Cardioprotective effect of rosvustatinin vivo is dependent on inhibition of geranylgeranyl pyrophosphate and altered RhoA membrane translocation

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Bulhak A, Roy J, Hedin U, Sjöquist PO, Pernow J. Cardioprotective effect of rosvustatin in vivo is dependent on inhibition of geranylgeranyl pyrophosphate and altered RhoA membrane translocation. Am J Physiol Heart Circ Physiol 292: H3158–H3163, 2007. First published February 23, 2007; doi:10.1152/ajpheart.01354.2006.—Hydroxymethyl glutaryl (HMG)-coenzyme A (CoA) reductase inhibitors (statins) protect the myocardium against ischemia-reperfusion injury via a mechanism unrelated to cholesterol lowering. Statins may inhibit isoprenylation and thereby prevent activation of proteins such as RhoA. We hypothesized that statins protect the myocardium against ischemia-reperfusion injury via a mechanism involving inhibition of geranylgeranyl pyrophosphate synthesis and translocation of RhoA to the plasma membrane. Sprague-Dawley rats were given either the HMG-CoA reductase inhibitor rosvustatin, geranylgeranyl pyrophosphate solvate in methanol, the combination of rosvustatin and geranylgeranyl pyrophosphate, rosvustatin and methanol, or distilled water (control) by intraperitoneal injection for 48 h before ischemia-reperfusion. Animals were anesthetized and either subjected to 30 min of coronary artery occlusion followed by 2 h of reperfusion whereat infarct size was determined, or the expression of RhoA protein was determined in cytosolic and membrane fractions of nonischemic myocardium. There were no significant differences in hemodynamics between the control group and the other groups before ischemia or during ischemia and reperfusion. The infarct size was 80 ± 3% of the area at risk in the control group. Rosuvustatin reduced infarct size to 64 ± 2% (P < 0.001 vs. control). Addition of geranylgeranyl pyrophosphate (77 ± 2%, P < 0.01 vs. rosvustatin) but not methanol (65 ± 2%, not significant vs. rosvustatin) abolished the cardioprotective effect of rosvustatin. Geranylgeranyl pyrophosphate alone did not affect infarct size per se (84 ± 2%). Rosuvustatin increased the cytosol-to-membrane ratio of RhoA protein in the myocardium (P < 0.05 vs. control). These changes were abolished by addition of geranylgeranyl pyrophosphate. We conclude that the cardioprotection and the increase of the RhoA cytosol-to-membrane ratio induced by rosvustatin in vivo are blocked by geranylgeranyl pyrophosphate. The inhibition of geranylgeranyl pyrophosphate formation and subsequent modulation of cytosol/membrane-bound RhoA are of importance for the protective effect of statins against myocardial ischemia-reperfusion injury.

hydroxymethyl glutaryl-coenzyme A reductase inhibitor; ischemia-reperfusion injury; myocardium; rats

HYDROXYMETHYL GLUTARYL (HMG)-coenzyme A (CoA) reductase inhibitors (statins) have been used for the treatment of hypercholesterolemia for 20 years (17). The effects of statins are mediated via blockade of mevalonate formation, which results in reduction of cholesterol synthesis. Statin treatment reduces the risk of coronary events and mortality in patients with coronary artery disease (26). These clinically beneficial effects are believed to be the result of cholesterol-lowering mechanisms. However, several studies have demonstrated that statins exert beneficial cardiovascular effects that may be independent of cholesterol lowering (24, 30, 36). Inhibition of mevalonate formation results in reduced levels of isoprenoid derivatives, including geranylgeranyl pyrophosphate (GGPP; see Ref. 11), which play important roles in the isoprenylation of small GTPases of the Rho/Rac/Cdc42 family, including RhoA (3, 19, 31). Isoprenylation facilitates activation and translocation of these proteins from the cytosol to the plasma membrane and thereby supports the function of these intracellular signaling pathways, which are necessary for many cellular processes in the cardiovascular system such as cell survival, cell growth, cell migration, and inflammatory processes (3, 23, 32). Both lipophilic and hydrophilic statins have been shown to inhibit RhoA isoprenylation in human endothelial cells (9, 20, 22) and to increase nitric oxide (NO) production and release (7, 14, 15, 28). Accordingly, administration of GGPP and its substrate geranylgeraniol has been demonstrated to reverse the effects of statins on endothelial cells (14, 16, 18, 27, 33). Thus statins may influence cellular function by preventing RhoA signaling (8, 21, 29).

