Circulation Journal
Official Journal of the Japanese Circulation Society
http://www.j-circ.or.jp

Ezetimibe Improves Endothelial Function and Inhibits Rho-Kinase Activity Associated With Inhibition of Cholesterol Absorption in Humans

Kotaro Nochioka, MD, PhD; Shin-ichi Tanaka, BSc; Masanobu Miura, MD; Do.e Zhulanqiqige, MD, PhD; Yoshihiro Fukumoto, MD, PhD; Nobuyuki Shiba, MD, PhD; Hiroaki Shimokawa, MD, PhD

Background: Ezetimibe is an inhibitor of cholesterol absorption in the intestine. We examined whether ezetimibe improves endothelial function, and if so, what mechanisms are involved.

Methods and Results: Nineteen healthy subjects (male/female 14/5; mean age, 31±3 [SD] years-old) were randomized to receive ezetimibe (10 mg/day) or pravastatin (10 mg/day) for 4 weeks in a cross-over manner with a 4-week washout interval. Lipid profiles, flow-mediated dilatation (FMD) and Rho-kinase activity of circulating leukocytes (the extent of phosphorylation of myosin binding subunit, a Rho-kinase substrate) were examined. We also evaluated remnant-like particle cholesterol (RLP-C) known as an up-regulator of Rho-kinase and cholesterol absorption status by measuring cholestanol and campesterol/lathosterol ratio (CLR) (both absorption markers). Although ezetimibe and pravastatin equally reduced low-density lipoprotein cholesterol (E: –25% vs. P: –21%), the CLR was reduced by ezetimibe but was rather increased by pravastatin (E: –41% vs. P: +37%; P<0.01). Reduction in RLP-C by ezetimibe was greater compared with pravastatin (E: –33% vs. P: –14%; P<0.05). Importantly, ezetimibe significantly improved FMD (26%, P<0.05) and reduced Rho-kinase activity (–21%, P<0.05), whereas pravastatin had no such effects. A significant correlation was noted between the reduction in cholestanol and the improvement in FMD (P<0.05).

Conclusions: These results indicate that ezetimibe improves endothelial function and inhibits Rho-kinase activity associated with the inhibition of cholesterol absorption, suggesting novel anti-atherogenic effects of the agent in humans. (*Circ J* 2012; **76:** 2023–2030)

Key Words: Ezetimibe; Flow-mediated dilatation; LDL cholesterol; Rho-kinase

Ezetimibe is a lipid-lowering agent that selectively inhibits cholesterol absorption by binding to the Niemann-Pick C1 Like 1 (NPC1L1) protein.[1] Inhibition of cholesterol absorption in the small intestine reduces the events of myocardial infarction and death due to coronary artery disease in patients with prior myocardial infarction and hyperlipidemia, suggesting the beneficial effects of ezetimibe by lipid modification.[2] However, the ENHANCE trial (2008) evoked a controversy regarding the clinical benefit of ezetimibe for preventing atherosclerosis,[3] as ezetimibe (10 mg/day) or simvastatin (80 mg/day) for 24 months failed to suppress the progression of carotid intima/medial thickness in patients with an early stage of familial hypercholesterolemia.[3] Subsequently, however, the SHARP trial (2011) demonstrated the beneficial effects of a combination therapy with ezetimibe and simvastatin on the long-term prognosis of patients with chronic kidney disease.[4]

Editorial p1836

Endothelial dysfunction is one of the initial steps of atherosclerosis.[5] Rho-kinase, a small GTP-binding protein, has been

Received March 12, 2012; revised manuscript received March 28, 2012; accepted March 29, 2012; released online April 28, 2012
Time for primary review: 14 days

Department of Cardiovascular Medicine (K.N., S.T., M.M., D.Z., Y.F., H.S.), Department of Evidence-based Cardiovascular Medicine (N.S.), Tohoku University Graduate School of Medicine, Sendai, Japan

The Guest Editor for this article was Kenichi Hirata, MD.

Current address (N.S.): Department of Cardiology, International University of Health and Welfare, 537-3 Iguch, Nasu-Shiobaru 329-2763, Japan.
Mailing address: Hiroaki Shimokawa, MD, PhD, Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan. E-mail: shimo@cardio.med.tohoku.ac.jp

ISSN-1346-9843 doi:10.1253/circj.CJ-12-0331

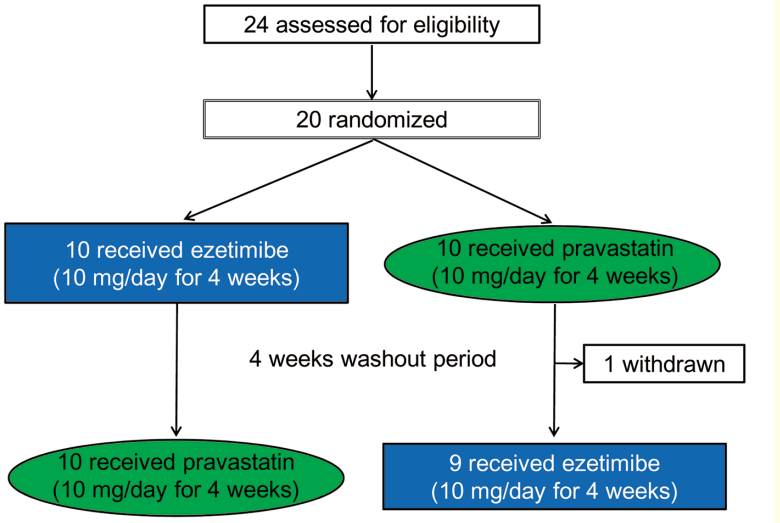

Figure 1. Diagram of the study participants.

