Remnant Lipoproteins From Patients with Sudden Cardiac Death Enhance Coronary Vasospastic Activity Through Upregulation of Rho-kinase

Keiji Oi, Hiroaki Shimokawa, Junko Hiroki, Toyokazu Uwatoku, Kohtaro Abe, Yasuharu Matsumoto, Yasuhiro Nakajima, Katsuyuki Nakajima, Sanae Takeichi, Akira Takeshita

Objective—Sudden cardiac death (SCD) still remains a serious problem. We have previously shown that remnant-like particles (RLP) are the major risk factor for SCD and that Rho-kinase plays a central role in the molecular mechanism of coronary vasospasm. In this study, we examined whether RLP from patients with SCD upregulate Rho-kinase associated with an enhanced coronary vasospastic activity.

Methods and Results—We isolated RLP and non-RLP in very-low-density lipoprotein (VLDL) fraction from SCD patients without coronary stenosis. We performed in vivo study in which we treated the coronary artery with RLP or non-RLP fraction at the adventitia in pigs. After 1 week, intracoronary serotonin caused marked coronary hyperconstriction at the segment treated with RLP fraction but not with non-RLP fraction \((P<0.001, n=6)\), and hydroxyfasudil, a selective Rho-kinase inhibitor, dose-dependently inhibited the spasm in vivo. In organ chamber experiments, serotonin caused hypercontraction of vascular smooth muscle cells (VSMC) from RLP-treated segment, which was significantly inhibited by hydroxyfasudil \((P<0.001, n=6)\). In cultured human coronary VSMC, the treatment with RLP significantly enhanced the expression and activity of Rho-kinase \((P<0.05, n=6)\).

Conclusions—These results indicate that RLP from SCD patients upregulate Rho-kinase in coronary VSMC and markedly enhance coronary vasospastic activity. (Arterioscler Thromb Vasc Biol. 2004;24:1-6.)

Key Words: sudden cardiac death  lipoproteins  coronary vasospasm

Although a significant progress has been made in the treatment of ischemic heart disease, sudden cardiac death (SCD) still remains a serious problem. Furthermore, there are many cases of out-hospital SCD without significant coronary stenosis. Although coronary vasospasm has been postulated as one of the major causes of SCD,1,2 the triggers for the spasm still remain to be elucidated.

Recently, a new method has been developed to isolate remnant-like particles (RLP), a major component of remnant lipoproteins mainly detected in very-low-density lipoprotein (VLDL) fraction, by using immunoadfinity gels coupled to anti-apoA-1 and anti-apoB-100 antibodies. With this method, it has been shown that plasma RLP level is an independent risk factor for coronary artery disease (CAD).3,4 Furthermore, we have demonstrated that RLP are associated with severity of coronary atherosclerosis and also are the most significant risk factor for SCD without coronary stenosis in our postmortem studies.5,6 RLP also are a major risk factor for myocardial infarction in patients with vasospastic angina with nearly normal coronary artery.7,8 These findings suggest that RLP are substantially involved in the fatal events, such as coronary vasospasm and SCD.

Recent studies have shown the important role of small GTPase Rho and its effector, Rho-kinase, in Ca-independent regulation of smooth muscle contraction.9,10 The Rho/Rho-kinase pathway modulates the phosphorylation level of myosin light chain (MLC) through inhibition of myosin phosphatase and contributes to the agonist-induced Ca-sensitization in smooth muscle contraction.10 We have demonstrated that increased Rho-kinase activity in vascular smooth muscle cells (VSMC) plays a central role in the pathogenesis of coronary vasospasm in both animal models11,12 and patients with vasospastic angina. Thus, in this study, we examined whether RLP from patients with SCD without significant coronary stenosis upregulate Rho-kinase, resulting in enhanced coronary vasospastic activity in pigs.
Serum Lipid Profiles in the SCD Patients and Controls.

