atherosclerosis, independent of cholesterol reduction. It is not known whether statins inhibit ROCK activity in humans with atherosclerosis.

Methods—We investigated 35 patients with stable atherosclerosis in a randomized, double-blind study comparing treatment with high-dose (80 mg/d) or low-dose (10 mg/d) atorvastatin to placebo for 28 days. Blood samples for leukocyte ROCK activity, fasting lipids, and high-sensitivity C-reactive protein (hs-CRP) were obtained on days 0, 7, 14, and 28 after randomization and change over time with the 2 statin treatments relative to placebo was examined.

Results—Atorvastatin 80 mg/d reduced ROCK activity ($p=0.002$ vs. placebo). This decline was rapid and significant within 2 weeks of treatment. The inhibition of ROCK by atorvastatin (80 mg/d) remained significant even after controlling for changes in low-density lipoprotein cholesterol (LDL-C) and triglycerides ($p=0.01$). Furthermore, there was no correlation between changes in ROCK activity and changes in LDL-C ($r=0.2$, $p=0.25$) or triglycerides ($r=0.1$, $p=0.55$). There was a modest correlation between ROCK inhibition and change in hs-CRP among patients randomized to atorvastatin 80 mg/d ($r=0.6$, $p=0.07$).

Conclusions—These first-in-man findings demonstrate that high-dose atorvastatin rapidly inhibits the pro-atherogenic Rho/ROCK pathway, independent of cholesterol reduction. This inhibition may contribute to the clinical benefits of statins. Rho/ROCK may provide a useful therapeutic target in patients with atherosclerosis.

Keywords

Atherosclerosis; lipids; statins; Rho kinase

Address for Correspondence: Peter Ganz, MD, Chief, Cardiovascular Division, San Francisco General Hospital, 1001 Potrero Avenue, San Francisco, CA 94110 Tel: (415) 206-3024 Fax: (415)-206-8965 Email: E-mail: ganzp@medsfgh.ucsf.edu.

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INTRODUCTION

3-Hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins) are accepted first line agents for the treatment of hyperlipidemia to reduce the risk of adverse cardiovascular events. Statins lower serum cholesterol by inhibiting HMG-CoA reductase, the rate-limiting enzyme in the mevalonate pathway that is responsible for cholesterol synthesis [1]. Mevalonate is also essential for the formation of isoprenoid intermediates such as farnesylpyrophosphate and geranylgeranylpyrophosphate [1]. These isoprenoid intermediates are important for membrane translocation and activation of small guanosine triphosphate (GTP) binding proteins, including Rho [2]. Rho and its downstream effector, Rho kinase (ROCK), play an important role in regulating the actin cytoskeleton and thereby affect intracellular transport, gene transcription, and messenger RNA expression and stability [3]. In cultured cells and in animals, inhibition of Rho/ROCK by statins exerts an anti-atherogenic effect that is independent of cholesterol reduction. Yet in animals, the doses of statins reported to inhibit Rho/ROCK *in vivo* are far higher (1–2 log units higher on a per kilogram basis) than those employed in clinical practice [4], raising doubt about the relevance of these findings to humans.

Development of an assay of ROCK activity[5,6] has permitted us to test the hypotheses that 1) statins used in approved doses inhibit the Rho/ROCK pathway in subjects with atherosclerosis, 2) that this inhibition occurs rapidly, 3) that it is particularly pronounced with the intensive dosing of statins found to be advantageous in recent clinical trials[7], and 4) that any inhibition of ROCK by statins correlates with an anti-inflammatory effect (assessed by high-sensitivity C-reactive protein).

METHODS

Study Subjects

The Human Research Committee at Brigham and Women's Hospital approved this study. We enrolled subjects with stable atherosclerosis who met the modified NCEP ATP III guideline criteria for initiation of statin therapy [8]. A complete history, physical examination, and laboratory evaluation was performed for each subject. Atherosclerosis was defined by the presence of $\geq 50\%$ stenosis in at least one coronary artery by cardiac catheterization, prior myocardial infarction, prior revascularization, prior thromboembolic stroke, or documented peripheral arterial disease. Exclusion criteria included an unstable coronary syndrome, revascularization, or severe heart failure within 3 months of study enrollment, malignancy, chronic inflammatory disease, chronic infection, pregnancy, low-density lipoprotein cholesterol (LDL-C) < 2.6 mmol/L (100 mg/dL) off statin therapy, prior intolerance to statins, liver transaminases > 2 times normal, creatine phosphokinase > 3 times normal, serum creatinine > 3 mg/dL, and reluctance to discontinue statins for the duration of the study. Subjects were encouraged to continue all their cardiac medications, except statins, throughout the study. All study blood samples were drawn after an overnight fast, before subjects had taken their medications.