Table 1. Baseline Characteristics of the Study Subjects

	Total (n=19)
Age (years)	30.9 (2.7)
Male (%)	14 (73.7)
Height (cm)	169.9 (7.8)
Weight (kg)	64.5 (10.8)
BMI (kg/m^2)	22.5 (2.3)
Waist circumference (cm)	79.8 (8.0)
Systolic BP (mmHg)	119.7 (11.4)
Diastolic BP (mmHg)	73.1 (7.3)
Heart rate (/min)	72.7 (11.7)
FBS (mg/dl)	92.5 (11.4)

Results are expressed as mean (SD).
BMI, body mass index; BP, blood pressure; FBS, fasting blood sugar.

implicated as one of the major causes of endothelial dysfunction and atherosclerosis.[6,7] Therefore, the inhibition of Rho-kinase might be a possible therapeutic option in preventing cardiovascular diseases.[6,7] Previous studies showed that ezetimibe inhibits intestinal cholesterol absorption and reduces the level of remnant-like particle cholesterol (RLP-C),[8] which is known to enhance Rho-kinase activity.[9] Furthermore, ezetimibe also inhibits absorption of oxidized cholesterol that is incorporated in atherogenic oxidized lipoproteins.[10]

In the present study, we thus evaluated whether ezetimibe improves endothelial function and inhibits Rho-kinase activity, in comparison with the effects of pravastatin that showed a similar low-density lipoprotein cholesterol (LDL-C) lowering effect, in humans.

Methods

Study Protocol

The study protocol was approved by the ethics committee of the Tohoku University Graduate School of Medicine and written informed consent was obtained from all subjects. The study conformed to the Declaration of Helsinki and was registered in the University Hospital Medical Information Network (UMIN000002946).

The present single center, randomized, cross-over study was performed at the Tohoku University Hospital in Sendai, Japan. A total of 20 healthy subjects were randomized to receive either ezetimibe (10 mg/day) or pravastatin (10 mg/day) for 4 weeks. After a 4-week washout period, the subjects were switched to take the alternate agent for an additional 4 weeks (Figure 1). It was confirmed that a 4-week wash out period was enough to restore the pre-treatment condition for both statin and ezetimibe.[11,12] The participants were requested not to change their life-style behavior including diet and physical exercise during the study period. The study medicines (ezetimibe and pravastatin) were purchased from Bayer-HealthCare Co (Osaka, Japan) and Daiichi-Sankyo Co (Tokyo, Japan), respectively. A venous blood sample was drawn and flow-mediated dilatation (FMD) was measured at 0, 4, 8, and 12 weeks after the randomization. Primary outcomes were the changes in FMD and Rho-kinase activity caused by each intervention and the differences between the 2 treatments. We also examined the relationships between the outcomes and the following parameters: serum levels of total cholesterol (TC), LDL-C, high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), and RLP-C. We also measured cholesterol absorption markers (cholestanol, campesterol and sitosterol), a cholesterol synthesis marker (lathosterol) and the campesterol/lathosterol ratio (CLR), high-sensitivity C-reactive protein (hsCRP) and the homeostasis model assessment of insulin resistance (HOMA-IR).

Study Subjects

Healthy individuals aged ≥20 years old were enrolled in the present study. Individuals who had at least 1 of the following conditions were excluded: (1) a history of receiving lipid-lowering medicines; (2) active diseases; (3) taking any kind of medicines or supplements; (4) former or current smokers; (5) liver and/or renal dysfunction; and (6) eating disorder.

All participants were recruited by posted notices on bulletin boards in the Tohoku University from December 2009 to August 2010. We screened 24 subjects and excluded 4 subjects; 2 subjects for liver dysfunction and dyslipidemia, 1 for a high hsCRP level due to a sinus infection, and 1 for being a current smoker. There were no significant side-effects related to ezetimibe or pravastatin, but 1 subject showed a low LDL-C level

Table 2. Effects of Ezetimibe and Pravastatin on Lipid Profile, Cholesterol Absorption/Synthesis Markers, hsCRP and HOMA-IR

	Ezetimibe (10 mg/day)				Pravastatin (10 mg/day)			
	Baseline	After 4 weeks	%Change	P value	Baseline	After 4 weeks	%Change	P value
Fasting lipids (mg/dl)								
Total cholesterol	199 (31)	164 (24)	−17.2	<0.001	201 (37)	177 (26)	−12.2	<0.001
LDL-C	121 (24)	90 (18)	−25.4	<0.001	120 (27)	95 (19)	−20.8	<0.001
HDL-C	60 (15)	57 (12)	−5.1	0.04	61 (16)	63 (15)	3.0	0.15
TG	85 (34)	83 (22)	−3.0	0.72	84 (25)	76 (30)	−9.0	0.23
Cholesterol absorption marker (μg/ml)								
Cholestanol	3.0 (0.8)	2.4 (0.5)	−37.5	0.001	3.1 (0.7)	2.7 (0.8)	−14.0	0.001
Campesterol	5.6 (2.8)	3.5 (2.3)	−37.4	<0.001	5.4 (3.0)	5.3 (3.4)	−0.03	0.63
Sitosterol	3.5 (2.0)	2.3 (1.5)	−35.0	<0.001	3.6 (2.0)	3.3 (1.9)	−10.0	0.16
Cholesterol synthesis marker (μg/ml)								
Lathosterol	3.6 (1.3)	4.3 (1.7)	20.2	<0.001	3.5 (1.3)	2.4 (0.9)	−33.5	<0.001
RLP-C (mg/dl)	3.9 (1.5)	2.6 (1.0)	−33.2	0.002	3.6 (1.1)	3.1 (0.8)	−14.2	0.07
hsCRP (mg/dl)	0.4 (0.7)	0.3 (0.3)	−21.0	0.58	0.5 (0.8)	0.2 (0.1)	−58.8	0.13
HOMA-IR	2.0 (0.8)	2.1 (1.1)	8.6	0.30	1.9 (0.8)	1.8 (0.9)	−6.3	0.10

Results are expressed as mean (SD).
hsCRP, high-sensitivity C-reactive protein; HOMA-IR, homeostasis model assessment-insulin resistance; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride ; RLP-C, remnant-like particles cholesterol.