We have previously shown that pretreatment with clinically relevant doses of rosvustatin (RSV) protects against myocardial ischemia-reperfusion injury in the pig (4). Both lipophilic (simvastatin, atorvastatin; see Refs. 2 and 30) and hydrophilic (RSV, pravastatin; see Refs. 4 and 13) statins appear to protect against myocardial ischemia-reperfusion injury in vivo. The effect of RSV was blocked by a NO synthase (NOS) inhibitor, which suggests involvement of NO in the cardioprotective effect. It is not known, however, whether the protective effect of RSV against ischemia-reperfusion injury in vivo involves inhibition of Rho signaling. We hypothesized that statins exert cardioprotection against ischemia-reperfusion injury by preventing RhoA activation and tested whether the protection induced by RSV is dependent on GGPP and subsequent reduction in RhoA translocation to the plasma membrane.

METHODS

Treatment groups. The study was approved by the regional ethical committee for laboratory animal experiments and conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

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Male Sprague-Dawley rats (250–300 g) were used in two experimental protocols (Fig. 1). In protocol I, rats were randomly divided into five groups 48 h before the onset of ischemia. The first group received 10 mg/kg RSV dissolved in distilled water (n = 8), the second group received 1 mg/kg GGPP dissolved in methanol (n = 8), the third group received the combination of RSV and GGPP (RSV + GGPP; n = 7), the fourth group received RSV and methanol (RSV + methanol; n = 7), and the fifth group received distilled water (control; n = 9). All treatments were given as intraperitoneal injections. RSV and distilled water were given every 12 h; GGPP and methanol were given every 24 h during a 48-h treatment period.

In protocol II, rats were divided into three groups (groups 6, 7, and 8): control, RSV, and RSV + GGPP groups (n = 6 in each group), respectively, treated as in protocol I (Fig. 1).

Experimental protocols. Animals in protocol I were anesthetized with pentobarbital sodium (50 mg/kg ip followed by continuous infusion of 5 mg·kg⁻¹·h⁻¹ iv), tracheotomized, intubated, and ventilated with air by a rodent ventilator (50 strokes/min, 8–10 ml/kg tidal volume). Rectal temperature was maintained at 38.0 ± 0.5°C by a heated operation table. The right carotid artery was cannulated and connected to a pressure transducer (Statham P23Db) for measurement of mean arterial pressure (MAP), which was continuously recorded on a Grass polygraph (model 7D; Grass Instruments, Quincy, MA). Heart rate (HR) was determined from the arterial pressure curve. The left jugular vein was cannulated for administration of anesthetics and Evans blue at the end of the experiment. The heart was exposed via a left thoracotomy. A ligature was placed around the left coronary artery. After completion of the surgical preparation, the rats were allowed to stabilize for 15 min before the onset of ischemia. The coronary artery was occluded by tightening the ligature. This was associated with a reduction in MAP and appearance of a cyanotic color of the myocardial area at risk. Reperfusion was initiated after 30 min of ischemia by removal of the snare and was maintained for 2 h. The reperfusion was associated with hyperemia and disappearance of the cyanotic color of the myocardium.

Determination of infarct size. Infarct size was measured as previously described (5). Briefly, after 2 h of reperfusion, the coronary artery was reoccluded and 1.5 ml of 2% Evans blue was injected in the right atrium via the left jugular vein to outline the ischemic myocardium (area at risk). The rats were killed with an overdose of anesthetics, and the heart was rapidly excised. The atria and the right ventricle were removed. The left ventricle was cut into 1- to 1.5-mm thick slices perpendicular to the heart base-apex axis. The slices were scanned from both sides for determination of the area at risk, weighed, and put in 0.8% triphenyltetrazolium chloride for 15 min at 37°C to distinguish the viable myocardium from the necrotic (10). After 24 h of incubation in 4% formaldehyde, slices were again scanned from both sides, and the extent of myocardial necrosis and the area at risk were determined by planimetry of computer images (Photoshop 6.0; Adobe Systems, San Jose, CA).