Study Design

The study was a randomized, double-blind, placebo-controlled, 3 parallel-arms trial. Subjects who signed informed consent and met the inclusion/exclusion criteria were asked to discontinue statins for a minimum of 2 weeks. This period is sufficient to restore the lipid profile to pre-statin treatment levels [9]. Subjects were then randomized to receive 1 month of low-dose atorvastatin (10 mg), high-dose atorvastatin (80 mg), or placebo once daily. Blood for leukocyte ROCK activity, fasting lipids, high-sensitivity C-reactive protein (hs-CRP), and

safety assessment was collected on days 0, 7, 14, and 28 of randomized treatment. Subjects were instructed to resume their routine lipid-lowering therapy upon study completion.

We randomized a total of 45 subjects in this study. Four subjects were withdrawn on day 0 for LDL-C < 2.6 mmol/L (100 mg/dL), 1 subject was withdrawn on day 0 for creatine phosphokinase > 3 times normal, 1 subject withdrew consent after day 0, and 3 additional subjects were withdrawn for acute cholecystitis, elevation in liver enzymes on study drug, and medication non-compliance. A total of 36 subjects completed the study and all except one subject with diabetes but no documented evidence of atherosclerosis were included in the analysis. Only one subject had missing data on day 7.

Measurement of Rho Kinase Activity

ROCK activity was assayed in peripheral blood leukocytes as the proportion of phospho-Thr⁸⁵³ in the myosin binding subunit (MBS) of myosin light chain phosphatase [5]. Blood was collected at room temperature in heparinized tubes (20U/ml) containing 10mM fasudil (Asahi Chemical Industry Co. Ltd., Japan). Fasudil was added to inhibit ROCK activity, and hence further formation of phospho-Thr⁸⁵³ MBS *ex vivo* [5]. In our experience, there is no appreciable dephosphorylation of phospho-Thr⁸⁵³ at room temperature [5]. Leukocytes were isolated from peripheral blood as described previously [5,6]. The leukocyte pellet was suspended in Media 199 solution (M199) (Sigma Chemical, IL) and then diluted to achieve 5×10^6 cells/mL. Fixative solution (50% trichloroacetic acid (Sigma Chemical, IL), 50 mmol/L dichlorodiphenyltrichloroethane (Sigma Chemical, IL), and protease inhibitors (Calbiochem, EMD Biosciences, Inc, Darmstadt, Germany) were then added to the solution and the resulting precipitate was stored at -80°C for Western blot analysis.

Western blot analysis was performed as described previously [5] using rabbit anti-phospho-specific Thr⁸⁵³-MBS polyclonal antibody (generous gift of Dr. Ikebe, Worcester, MA) or rabbit anti-MBS polyclonal antibody (Covance Laboratories, IN). NIH 3T3 cell lysates were used as a positive control. Rho kinase activity was expressed as the ratio of phosphorylation levels of myosin binding subunit (pMBS) in each sample per pMBS in each positive control divided by MBS in each sample per MBS in each positive control [6].

Measurement of Lipids and High-Sensitivity C-Reactive Protein

Fasting serum lipids (total and high-density lipoprotein (HDL-C) cholesterol and triglycerides) were measured with an Olympus AU400 analyzer using enzymatic methods. LDL-C was calculated according to the methods of Friedewald et al. [10]. Serum hs-CRP was quantified by latex-enhanced nephelometry on the BNTMnephelometry system (Dade Behring, Deerfield, IL).

Drugs

Atorvastatin (10 mg and 80 mg) was obtained from Pfizer, Inc. (Morris Plains, NJ). The different strengths of atorvastatin and placebo tablets were encapsulated and matched to facilitate blinding. Randomization and drug administration were performed by the investigational drug pharmacy at Brigham and Women's Hospital.