(56 mg/dl) at 4 weeks after the treatment with pravastatin and was excluded from the study. Thus, the final analyses were conducted for the 19 subjects who completed the study protocol (Figure 1). There were no significant changes in bodyweight, blood pressure or heart rate after each treatment during the study period.

Biochemical Analysis

Serum levels of TC, LDL-C, HDL-C and TG were determined by using enzymatic methods at the central laboratory of the Tohoku University Hospital. Serum was obtained by centrifugation at 1,400 g for 30 min at 4°C and the aliquots were carried immediately to the Special Reference Laboratory Inc., Tokyo, in containers at –80°C for the assays of RLP-C, cholestanol, campesterol, sitosterol, and lathosterol. RLP-C was measured by using the immunoadsorption method (RLP MIXER J-100, Otsuka Electronics, Japan) and cholestanol, campesterol, sitosterol and lathosterol were measured by using a gas chromatograph (GC-17A, Shimadzu, Kyoto, Japan). The HOMA-IR was calculated by using the international formula: fasting glucose (mmol/L)×fasting insulin (mU/L)/22.5.[13]

Rho-Kinase Activity Assay

Rho-kinase activity was evaluated by Western blot analysis using circulating leukocytes.[12,14] The activity was quantified by calculating the ratio of the levels of the phosphorylated myosin-binding subunit (p-MBS) and the total MBS (t-MBS), substrates of Rho-kinase.[12,14] Circulating leukocytes were isolated from blood samples as previously described.[12,14] Cell extracts were loaded on 7.5% SDS-PAGE gel and immunoblotted for the detection of Rho-kinase activity, using rabbit polyclonal anti-phospho-myosin phosphatase 1 (MYPT-1) (Thr696) (Upstate) for p-MBS and mouse monoclonal anti-MYPT1 (BD Biosciences) for t-MBS.[12,14] All Western blot analyses were performed by 1 technician in a blinded manner. Because the bands of p-MBS were not detected in 2 samples, analyses were performed for the remaining 17 subjects.

Measurement of FMD

Examinations of FMD were performed using UNEXEF18G (UNEX, Aichi, Japan)[15] by 1 trained ultrasonographer in a blinded manner. The subjects were prohibited from diet, exercise, or caffeine/alcohol intake at least 9 h before the examination. The procedure was performed in a quiet, dark, temperature-controlled examination room according to the guidelines of the International Brachial Artery Reactivity Task Force.[16] Briefly, the right brachial diameter was measured at rest after 10 min of bed rest and during reactive hyperemia above the antecubital fossa with a 10-MHz linear array transducer.[16] Reactive hyperemia was induced by inflating a sphygmomanometiric cuff, which is equipped in UNEXEF18G, on the proximal portion of the arm at least 50 mmHg above the systolic blood pressure to occlude arterial inflow for 5 min. End-diastolic images of the brachial artery were obtained and diameters were measured with R-wave synchronized automated edge-detection software automatically.[15] The values of FMD were calculated with the following formula:

(diameter at peak hyperemia − diameter at rest) / (diameter at rest) × 100.[16]

Statistical Analysis

All continuous variables are reported as mean ± standard deviation (SD) unless otherwise stated. We assessed the differences in measured parameters before and after the treatment within each arm using the paired t-test. We also compared the changes caused by each intervention between the 2 arms using an unpaired t-test. The Spearman rank correlation test was used to evaluate the statistical significance in the correlation between the changes in FMD or Rho-kinase activity and those in LDL-C, RLP-C or cholestanol after each treatment. We performed all analyses using IBM SPSS Statistics 18.0 (New York, NY, USA). A 2-sided P value of <0.05 was considered to be statistically significant.

Results

Baseline Characteristics and Changes in Lipid Profiles, hsCRP, and HOMA-IR

Table 1 shows the baseline characteristics of the study sub-

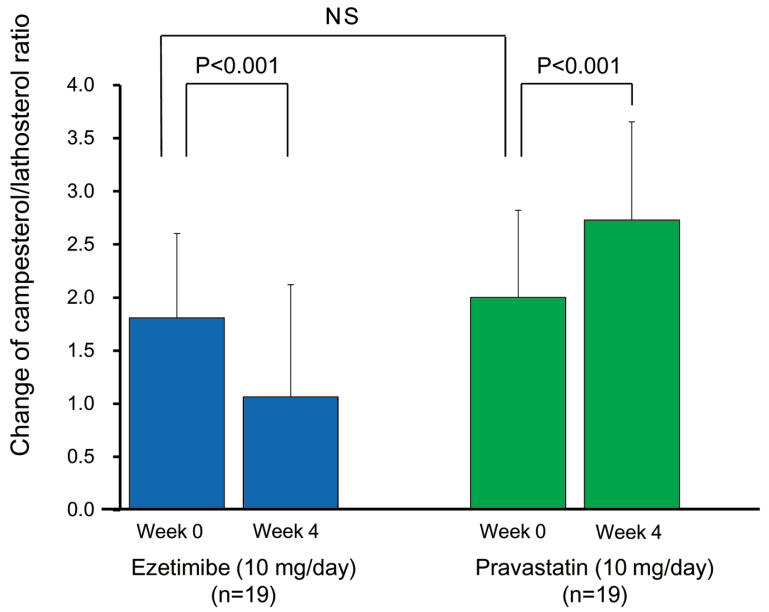

Figure 2. Changes of the campesterol/lathosterol ratio before and after a 4-week treatment with ezetimibe (10 mg/day) or pravastatin (10 mg/day). A higher level reflects increased cholesterol absorption.