Determination of RhoA expression and translocation to the membrane. Animals from protocol II were killed 48 h after the start of pretreatment, without being subjected to ischemia-reperfusion, with an overdose of anesthetics, and the heart was rapidly excised, frozen, and homogenized in cold homogenization buffer containing (in mM) 100 Tris·HCl (pH 7.4), 1 EGTA, 1 EDTA, 1 phenylmethylsulfonyl fluoride, and 1 Na₃VO₄. The homogenates were centrifuged at 100,000 g and 4°C for 60 min in a Beckman Optima LE-80 ultracentrifuge with type 50.3 Ti rotor (GMI, Ramsey, MN). The supernatant (cytosolic fraction) was collected, and the pellet (membrane fraction) was resuspended in homogenized buffer containing 1% Triton X-100 (12). Protein concentrations were determined with a bicinchoninic acid kit (Pierce Biotechnology, Rockford, IL) with a multilabel counter (Victor2, Turcu, Finland). RhoA protein expression was determined in cytosolic and membrane fractions by Western blotting. Equal amounts of protein (50 µg/lane) were loaded on a 12% SDS gel and separated by electrophoresis. Extracts from two separate groups were loaded on one gel and compared accordingly. Proteins were transferred to nitrocellulose membranes (Hybond-C pure; Amersham Biosciences, Little Chalfont, UK), and Ponceau solution was used to visualize protein loading. Thereafter, the membranes were washed and incubated with a rabbit polyclonal anti-RhoA antibody (sc-179; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1,000. Peroxidase-conjugated goat anti-rabbit IgG (sc-2004; Santa Cruz Biotechnology) and the enhanced chemiluminescence advance Western blotting detection kit (Amersham Biosciences) were used for visualization. The bands were digitally scanned, and the band densities were calculated (Quantity One 4.5.1; Bio-Rad Laboratories, Hercules, CA). After that, membranes were stripped and rebotted with rabbit polyclonal anti-pan-cadherin antibody (4068; Cell Signaling Technology, H3159 STATIN AND MYOCARDIAL ISCHEMIA-REPERFUSION
Danvers, MA) to verify adequate separation of membranes from the cytosol.

Chemicals and statistical analysis. Pentobarbital sodium was purchased from Apoteksbolaget (Stockholm, Sweden), GGPP and Poncova from Sigma (St. Louis, MO), and methanol from Merck (Darmstadt, Germany). RSV was a kind gift from AstraZeneca.

All values are presented as means ± SE. Differences between the groups were calculated using one-way ANOVA followed by the Tukey-Kramer multiple-comparisons test. Changes in hemodynamics were analyzed using ANOVA for repeated measurements. Because protein amounts were compared in pairs, differences in RhoA protein expression between groups were determined using unpaired t-test. P < 0.05 was considered statistically significant.

Exclusion criteria. Exclusion criteria were established before the start of the study; these included MAP < 70 mmHg before ischemia, ventricular fibrillation (VF) lasting > 3 min, and problems with the injection of dye leading to difficulties in assessment of the area at risk.

RESULTS

Mortality and exclusions from the study. Of forty-six rats entering protocol 1, seven animals were excluded from the study: two in the control group because of problems with injection of Evans blue and totally unmarked area at risk; two rats died because of irreversible VF (1 in the RSV group and 1 in the RSV + GGPP group); and three animals (1 in the RSV group, 1 in RSV + GGPP group, and 1 in the RSV + methanol group) were excluded because of low MAP (< 70 mmHg) before ischemia. Thus thirty-nine rats were included in protocol 1.

Hemodynamics. MAP, HR, and rate-pressure product (RPP) before ischemia and before and during reperfusion in all experimental groups are presented in Table 1. There were no statistically significant differences in basal MAP and RPP between the groups. HR before ischemia was lower in the RSV group than in the RSV + GGPP (P < 0.05), GGPP (P < 0.05), and RSV + methanol (P < 0.01) groups but not different between the RSV and the control groups. Moreover, before reperfusion, HR was significantly lower in the RSV group than in the RSV + methanol group (P < 0.05). Reperfusion led to significant reductions in MAP, HR, and RPP from preischemic values in all groups, except in the GGPP group, in which MAP and RPP were non-significantly reduced from preischemic values. RPP at the end of reperfusion was significantly lower in the RSV group than in the GGPP group (P < 0.05; Table 1).

Effect of RSV on infarct size. There were no significant differences in the areas at risk between any of the groups (Fig. 2A). The infarct size was 80 ± 3% of the area at risk in the control group (Fig. 2B), and RSV induced a significant reduction in infarct size to 64 ± 2% (P < 0.001). Administration of GGPP reversed the cardioprotective effect of RSV (infarct size 77 ± 3%; P < 0.01 vs. RSV), whereas GGPP alone did not affect infarct size [infarct size 84 ± 2%; not significant (NS) vs. control]. Methanol (vehicle for GGPP) had no influence on the cardioprotective effect of RSV (65 ± 2%; NS vs. RSV).