<table>
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<tr>
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<th>n</th>
<th>TC</th>
<th>TG</th>
<th>LDL</th>
<th>HDL</th>
<th>RLP-C</th>
<th>RLP-TG</th>
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<tbody>
<tr>
<td>SCD patients</td>
<td>15</td>
<td>223±15</td>
<td>211±27</td>
<td>139±9</td>
<td>40±4</td>
<td>23±3†</td>
<td>131±20†</td>
</tr>
<tr>
<td>Controls</td>
<td>5</td>
<td>177±27</td>
<td>148±30</td>
<td>159±6</td>
<td>41±7</td>
<td>13±4</td>
<td>29±8</td>
</tr>
</tbody>
</table>

TC indicates total cholesterol; TG, triglyceride; LDL, low-density lipoproteins; HDL, high-density lipoproteins; RLP-C and RLP-TG, remnant-like particles cholesterol and triglyceride, respectively. Results are expressed as mean±SEM (mg/dL).

*P<0.05, †P<0.01 vs controls.

Methods

All procedures were approved by the Institutional Animal Care and Use Committee and were conducted in conformity with the institutional guidelines of the Kyushu University.

Animal Preparation

Twenty male domestic pigs (2- to 4-month-old and weighing 25 to 30 kg) were used. We anesthetized the animals with ketamine hydrochloride (15 mg/kg, intramuscular) and sodium pentobarbital (25 mg/kg, intravenous), ventilated with room air while oxygen was supplemented via a positive pressure respirator (Shinano, Tokyo, Japan). Under aseptic conditions, the proximal segments of the left anterior and the circumflex coronary arteries were carefully dissected and were gently wrapped with a cotton mesh after absorbing 0.1 mL of RLP or non-RLP in VLDL fraction in a randomized manner.14

Preparation of RLP and Non-RLP Fraction

The plasma was obtained from 15 SCD patients without significant coronary stenosis (13 males and 2 female, age 19 to 62 years) and 5 healthy volunteers (5 males, age 49 to 76 years). Among the 15 SCD patients, 14 had no obvious diseases and had not taken any medications before death. The remaining 1 patient was hypertensive but was without any antihypertensive medication. An informed consent was obtained from all the family of the patients. The mean elapsing time from SCD to the plasma collection was 8.5 hours. We have previously confirmed that there is no postmortem qualitative change in the plasma RLP within 12 hours after death.2 and RLP and non-RLP in VLDL fraction were isolated by the method by Nakajima et al with immunoaffinity chromatography using anti-apoA-I and anti-apoB-100 monoclonal antibodies. Briefly, VLDL (d < 1.006 kg/L) was isolated by density gradient ultracentrifugation from plasma samples. RLP (unbound fraction) were then isolated from VLDL fraction by immunoaffinity mixed gels containing 2 clones of monoclonal antibodies: Non-RLPs (bound fraction) were isolated from the gel with 5 mL of 3 mol/L sodium thiocyanate solution containing 0.1% bovine serum albumin (pH 7.4). RLP and non-RLP fractions were dialyzed against 5 L of PBS (pH 7.4) for 24 hours.18 RLP and non-RLP in VLDL were concentrated by ultracentrifugation.

Coronary Angiography

Coronary angiography was performed 1 week after the operation, using the quantitative cineangiography (QCA) system (Toshiba Medical). Coronary diameters at end-diastole were measured by computer-assisted QCA system in a blind manner. Coronary vasoconstrictor response to serotonin was expressed as a percent decrease in luminal diameter from the control level.14 The inhibitory effect of hydroxyfasudil, a specific Rho-kinase inhibitor (Asahi Kasei),13 was also examined.

Organ Chamber Experiments

The porcine coronary segments treated with either RLP or non-RLP fraction were carefully isolated in physiological salt solution. The rings without endothelium were then mounted vertically between 2 hooks in organ chamber myographs (Medical Supply). Isometric tension was measured with force transducers (Nihon Kohden). Each preparation was stretched in a stepwise manner to an optimal length where the force induced by 118 mmol/L KCl became maximal and constant. After equilibration for 30 minutes, contractions to serotonin (10⁻⁴ to 10⁻⁵ mol/L) were examined.14 The acute inhibitory effect of hydroxyfasudil (10⁻⁵ mol/L) was also examined.

Western Blot Analysis

The ERM family, a substrate of Rho-kinase, is phosphorylated by the kinase at T567 (ezrin), T5648 (radixin), and T558 (moesin).19,20 The regions containing ERM family proteins were visualized by ECL Western blotting luminal reagent (Santa Cruz Biototechnology). Isolated coronary rings without endothelium and adventitial tissue were subjected to SDS-PAGE immunoblot analysis 1 week after the treatment. Phosphorylation of ERM was measured when the serotonin-induced (10⁻⁵ mol/L) contraction reached a maximum.16

Cell Culture

Human coronary VSMC (hcVSMC) were obtained from Bio Witsaker. The hcVSMC were grown to confluence, growth-arrested in DMEM with 0.1% BSA for 2 days; and used for the experiments. Passages 4 to 10 were used.