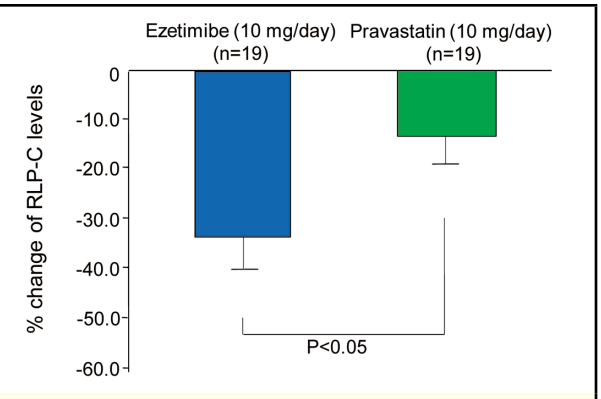

Figure 3. Changes of remnant-like lipoprotein cholesterol (RLP-C) before and after a 4-week treatment with ezetimibe (10 mg/day) or pravastatin (10 mg/day).

jects. The mean age was 30.9±2.7 years and males accounted for 73.7%. No participants had a body mass index (BMI) ≥25.0 kg/m^2. Table 2 shows the effects of ezetimibe (10 mg/day) (E) or pravastation (10 mg/day) (P) on lipid profiles after the 4-week treatment. There was no significant difference in the lipid profiles at baseline between the ezetimibe and pravastatin treatment arms. Both ezetimibe and pravastatin caused a comparable extent of reduction in LDL-C after each treatment (E: –25.4% vs. P: –20.8%). A mild but significant reduction in HDL-C was observed in the ezetimibe treatment (E: –5.1% vs. P: +3.0%), whereas there was no significant reduction in TG in either treatment (E: –3.0% vs. P: –9.0%). Ezetimibe reduced RLP-C to a greater extent compared with pravastatin (E: –33.2% vs. P: –14.2%; P<0.05). Both ezetimibe and pravastatin tended to decrease hs-CRP levels, but the changes did not reach statistical significance in either group. HOMA-IR also did not show significant changes after the treatment, suggesting that neither ezetimibe nor pravastatin altered insulin sensitivity during the 4-week study period (Table 2).

Changes in Cholesterol Absorption and Synthesis

The changes in cholesterol absorption/synthesis markers are shown in Table 2. Ezetimibe decreased cholesterol absorption markers, such as cholestanol, campesterol and sitosterol levels. Lathosterol, a cholesterol synthesis marker, was increased by ezetimibe, but decreased by pravastatin. Ezetimibe significantly decreased CLR (–41.1%, P<0.001), whereas pravastatin significantly increased it (+36.5%, P<0.001; Figure 2).

Changes in RLP-C

Figure 3 shows the effects of ezetimibe and pravastatin on RLP-C levels. Ezetimibe significantly decreased the RLP-C level (–1.3±0.5 mg/dl, P<0.01), whereas pravastatin was without the effect (–0.5±0.3 mg/dl, NS), and the extent of the reduction was significantly greater with ezetimibe treatment (E: –33.2% vs. P: –14.2%; P<0.05; Figure 3).

Effect of Ezetimibe and Pravastatin on Rho-Kinase Activity

Figure 4 shows the changes in Rho-kinase activity of circulating leukocytes after each treatment. Baseline Rho-kinase activity did not differ significantly between the 2 treatment arms (E: 1.03±0.44 vs. P: 0.97±0.28). Importantly, after the 4-week treatment, ezetimibe significantly inhibited the Rho-kinase activity (–21%, P<0.05), whereas pravastatin was without the effect (+6.2%, NS).

Effect of Ezetimibe and Pravastatin on FMD

Figure 5 shows the effect of ezetimibe and pravastatin on FMD. No significant difference in baseline FMD was observed between the 2 treatment arms (E: 6.8±2.6% vs. P: 7.5±2.1%). FMD was significantly improved by ezetimibe (+25.5%, P<0.05), whereas pravastatin was without the effect (+4.1%, NS).

Correlations Between LDL-C, RLP-C, Cholestanol, Rho-Kinase Activity, and FMD

There were no significant correlations between the changes in Rho-kinase activity and those in LDL-C, RLP-C, or cholestanol level after the treatments (Figures 6A–C). Similarly, no