Effect of RSV on RhoA expression and translocation. RhoA protein was determined by immunoblotting of cytosolic and membrane fractions isolated from myocardium of control, RSV, and RSV + GGPP pretreated rats. In all groups, RhoA protein was detected in both cytosolic and membrane fractions, with consistently higher content of RhoA in the latter (Fig. 3A). Cadherin proteins (membrane marker) were detected in the membrane fraction only and not in the cytosolic fraction (Fig. 3A). Quantification of RhoA protein by densitometry revealed that RSV increased the cytosol-to-membrane ratio of RhoA (P < 0.05 vs. control), whereas administration of GGPP abolished the effect of RSV on RhoA protein translocation (P < 0.001 vs. RSV; Fig. 3B). No difference was observed between the control and the RSV + GGPP groups. The total amount of RhoA protein was unchanged in the RSV-treated group.

DISCUSSION

The results of the present study demonstrate that cardioprotection against ischemia-reperfusion in vivo induced by a statin is mediated via inhibition of GGPP and associated with an increased cytosolic/membrane fraction of RhoA protein. Co-administration of RSV and GGPP abolished the cardioprotective effect and the inhibitory effect of RSV on translocation of RhoA to the plasma membrane. This suggests that RSV in-

Table 1. Hemodynamics in rats subjected to 30 min ischemia and 120 min of reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>Before Ischemia</th>
<th>Before Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Control (n = 9)</td>
<td>MAP</td>
<td>102 ± 6</td>
<td>106 ± 5</td>
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<tr>
<td></td>
<td>HR</td>
<td>425 ± 9</td>
<td>438 ± 11</td>
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<tr>
<td></td>
<td>RPP</td>
<td>43,397 ± 3,144</td>
<td>46,359 ± 2,449</td>
</tr>
<tr>
<td>RSV (n = 8)</td>
<td>MAP</td>
<td>95 ± 9</td>
<td>98 ± 7</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>411 ± 6</td>
<td>419 ± 6</td>
</tr>
<tr>
<td></td>
<td>RPP</td>
<td>39,031 ± 3,591</td>
<td>40,869 ± 2,777</td>
</tr>
<tr>
<td>RSV + GGPP (n = 7)</td>
<td>MAP</td>
<td>96 ± 5</td>
<td>98 ± 7</td>
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<tr>
<td></td>
<td>HR</td>
<td>444 ± 4</td>
<td>442 ± 9</td>
</tr>
<tr>
<td></td>
<td>RPP</td>
<td>42,565 ± 2,609</td>
<td>43,591 ± 3,896</td>
</tr>
<tr>
<td>GGPP (n = 8)</td>
<td>MAP</td>
<td>94 ± 7</td>
<td>101 ± 6</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>439 ± 11</td>
<td>452 ± 13</td>
</tr>
<tr>
<td></td>
<td>RPP</td>
<td>41,581 ± 3,958</td>
<td>45,732 ± 3,443</td>
</tr>
<tr>
<td>RSV + methanol (n = 7)</td>
<td>MAP</td>
<td>103 ± 4</td>
<td>105 ± 6</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>453 ± 6</td>
<td>461 ± 6*</td>
</tr>
<tr>
<td></td>
<td>RPP</td>
<td>46,712 ± 2,151</td>
<td>48,667 ± 2,994</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. MAP, mean arterial pressure (in mmHg); HR, heart rate (in beats/min); RPP, rate-pressure product; RSV, rosuvastatin; GGPP, geranylgeranyl pyrophosphate. Significant differences from preischemic values (*P < 0.05 and †P < 0.01) and from the RSV group (**P < 0.05 and §P < 0.01) are shown.

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produces protection against ischemia-reperfusion injury by a mechanism involving inhibition of RhoA signaling.

Statin treatment has previously been shown to protect the myocardium against ischemia-reperfusion injury (24, 30, 36), and we have recently demonstrated that RSV reduces infarct size via a mechanism that is independent of cholesterol lowering but involves NO production (4). Inhibition of the mevalonate pathway by statins not only prevents cholesterol synthesis but also inhibits formation of isoprenoid derivatives, including GGPP (19, 25). However, isoprenoids are derivatives generated downstream of mevalonate but on a pathway that is separate from cholesterol synthesis. Thus the observation that GGPP can abolish the cardioprotective effect of RSV suggests that inhibition of isoprenoid synthesis is an important mechanism behind the protective cholesterol-independent effect of statins.