Northern Blot Analysis

Total RNA was isolated from cultured hcVSMC treated with either RLP or non-RLP in VLDL fraction from SCD patients for 30 minutes to 24 hours. The sequence of the primer for reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of human Rho-kinase α and β was amplified from a human blood cDNA library. We obtained direct purification products of DNA from these PCR amplifications used by the Wizard PCR Prep DNA purification system. A human Rho-kinase α/β cDNA was used as a probe. Northern blot analysis was performed as previously described.21

Statistical Analysis

All results are expressed as the mean±SEM. Differences in all parameters were evaluated by ANOVA, followed by Fisher post-hoc test. A P<0.05 was considered to be statistically significant.

Results

Serum Lipid Profiles and Composition of Isolated RLP in the SCD Patients and Controls

Plasma concentrations of RLP, especially those of RLP-triglyceride, were significantly higher in the SCD patients compared with the healthy volunteers, whereas there was no difference in other lipid profiles between the 2 groups (Table). Isolated RLP from the SCD patients contained a significantly higher cholesterol level as compared with controls (58±9 versus 19±9 mg/dL, P<0.05) and tended to do so for triglyceride level (252±34 versus 126±52 mg/dL, P=0.06).

RLP From Patients With SCD Enhance Coronary Vasospastic Activity in Pigs

In the coronary angiography study 1 week after the treatment, intracoronary serotonin caused marked coronary hypercon-
striction at the segment treated with RLP but not at that with non-RLP fraction from the SCD patients without significant coronary stenosis (Figure 1A through 1D). The serotonin-induced coronary hyperconstrictions were dose-dependently inhibited by pretreatment with hydroxyfasudil, a specific Rho-kinase inhibitor (Figure 1C and 1D). Histological examination demonstrated that there was no obvious intimal thickening or mural thrombus formation at the spastic coronary segment treated with RLP, except for inflammatory cell infiltration at the adventitia (Figure 1E and 1F). By contrast, intracoronary serotonin caused a comparable extent of coronary vasoconstriction at the segments treated with RLP (31% ± 17%) and non-RLP (32% ± 28%) from the normal volunteers (n = 3).

To examine the vasoconstrictor responses of VSMC, we performed organ chamber experiments at 1 week after the treatment with RLP and non-RLP from SCD patients. Serotonin (10⁻⁹ to 10⁻³ mol/L) caused concentration-dependent contractions of isolated coronary rings without endothelium. The serotonin-induced contractions were significantly augmented at the RLP-treated site as compared with the non-RLP-treated site and hydroxyfasudil significantly suppressed those contractions to serotonin only at the RLP-treated site (Figure 2A). To quantify the Rho-kinase activity of the porcine coronary arteries, we performed Western blot analysis for phosphorylated ERM (ezrin, radixin, and moesin) family, a substrate of Rho-kinase.¹⁹,²⁰ The extent of ERM phosphorylation was measured when the serotonin-induced contraction of each ring without endothelium reached maximum. The extent of ERM phosphorylation was significantly increased in the RLP-treated segment compared with non-RLP-treated segment and was again inhibited by hydroxyfasudil (Figure 2B).

There was a significant correlation between the extent of coronary vasoconstriction to serotonin in vitro and RLP-C (P < 0.001), whereas such a tendency was noted between the former and RLP-TG (Figure 3).

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**Figure 1.** RLP from patients with SCD markedly enhance coronary vasospastic activity in pigs. A to C, Coronary angiograms before (A) and after intracoronary serotonin without (B) and with hydroxyfasudil (C). Black arrows indicate RLP site; white arrows, non-RLP site. D, Inhibitory effect of hydroxyfasudil on serotonin (5HT)-induced coronary hyperconstrictions. HF30 and HF100, hydroxyfasudil (30 and 100 μg/kg intracranially). Results are expressed as mean ± SEM. E and F, H&E staining of a coronary segment treated with RLP. The bar indicates 1 mm (E) and 100 μm (F). I indicates intima; M, media; A, adventitia.