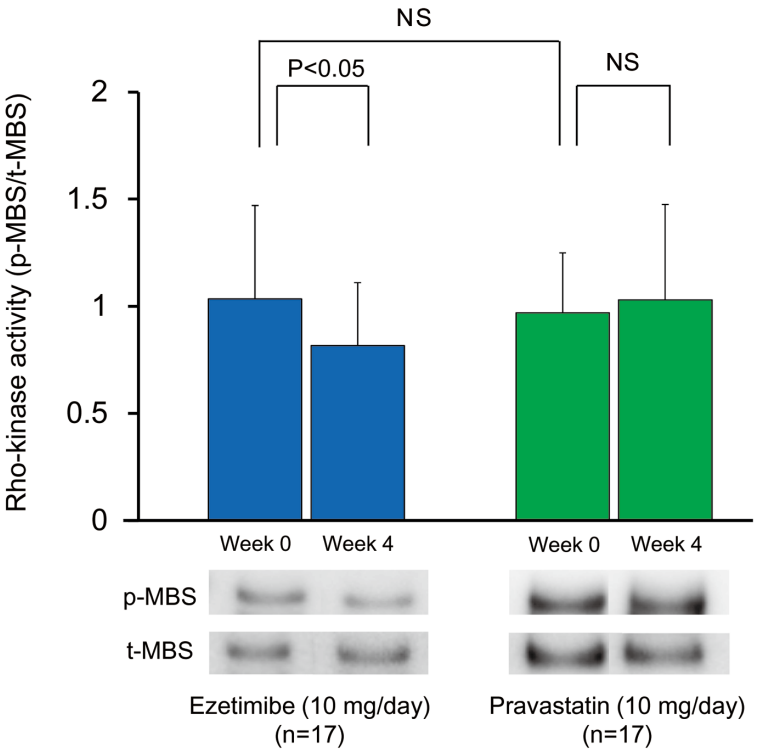

Figure 4. Rho-kinase activity of circulating leukocytes before and after a 4-week treatment with ezetimibe (10 mg/day) or pravastatin (10 mg/day). p-MBS, phosphorylated myosin-binding subunit; t-MBS, total myosin-binding subunit.

significant correlation was noted between the changes in FMD and those in LDL-C or RLP-C (Figures 6D–E). Importantly, however, there was a significant correlation between the changes in FMD and those in cholestanol levels (Figure 6F). No significant correlation was noted between the changes in FMD and those in Rho-kinase activity (Figure 6G).

Discussion

In the present study, we were able to demonstrate that the treatment with ezetimibe (10 mg/day for 4 weeks) significantly inhibited the Rho-kinase activity of circulating leukocytes in healthy subjects and that the ezetimibe treatment significantly enhanced FMD, where the changes in FMD were significantly associated with those in the serum cholestanol level, a cholesterol absorption marker. These results suggest that ezetimibe has novel anti-atherogenic effects associated with its inhibition of cholesterol absorption, which could explain, at least in part, the beneficial effects of the agent in the SHARP trial.[4]

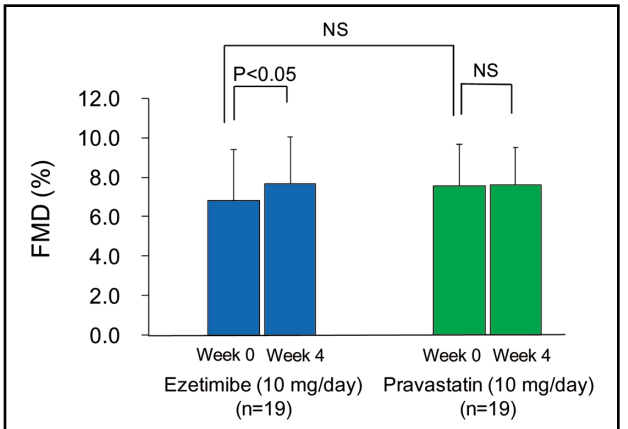

Figure 5. Changes in flow-mediated dilatation (FMD) before and after a 4-week treatment with ezetimibe (10 mg/day) or pravastatin (10 mg/day).

Effects of Ezetimibe on FMD

In the present study, ezetimibe significantly improved FMD in healthy volunteers. Because FMD has been demonstrated as an established surrogate marker of endothelial function,[16–18] the beneficial effect of ezetimibe on endothelial function might exert anti-atherogenic effects in humans. In contrast, pravastatin did not significantly improve FMD, although both treatments caused a comparable extent of LDL-C reduction in the present study. We consider that the difference in the effect on endothelium function between ezetimibe and pravastatin was mainly caused by the difference in the alteration in cholesterol absorption/synthesis status as follows.

In the present study, we evaluated the cholesterol absorption/synthesis status by examining the surrogate markers, cholestanol and plant sterols (campesterol, lathosterol and sitosterol) levels, as it is difficult to directly examine the cholesterol absorption/synthesis status in humans. Interestingly, we noted the significant correlation between the change in FMD and that in cholestanol level, a cholesterol absorption marker. Cholestanol is the 5α-saturated derivative of cholesterol and serum cholestanol levels usually remain constant.[19] The cholestanol production is generally at a low level (<20 mg/day),[19] and serum cholestanol is absorbed from the gut in humans.[19,20] It was reported that in middle-aged normal males, high levels of serum cholestanol reflect the status with high cholesterol ab-