It is known that geranylgeranylation is required for the activation and membrane targeting of small GTPases, including RhoA and Rac1 (3, 19, 31). The active membrane-bound form of RhoA elicits downstream signaling, which influences cytoskeleton organization, intracellular transport, gene transcription, and mRNA stability (23, 32). Therefore, HMG-CoA inhibitors that block synthesis of isoprenoids and prevent translocation of small GTP proteins, including RhoA, to the plasma membrane can modulate intracellular signaling. Indeed, statins have been shown to restore endothelial NOS (eNOS) mRNA stability and eNOS protein expression via inhibition of Rho isoprenylation in vitro (9, 18, 20, 22). Accordingly, addition of mevalonate, or GGPP, was shown to inhibit the ability of statins to activate eNOS and increase NO production in vascular endothelial cells (14, 18, 33). In addition, the GGPP substrate geranylgeraniol inhibits NO-dependent anti-inflammatory effects of statins in endothelial cells (16, 27). These findings are in accordance with the observation that RSV protects against ischemia-reperfusion injury via an NO-dependent pathway (4). In the present in vivo study, RSV increased the cytosol-to-membrane ratio of RhoA without affecting total RhoA protein in the myocardium. GGPP completely abolished the effect induced by RSV, supporting the concept of involvement of GGPP in statin-induced reduction of RhoA membrane translocation. Thus the present data extend previous in vitro findings and suggest that statins protect against myocardial ischemia-reperfusion injury in vivo by inhibiting GGPP and subsequent membrane translocation of RhoA in the myocardium. Further support for the notion that RSV-induced cardioprotection via inhibition of GGPP and reduction of RhoA membrane translocation and activation is given by the timing of changes in RhoA expression. The changes were observed 48 h after the start of RSV treatment and thus conform to the time point of the measured infarct size reduction.

The signaling pathway involving isoprenoids, such as GGPP, and small GTP proteins has previously been shown to be of importance during ischemia-reperfusion. Inhibition of Rho-kinase, the downstream effector of RhoA protein, protects the heart against ischemia-reperfusion injury (1, 6) and leads to the activation of the phosphatidylinositol 3-kinase/protein kinase B/eNOS pathway (35). These data are compatible with ours and further support the notion that inhibition of RhoA translocation is of importance for protection against ischemia

Fig. 2. Area at risk (A) expressed as % of left ventricle, and infarct size (B), expressed as % of area at risk after 30 min of ischemia followed by 2 h of reperfusion. The rats were given distilled water (control; n = 9), RSV (n = 8), RSV + GGPP (n = 7), GGPP (n = 8), or RSV + methanol (n = 7) ip 48 h before ischemia. Data are presented as means ± SE. ***P < 0.01 and ###P < 0.001, significant differences from the control group. #P < 0.05 and ##P < 0.01, significant differences from the RSV + GGPP group.

Fig. 3. A: representative immunoblots with an anti-pan-cadherin (membrane marker) and anti-RhoA antibodies on protein extracts from rat heart. Extracts are cytosolic and membrane fractions of myocardium from animals given distilled water (control), RSV, or RSV + GGPP. B: quantification of the cytosol-to-membrane ratio of RhoA protein in rat myocardium. Animals were given distilled water (control), RSV, or RSV + GGPP (n = 6 in each group). Data are presented as means ± SE. *P < 0.05 and **P < 0.01, significant differences between the groups.
reperfusion injury. The link between statin-induced protection against ischemia-reperfusion in vivo and RhoA/Rho-kinase as demonstrated in the present study has, to our knowledge, not been addressed previously. It should also be noted that different mechanisms may be involved in the beneficial effects of statins downstream of GGPP and RhoA. Weinberg et al. (34) could not demonstrate an effect of RSV on myocardial expression of eNOS protein or mRNA in mice, which supports the concept that multiple signaling pathways are involved.

In the present study, rats belonging to the RSV group had a lower HR before ischemia than rats from the RSV + GGPP, GGPP, and RSV + methanol groups and compared with the RSV + methanol group before reperfusion. There was also significant difference in RPP between the RSV group and the GGPP group at the end of reperfusion. However, it is unlikely that these differences in hemodynamics between groups influenced the myocardial injury, since no differences were found in infarct sizes between rats given RSV and the combination of RSV and methanol, despite the most pronounced differences in hemodynamics between these groups.

In conclusion, administration of GGPP abolishes the protection against ischemia-reperfusion injury and the increase in the cytosol-to-membrane ratio of RhoA in vivo induced by RSV. This suggests that lowering of GGPP formation and subsequent inhibition of RhoA translocation is of importance for the protective effect of statins against myocardial ischemia-reperfusion injury in vivo.

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