Statistical Analysis

Descriptive and experimental measures are expressed as mean $\pm$ SD or median (25th percentile, 75th percentile) as appropriate. Baseline characteristics were compared between the 3 treatment groups using one-way ANOVA or the Kruskal-Wallis test for continuous variables and Fisher's exact test for discrete variables. Triglycerides and hs-CRP were not normally distributed and were log transformed for the regression analyses. The effect of treatment by randomization

subgroup on the average change in dependent variables (ROCK activity, lipids, and hs-CRP) from baseline over time was assessed using a random effects regression model for longitudinal data (PROC MIXED). We fit a repeated measures model with a random effect for subject and fixed effects for treatment, time, and their interaction. We controlled for baseline ROCK activity, lipids, and hs-CRP in the longitudinal models to account for any baseline differences in these parameters that may not have been statistically significant because of the modest sample size. Correlation between change in ROCK activity and change in LDL-C, triglycerides, and hs-CRP from baseline to each time point was assessed using Pearson's and Spearman's correlation coefficients, as appropriate. All analyses were conducted using SAS software (version 9.1). All statistical tests were two-sided, with an alpha level of 0.05.

RESULTS

Baseline Characteristics

Subjects in all 3 treatment arms were well-matched for age, gender, race, cardiac risk factors, and medications (Table 1).

Effect of Statin Therapy on Rho Kinase Activity

Baseline ROCK activity did not differ significantly between the 3 treatment groups (Table 1). Nonetheless, we controlled for baseline ROCK activity in the longitudinal analyses due to differences that may have been present but were not statistically apparent due to the modest sample size. ROCK activity did not change over time in subjects randomized to placebo ($p=0.29$ vs. baseline) and only trended to decline with atorvastatin 10 mg/d ($p=0.10$ vs. placebo) (Figure 1). Atorvastatin 80 mg/d decreased ROCK activity over time ($p=0.02$) and this reduction was highly significant compared to placebo ($p=0.002$) (Figure 1). The time course of ROCK inhibition by high-dose atorvastatin (Figure 1) revealed a rapid reduction in ROCK activity, becoming statistically significant within 14 days of treatment (change from baseline relative to placebo at day 7, $p=0.06$; day 14, $p=0.04$; and day 28, $p=0.003$). Representative Western blots depicting the change in ROCK activity over time are shown in Figure 2.

Effect of Statin Therapy on Lipids

Subjects in all 3 treatment groups had similar lipid profiles at randomization (Table 1). There was no change in any lipid parameter during the study period in subjects randomized to placebo (data in supplement). The stable lipid concentrations in the placebo arm indicate that the 2-week statin washout was sufficient to re-establish baseline levels. As expected, both doses of atorvastatin reduced total cholesterol and LDL-C compared to placebo ($p<0.0001$), with the high-dose (80 mg/d) being more effective than the low-dose (10 mg/d) ($p<0.05$). High-dose, but not low-dose atorvastatin, reduced triglycerides relative to placebo ($p=0.0002$). Neither dose of atorvastatin changed HDL-C compared to placebo.

Effect of Statin Therapy on High-Sensitivity C-Reactive Protein

High sensitivity CRP levels were similar at baseline in all 3 treatment groups (Table 1). Hs-CRP levels did not change significantly from baseline within any treatment group (data in supplement). However, the trend over time in subjects treated with atorvastatin 80 mg/d showed a significant decline in hs-CRP compared to those treated with placebo ($p=0.02$) or atorvastatin 10 mg/d ($p=0.04$).

Lipid Levels and Rho Kinase Activity

To test the hypothesis that ROCK inhibition by atorvastatin is independent of lipid-lowering, we controlled for changes in both LDL-C and triglycerides in the longitudinal model. The effect of treatment on ROCK activity remained significant after controlling for change in LDL-C

(atorvastatin 80 mg/d vs. placebo, $p=0.004$), change in triglycerides (atorvastatin 80 mg/d vs. placebo, $p=0.02$), and reductions in both LDL-C and triglycerides simultaneously (atorvastatin 80 mg/d vs. placebo, $p=0.01$). Furthermore, there was no correlation between change in ROCK activity and change in LDL-C or change in triglycerides at any time-point during the study or with either statin dose (Figure 3).

Rho Kinase Activity and High-Sensitivity C-Reactive Protein

To examine whether ROCK inhibition contributes to the anti-inflammatory effects of statin therapy, we correlated the change in ROCK activity with change in hs-CRP levels at each time point in the study. For the study population as a whole, there was no correlation between ROCK inhibition and change in hs-CRP at any time point during the study. However, among those randomized to atorvastatin 80 mg/d, there was a modest correlation between ROCK inhibition and reduction in hs-CRP after one month of therapy ($r=0.6$, $p=0.07$) (Figure 4). Furthermore, there was no correlation between change in LDL-C and hs-CRP levels throughout the study.