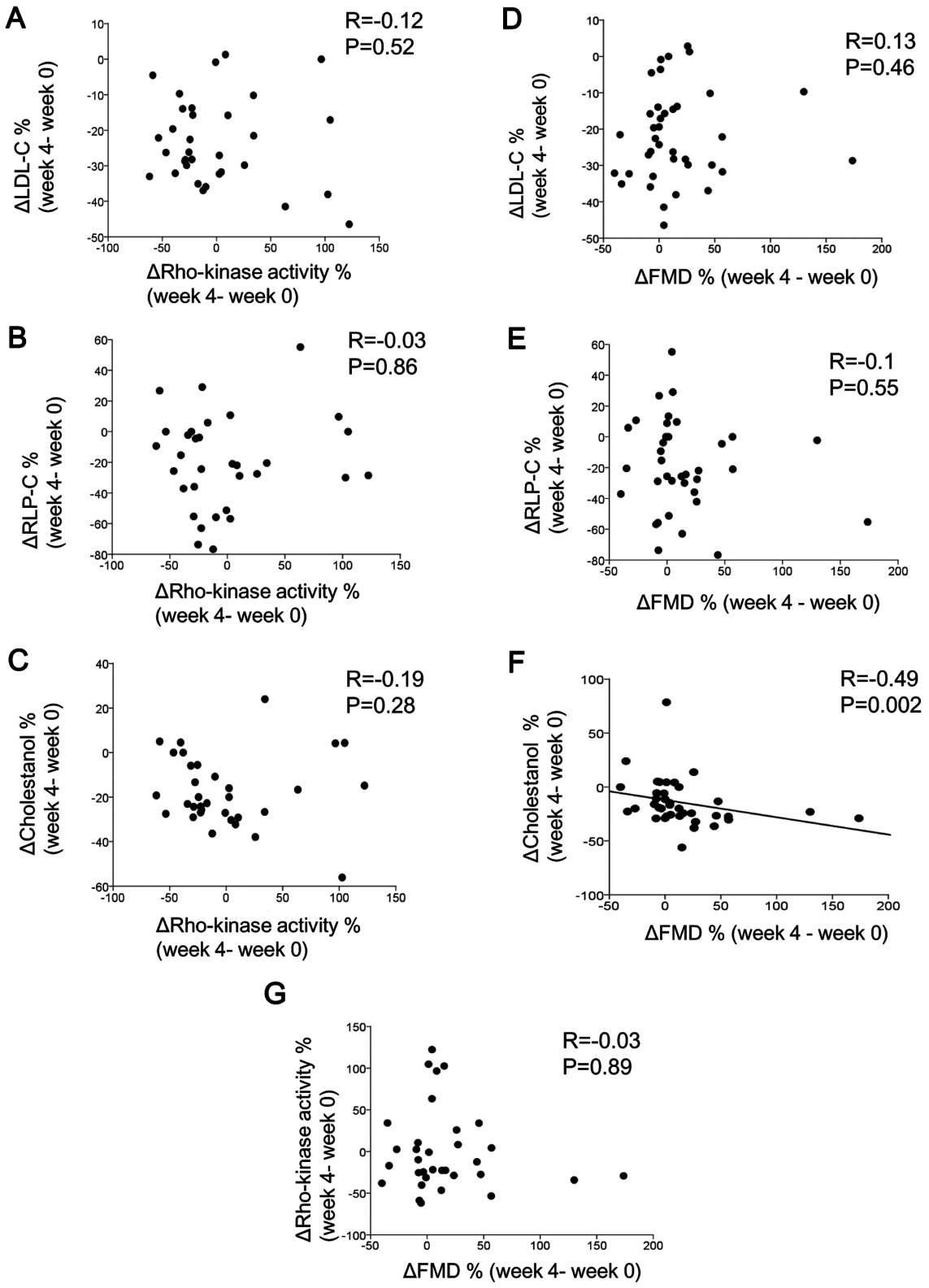

Figure 6. Correlations between changes in Rho-kinase activity of circulating leukocytes and those in low-density lipoprotein cholesterol (LDL-C) (**A**), remnant-like lipoprotein cholesterol (RLP-C) (**B**), cholestanol (**C**), and correlations between changes in flow-mediated dilatation (FMD) and those in LDL-C (**D**), RLP-C (**E**), cholestanol (**F**), and Rho-kinase activity (**G**). A linear regression line was plotted to visualize the statically significant relationship (**F**).

sorption and low cholesterol synthesis.[21] Thus, the serum cholestanol level has been used as a surrogate marker of cholesterol absorption. Indeed, it was previously reported that the baseline serum cholestanol level is the predictor of recurrent coronary events in patients with coronary artery disease.[22]

In the present study, CLR as a marker of cholesterol absorption/synthesis status,[21] was also decreased by ezetimibe but was rather significantly increased by pravastatin. It was previously shown that ezetimibe inhibits the absorption of dietary oxidized cholesterol and reduces its incorporation into lipoproteins.[10] Although the direct assay of atherogenic cholesterols, such as circulating oxidized cholesterol, was not performed in the present study, it is highly possible that ezetimibe improved endothelial function through inhibition of atherogenic cholesterol absorption in the intestine.

The present study results are in contrast to those reported by Landmesser et al, as they reported that simvastatin (10 mg/day for 4 weeks) significantly improved FMD, whereas ezetimibe was without the effects in chronic heart failure patients.[23] It should be noted that they enrolled patients with established heart failure and lower LDL-C levels (simvastatin group, 106± 8 mg/dl; ezetimibe group, 109±6 mg/dl), whereas we enrolled normal volunteers in the present study. Furthermore, simvastatin is a lipophilic statin that has a more potent LDL-C lowering effect compared with a hydrophilic statin, pravastatin.

Possible Mechanisms of Rho-Kinase Inhibition by Ezetimibe in Humans

In the present study, ezetimibe significantly inhibited the Rho-kinase activity of circulating leukocytes in healthy volunteers, independent of LDL-C. It was reported that the Rho-kinase of circulating leukocytes is the primary determinant of leukocyte recruitment to the vessel wall and is the critical mediator of neointimal proliferation following vascular injury.[24] Thus, the inhibitory effect of ezetimibe on Rho-kinase activity might be one of the important mechanisms of its anti-atherogenic effects.