**Figure 2.** RLP enhance VSMC contractions and Rho-kinase activity. A, VSMC contractions to serotonin were significantly augmented at the RLP site (●) compared with the non-RLP site (○). Hydroxyfasudil significantly inhibited the VSMC hypercontractions only at RLP-site (●). B, The extent of the ERM phosphorylation of the coronary artery. Hydroxyfasudil significantly suppressed the enhanced ERM phosphorylation at RLP site. Results are expressed as mean ± SEM.
RLP From Patients With SCD Upregulate Rho-kinase in hcVSMC

We examined the effect of RLP and non-RLP in VLDL fraction from SCD patients on Rho-kinase expression and activity in cultured hcVSMC in vitro. Northern blot analysis revealed that mRNA expression of Rho-kinase α (ROCK2) and Rho-kinase β (ROCK1) was significantly increased in response to RLP but not to non-RLP in VLDL fraction (Figure 4A and 4B). Western blot analysis also revealed that the extent of phosphorylated ERM was significantly increased in response to RLP but not to non-RLP (Figure 4C). These results demonstrate that RLP, but not non-RLP in VLDL, exert a potent enhancing effect on the expression and activity of Rho-kinase.

Discussion

The novel findings of this study were that RLP from SCD patients without significant coronary stenosis upregulate Rho-kinase, enhancing the coronary vasospastic activity both in vivo and in vitro, and a specific Rho-kinase inhibitor, hydroxyfasudil, suppressed the coronary vasospastic activity both in vivo and in vitro. To the best of our knowledge, this is the first study that demonstrates the important role of RLP and coronary vasospasm in the pathogenesis of SCD.

Coronary vasospasm has been postulated to play an important role in SCD, although a direct demonstration for the hypothesis is still lacking. Likewise, although our previous postmortem studies demonstrated that RLP may be the major risk factor for SCD, the mechanism for RLP-mediated SCD remains to be elucidated. In this study, we were able to demonstrate the close relation between RLP and coronary vasospasm that is mediated by upregulated Rho-kinase. We have previously shown that the expression and the activity of Rho-kinase are enhanced at the inflammatory coronary lesions in our porcine model with interleukin-1β. The present study demonstrates that RLP from SCD patients also exert a potent upregulating effect on Rho-kinase in hcVSMC.

RLP exert several proinflammatory effects, including impairment of endothelium-dependent relaxation, monocyte adhesion to the endothelium, and VSMC proliferation. It has been recently reported that postprandial increase in RLP is closely associated with postprandial inflammatory response. RLPs are unique in dramatically increasing after a meal and remaining thereafter in the circulation for some time.
time, although a wide individual variation appears to be present in the postprandial response. Thus, it is conceivable that the adverse cardiovascular effects of RLP increase in the postprandial phase as compared with the fasting phase. Indeed, clinical studies have demonstrated that postprandial increase in RLP is closely related to early atherosclerosis in healthy individuals.

In this study, no appreciable atherosclerotic lesion was noted at the RLP-treated site, indicating that functional alteration precedes the morphological one in coronary VSMC in response to RLP.

The present study also demonstrates the important proinflammatory effects of RLP to upregulate Rho-kinase. The important question arises as to whether Rho-kinase is upregulated by quantitative and/or qualitative alterations in RLP in SCD patients. The positive correlation between coronary vasoinconstriction and RLP-C from the SCD patients and from the normal volunteers suggests that quantitative alteration in RLP-C is involved in the Rho-kinase upregulation. However, possible qualitative alteration in RLP in SCD patients remains to be examined. It has been recently reported that sphingosine 1-phosphate (S1P) and sphingosylphosphorylcholine, present in serum lipoproteins, behave as lipid mediator and cause vasoinconstriction through upregulation of Rho/Rho-kinase pathway. The possible role of S1P and sphingosylphosphorylcholine in RLP fraction remains to be elucidated in a future study.

In summary, this study provides the evidence that the elevated RLP level and the consequent upregulation of Rho-kinase are substantially involved in the pathogenesis of SCD. These results suggest that the detection of postprandial sustained increase in RLP is important to identify the subjects at high-risk for SCD and that the use of a Rho-kinase inhibitor could be a promising approach to prevent the fatal disorder.

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Arterioscler Thromb Vasc Biol. published online March 25, 2004; Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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