DISCUSSION

The present study demonstrates for the first time in humans that statins inhibits the pro-atherogenic Rho/ROCK pathway *in vivo*, in a dose- and time-dependent manner. While both low (10 mg/d) and intensive (80 mg/d) dose atorvastatin lowered serum cholesterol, only the intensive dose achieved significant ROCK inhibition relative to placebo. This inhibition was significant within two weeks of therapy. The effect of atorvastatin on ROCK activity remained significant even after controlling for changes in LDL-C and triglycerides. Inhibition of ROCK in patients on atorvastatin 80 mg/d correlated modestly with a reduction in C-reactive protein. Accordingly, these data demonstrate that clinically approved doses of statins, and in particular intensive doses, inhibit ROCK activity in humans with atherosclerosis. Inhibition of Rho/ROCK, an anti-atherogenic mechanism elucidated in animal studies, may provide the mechanistic underpinning for the rapid clinical benefits of statin therapy that are independent of lipid-lowering and potentially related to an anti-inflammatory effect manifest by a reduction in hs-CRP.

Lipid-Independent Actions of Statins

Evidence for the lipid-independent “pleiotropic” effects of statins derives partly from statistical analyses of clinical trials demonstrating that improved outcomes correlate as much with anti-inflammatory actions of statins as with LDL-C lowering [11–13]. Furthermore, when similar reductions in LDL-C are achieved, only statins but not ezetimibe, a drug that lowers LDL-C without inhibiting HMG-CoA reductase, reverse endothelial dysfunction [14], reduce platelet reactivity [15], and inhibit pro-inflammatory cytokines [15].

Rho/Rho Kinase Pathway

In experimental studies, inhibition of Rho/ROCK has been implicated as a potential mechanism for many of the lipid-independent “pleiotropic” benefits of statins. By reducing mevalonate synthesis, statins prevent the formation of isoprenoid intermediates required for the membrane translocation and GTP binding activity of small GTPases such as Rho, Ras, and Rac [2]. Direct inhibition of Rho or ROCK is anti-atherogenic by augmenting endothelial nitric oxide synthesis [16], decreasing vascular smooth muscle cell contraction and proliferation [17], decreasing cytokine formation and inflammatory cell trafficking and proliferation [18,19], and reducing thrombogenicity of the vessel wall [20]. Statins replicate these vascular benefits of selective Rho/ROCK inhibitors *in vitro* via inhibition of Rho [19,21–23]. Yet *in vivo* in animals, doses of statins reported to inhibit Rho/ROCK are far higher than those used by clinicians raising legitimate concerns about the relevance of these experimental findings to practice [4]. The

development of a quantitative assay of ROCK activity in our laboratory has facilitated investigations of this pathway in humans [5,6].

Consequences of Rho Kinase Inhibition by Statins in Humans

In this study, atorvastatin 80 mg/d inhibited ROCK activity by approximately 49% compared to placebo. What might be the consequences of this magnitude of ROCK inhibition? Previously, we have treated atherosclerotic subjects with the direct ROCK inhibitor, fasudil, and have observed improved brachial artery endothelial function associated with 59% inhibition of ROCK activity [5]. This degree of ROCK inhibition was similar to that achieved in the present study with atorvastatin 80 mg/d. In the present study, we found a modest correlation between ROCK inhibition and an anti-inflammatory effect measured as reduction in hs-CRP ($r=0.6$, $p=0.07$). Although not conclusive, these results support the hypothesis that Rho/ROCK inhibition mediates the anti-inflammatory effects of statins and potentially explains the clinical benefit observed in the JUPITER study in patients with elevated CRP levels treated with intensive statin therapy [13].

Limitations

Due to the complexity of isolating leukocytes to measure ROCK activity at several time points per patient, this study is of necessity modest in size. Therefore, it is possible that we were inadequately powered to detect a significant difference in ROCK inhibition between low-dose atorvastatin and placebo.

The lipid-dependent and –independent actions of statins are difficult to separate. Previous studies have shown that oxidized LDL and triglycerides promote ROCK activity [6,24]. Thus, it is likely that in humans statins can affect Rho/ROCK activation by both lipid-dependent and –independent mechanisms. Yet, we have shown that high-dose atorvastatin inhibited ROCK activity even when controlling for reductions in LDL-C and triglycerides. Future studies will have to determine whether ezetimibe, a cholesterol-lowering agent that does not inhibit HMG CoA reductase, inhibits ROCK.