We consider that the possible mechanism of the inhibitory effect of ezetimibe on Rho-kinase activity involves, at least in part, the reductions in serum atherogenic cholesterols. Indeed, in the present study, the extent of the reduction in serum RLP-C level was significantly greater by ezetimibe compared with pravastatin. We have previously demonstrated that RLP-C enhances Rho-kinase activity in the coronary artery both in vitro and in vivo.[9] Thus, the reduction in RLP-C by ezetimibe might also exert a beneficial effect on endothelium function. In the present study, ezetimibe significantly reduced serum levels of circulating plant sterols (campesterol and sitosterol), biomarkers of cholesterol absorption, and increased those of lathosterol, a marker of cholesterol synthesis. It also was reported that increased serum levels of cholesterol absorption were significantly associated with cardiovascular diseases in the Framingham Offspring study.[25]

In the present study, however, no significant correlation was noted between the changes in Rho-kinase activity of circulating leukocytes and those in lipid profiles, but a significant correlation was noted between improvement of endothelial function and the extent of inhibition of cholesterol absorption. These findings could be explained by the fact that the baseline levels of RLP-C and plant sterols in the present normal volunteers were much lower than in patients with atherosclerosis,[9,26,27] which could have masked the association between atherogenic cholesterols and Rho-kinase activity. Furthermore, Rho-kinase activity could be upregulated not only by RLP-C or plant sterols but also by other mechanisms (eg, angiotensin-II and IL-1β).[28,29]

The present study findings might be in contrast to those reported by Liu et al, where they found in patients with dyslipidemia that simvastatin (40 mg/day for 4 weeks) significantly improved endothelial function and inhibited the Rho-kinase activity of circulating leukocytes, whereas ezetimibe (10 mg/day for 4 weeks) was without the effect.[30] Although the reason for the discrepancy between the present study and that by Liu et al remain to be elucidated, it is conceivable that the different dose of statins (pravastatin 10 mg/day vs. simvastatin 40 mg/day), the different study subjects (normal volunteers vs. patients with dyslipidemia) and the carry-over effects of statins in patients with cardiovascular diseases[31] might be involved.

Study Limitations

Several limitations should be mentioned for the present study. First, in the present study, we only enrolled normal healthy volunteers and thus the present study findings remain to be confirmed in patients with dyslipidemia and/or cardiovascular diseases. Furthermore, we cannot exclude the possibility that non-healthy subjects were included in the present study population. Second, we only examined the 4-week treatment with ezetimibe or pravastatin and the longer period of treatment would have provided more definite effects of the agents, especially on lipid profiles and endothelial function. Finally, we did not directly measure serum levels of atherogenic cholesterols, and the direct measurement of those profiles would reveal more detailed aspects of the anti-atherogenic effects of ezetimibe.

Conclusions

In the present study, we were able to demonstrate that ezetimibe improves endothelial function and inhibits Rho-kinase activity in humans, suggesting novel anti-atherogenic effects of the agent. The present study findings also suggest that the beneficial effect of ezetimibe is mediated by its inhibitory effects on absorption of atherogenic cholesterol.

Acknowledgments

The authors thank T. Suzuki for excellent echo assessment and F. Natsui for Western blot assays. This work was supported, in part, by the Grant-in-Aid for Scientific Research on Innovative Areas (Signaling Functions of Reactive Oxygen Species), the Grant-in-Aid for Tohoku University Global COE for Conquest of Signal Transduction Diseases with Network Medicine, and the Grants-in-Aid for Scientific Research, all of which are from the Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan.

Disclosures

Conflict of interest: None declared.

References

1. Altmann SW, Davis HR Jr, Zhu LJ, Yao X, Hoos LM, Tetzloff G, et al. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science* 2004; **303:** 1201–1204.
2. Buchwald H, Varco RL, Matts JP, Long JM, Fitch LL, Campbell GS, et al. Effect of partial ileal bypass surgery on mortality and morbidity from coronary heart disease in patients with hypercholesterolemia: Report of the Program on the Surgical Control of the Hyperlipidemias (POSCH). *N Engl J Med* 1990; **323:** 946–955.
3. Kastelein JJ, Akdim F, Stroes ES, Zwinderman AH, Bots ML, Stalenhoef AF, et al. Simvastatin with or without ezetimibe in familial hypercholesterolemia. *N Engl J Med* 2008; **358:** 1431–1443.
4. Baigent C, Landray MJ, Emberson J, Wheeler DC, Tomson C, Wanner C, et al. The effects of lowering LDL cholesterol with simvastatin plus ezetimibe in patients with chronic kidney disease (study of heart

and renal protection): A randomized placebo-controlled trial. *Lancet* 2011; **377:** 2181–2192.
5. Ross R, Glomset JA. Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesion of atherosclerosis. *Science* 1973; **180:** 1332–1339.
6. Shimokawa H, Takeshita A. Rho-kinase is an important therapeutic target in cardiovascular medicine. *Arterioscler Thromb Vasc Biol* 2005; **25:** 1767–1775.
7. Shimokawa H, Rashid M. Development of Rho-kinase inhibitors for cardiovascular medicine. *Trends Pharmacol Sci* 2007; **28:** 296–302.
8. Nozue T, Michishita I, Mizuguchi I. Effects of ezetimibe on remnant-like particle cholesterol, lipoprotein (a), and oxidized low-density lipoprotein in patients with dyslipidemia. *J Atheroscler Thromb* 2010; **17:** 37–44.
9. Oi K, Shimokawa H, Hiroki J, Uwatoku T, Abe K, Matsumoto Y, et al. Remnant lipoproteins from patients with sudden cardiac death enhance coronary vasospastic activity through upregulation of Rho-kinase. *Artherioscler Thromb Vasc Biol* 2004; **25:** 918–922.
10. Staprans L, Pan XM, Rapp JH, Moser AH, Feingold KR. Ezetimibe inhibits the incorporation of dietary oxidized cholesterol into lipoproteins. *J Lipid Res* 2006; **47:** 2575–2580.
11. Clarenbach JJ, Reber M, Lütjohann D, von Bergmann K, Sudhop T. The lipid-lowering effect of ezetimibe in pure vegetarians. *J Lipid Res* 2006; **47:** 2820–2824.
12. Rashid M, Tawara S, Fukumoto Y, Seto M, Yano K, Shimokawa H. Importance of Rac1 signaling pathway inhibition in the pleiotropic effects of HMG-CoA reductase inhibitors. *Circ J* 2009; **73:** 361–370.
13. Haffner SM, Miettinen H, Stern MP. The homeostasis model the San Antonio Heart Study. *Diabetes Care* 1997; **20:** 1087–1092.
14. Do.e Z, Fukumoto Y, Takaki A, Tawara S, Ohasi J, Nakano M, et al. Evidence for Rho-kinase activation in patients with pulmonary arterial hypertension. *Circ J* 2009; **73:** 1731–1739.
15. Watanabe K, Oba K, Suzuki T, Ouchi M, Suzuki K, Futami-Suda S, et al. Oral glucose loading attenuates endothelial function in normal individual. *Eur J Clin Invest* 2011; **41:** 465–473.
16. Corretti MC, Anderson TJ, Benjamin EJ, Celermajier D, Charbonnearu F, Creager MA, et al; International Brachial Artery Reactivity Task Force. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: A report of the international brachial artery reactivity task force. *J Am Coll Cardiol* 2002; **39:** 257–265.
17. Gokce N, Keaney JF Jr, Hunter LM, Watkins MT, Nedeljkovic ZS, Menzoian JO, et al. Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events with peripheral vascular disease. *J Am Coll Cardiol* 2003; **41:** 1769–1775.
18. Tomiyama H, Yamashina A. Non-invasive function tests: Their pathophysiological background and clinical application. *Circ J* 2010; **74:** 24–33.
19. Salen G, Grundy SM. The metabolism of cholestanol, cholesterol, and bile acids in cerebrotendinous xanthomatosis. *J Clin Invest* 1973; **52:** 2822–2835.
20. Bhattacharyya AK, Connor WE. Familial diseases with storage of sterols other than cholesterol. Cerebrotendinous xanthomatosis, and beta-sitosterolemia and xanthomatosisi. *In*: Stanbury JB, Wyngaarden JB, Fredrickson DS, editors. The Metabolic Basis of Inherited Disease. New York: McGraw-Hill, 1978; 656–669.
21. Miettinen TA, Tilvis RS, Kesäniemi YA. Serum cholestanol and plant sterol levels in relation to cholesterol metabolism in middle-aged men. *Metabolism* 1989; **38:** 136–140.
22. Miettinnen TA, Gylling H, Standberg T, Sarna S. Baseline serum chorestanol as predictor of recurrent coronary events in subgroup of Scandinavian simvastatin survival study. *BMJ* 1998; **316:** 1127–1130.
23. Landmesser U, Bahlmann F, Mueller M, Spiekermann S, Kirchhoff N, Schulz S, et al. Simvastatin versus ezetimibe: Pleiotropic and lipid-lowering effects on endothelial function in humans. *Circulation* 2005; **111:** 2356–2363.
24. Noma K, Rikitake Y, Oyama N, Yan G, Alcaide P, Liu PY, et al. ROCK1 mediates leukocyte recruitment and neointima formation following vascular injury. *J Clin Invest* 2008; **118:** 1632–1644.
25. Matthan NR, Pencina M, LaRocque JM, Jacques PF, D'Aqostino RB, Schaefer EJ, et al. Alternations in cholesterol absorption/synthesis makers characterize Framingham offspring study participants with CHD. *J Lipid Res* 2009; **50:** 1927–1935.
26. Sano K, Nakamura T, Hirano M, Kitta Y, Kobayashi T, Fujioka D, et al. Comparative study of bezafibrate and pravastatin in patients with coronary artery disease and high levels of remnant lipoprotein. *Circ J* 2010; **74:** 1644–1650.
27. Glueck CJ, Speris J, Tracy T, Streicher P, IIIiq E, Vandegriff J. Relationships of serum plant sterols (phytosterols) and cholesterol in 595 hypercholesterolemic subjects, and familial aggregation of phytosterols, cholesterol, and premature coronary heart disease in hyperphytosterolemic probands and their first-degree relatives. *Metabolism* 1991; **40:** 842–848.
28. Higashi M, Shimokawa H, Hattori T, Hiroki J, Mukai Y, Morikawa K, et al. Long-term inhibition of Rho-kinase suppresses angiotensin II-induced cardiovascular hypertrophy in rats in vivo: Effects on endothelial NAD(P)H oxidase system. *Circ Res* 2003; **93:** 767–775.
29. Hiroki J, Shimokawa H, Higashi M, Morikawa K, Kandabashi T, Kawamura N, et al. Inflammatory stimuli upregulate Rho-kinase in human coronary vascular smooth muscle cells. *J Mol Cell Cardiol* 2004; **37:** 537–546.
30. Liu PY, Liu YW, Lin LJ, Chen JH, Liao JK. Evidence for statin pleiotrophy in humans: Differential effects of statins and ezetimibe on Rho-associated coiled-coil containing protein kinase activity, endothelial function, and inflammation. *Circulation* 2009; **119:** 131–138.
31. Sever PS, Poulter NR, Dahlof B, Wedel H, Beevers G, Caulfield M, et al. The Anglo-Scandinavian cardiac outcomes trial lipid lowering arm: Extended observations 2 years after trial closure. *Eur Heart J* 2008; **29:** 499–508.