We have assessed ROCK activity in leukocytes as these cells are readily accessible with a blood draw. It is possible that circulating leukocytes do not accurately represent ROCK activity within the vessel wall. Yet, leukocytes are a very relevant to studies of atherosclerosis [25]. Trafficking of leukocytes to sites of atherosclerosis is controlled by their ROCK activity [18, 26,27]. Atherosclerosis is reduced in hypercholesterolemic mice whose bone marrows have been replaced with bone marrows from genetically deficient ROCK^{-/-} mice [27]. Deficiency of ROCK is associated with impaired chemotaxis and trafficking of monocyte derived macrophages to sites of injury and atherosclerosis [26,27].

Lastly, this paper did not assess the effect of statins on other members of the Ras superfamily of small GTPases, including Ras, Rac, and Cdc-42, that may also play a role in mediating the vascular abnormalities seen in patients with atherosclerosis [2]. Future analyses will assess the effect of statins on these GTPases as techniques to assess their downstream effects in humans become available.

In conclusion, this is the first study to demonstrate that in humans with atherosclerosis intensive treatment with statins rapidly inhibits ROCK activity, at least in part, independent of cholesterol reduction. The Rho/ROCK signal transduction pathway may be an important therapeutic target in patients with atherosclerosis and normal lipid levels. The clinical relevance of Rho/ROCK inhibition by statins or direct ROCK inhibitors deserves further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Effect of Statins on Rho Kinase Activity. Results are expressed as mean $\pm$ SE. ROCK activity is measured by Western blotting as staining of phosphorylated myosin binding subunit of myosin light chain phosphatase (pThr⁸⁵³-MBS) relative to staining of total myosin binding subunit of myosin light chain phosphatase (MBS). All comparisons control for baseline ROCK activity.

Figure 2.

Western Blot Analyses of Rho Kinase Activity. Shown are representative blots of ROCK activity (p-MBS/t-MBS) over time in patients randomized to placebo, atorvastatin 10 mg/d, and atorvastatin 80 mg/d, respectively. D = day, p-MBS = phosphorylated MBS, and t-MBS = total MBS.

Figure 3.

Correlation of Reduction in Rho Kinase Activity with Reduction in Lipid Parameters. For the entire cohort of 35 patients, there was no correlation between change in ROCK activity and change in LDL cholesterol (Panel A) or change in triglycerides (Panel B) at any time point during the study. The scatter-plot depicts the correlation in the change from day 28 to baseline for all parameters.

Figure 4.

Correlation of Change in Rho Kinase Activity with Change in hs-CRP. For the entire cohort of 35 patients, there was no correlation between change in ROCK activity and change in hs-CRP (Panel A). However, for patients randomized to atorvastatin 80 mg/d, there was a modest correlation between change in ROCK and hs-CRP (Panel B). The scatter-plot depicts the correlation in the change from day 28 to baseline.

TABLE 1

Baseline Characteristics

Characteristic	Placebo (n=14)	Atorvastatin 10mg (n=10)	Atorvastatin 80mg (n=11)
Age, y	60 ± 10	63 ± 8	61 ± 7
Male, n (%)	13 (93)	8 (80)	8 (73)
Caucasian, n (%)	13 (93)	9 (90)	9 (82)
Hypertension, n (%)	10 (71)	6 (60)	8 (73)
Diabetes, n (%)	3 (21)	3 (30)	3 (27)
Smoker, n (%)	5 (36)	2 (20)	0 (0)
Aspirin, n (%)	13 (93)	10 (100)	11 (100)
Beta-blocker, n (%)	11 (79)	8 (80)	9 (82)
Statin, n (%)	12 (86)	9 (90)	11 (100)
Cholesterol, mmol/L	6.1 ± 0.8	5.6 ± 0.6	6.0 ± 0.8
HDL cholesterol, mmol/L	1.1 ± 0.3	1.0 ± 0.2	1.2 ± 0.3
Triglycerides, mmol/L	2.5 [1.3, 3.0]	2.4 [1.2, 2.8]	1.5 [1.3, 2.9]
LDL cholesterol, mmol/L	3.9 ± 0.9	3.4 ± 0.8	3.9 ± 0.5
High sensitivity CRP, mg/L	1.8 [1.0, 6.4]	2.3 [0.9, 4.3]	1.2 [0.5, 5.2]
Rho Kinase Activity	1.3 ± 0.5	1.5 ± 0.7	1.1 ± 0.6

There are no statistically significant differences between the treatment groups. Data are presented as mean ± SD or median [25th, 75th percentile].