Simvastatin and GGTT-2133, a geranylgeranyl transferase inhibitor, increase erythrocyte deformability but reduce low O₂ tension-induced ATP release

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Clapp KM, Ellsworth ML, Sprague RS, Stephenson AH. Simvastatin and GGTT-2133, a geranylgeranyl transferase inhibitor, increase erythrocyte deformability but reduce low O₂ tension-induced ATP release. Am J Physiol Heart Circ Physiol 304: H660–H666, 2013. First published January 18, 2013; doi:10.1152/ajpheart.00635.2012.—Statins drugs inhibit 3-hydroxy-3-methylglutaryl CoA reductase, which reduces the synthesis of both cholesterol and isoprenoids (geranylgeranyl pyrophosphate and farnesyl pyrophosphate), with the latter being lipid molecules responsible for the posttranslational modification of small GTP-binding proteins such as Rho. Effects of statins, independent of lowering blood cholesterol levels, are thought to occur by inhibition of Rho/Rho kinase. The Rho kinase inhibitor Y-27632 has been reported to increase both erythrocyte deformability and low O₂ tension-induced ATP release. Here, we tested the hypothesis that by inhibiting Rho/Rho kinase, simvastatin would increase both erythrocyte deformability and low O₂ tension-induced ATP release. Male Sprague-Dawley rats were divided into two groups, control or simvastatin treated [simvastatin-supplemented chow (0.02%)], for 4 wk. Simvastatin treatment increased rat erythrocyte deformability compared with controls (n = 6, P < 0.05). However, erythrocytes of simvastatin-treated rats (n = 9, P < 0.05) exhibited impaired low O₂ tension-induced ATP release. Similarly, the geranylgeranyl transferase inhibitor GGTT-2133 (10 μM) also increased deformability and impaired low O₂ tension-induced ATP release in healthy human erythrocytes (P < 0.05). Interestingly, ATP release in response to mastoparan 7 (n = 7, P < 0.05), which directly activates Gi, and isoproterenol (n = 5, P < 0.05), which signals through Giα, was not altered by incubation with GGTT-2133. These results suggest that although statins increase erythrocyte deformability, likely by inhibiting geranylgeranyltransferase, the finding that both statins and a geranylgeranyl transferase inhibitor attenuated low O₂ tension-induced ATP release demonstrates that factors in addition to erythrocyte deformability are critical for ATP release in response to this physiological stimulus.

red blood cell; mastoparan 7; isoproterenol

The primary function of the erythrocyte is O₂ delivery to tissues. However, the erythrocyte has also been identified as a participant in the control of blood flow distribution within the skeletal muscle microcirculation (10). This control is achieved by the release of the vasodilator ATP from erythrocytes as O₂ dissociates from hemoglobin. Erythrocytes also release ATP when exposed to membrane deformation that directly activates Gi. Such a mechanism is consistent with studies (16, 37) in which shear stress was shown to activate Gi in other cell types and suggests that reduced membrane deformability would decrease low O₂ tension-induced activation of Gi. Importantly, previous studies have shown that reduced membrane deformability inhibits low O₂ tension-induced ATP release from erythrocytes (42, 46), whereas increased deformability of these cells augments ATP release in response to this stimulus (46).

Deformability of erythrocytes is determined by a variety of factors, including cytoskeletal protein composition and its regulation as well as membrane protein and lipid composition (29). The Rho/Rho kinase signaling pathway is known to regulate the organization of the actin cytoskeleton (31) and cellular stiffness in a variety of cell types (12, 13, 45). Rho kinase inhibition increases deformability in pulmonary endothelial cells, airway smooth muscle cells, and fibroblasts (1, 20, 21, 25). It has also been shown that the Rho kinase inhibitor Y-27632 increases deformability and low O₂ tension-induced ATP release in healthy human erythrocytes (46).

Simvastatin, a 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor, a member of the statin family of drugs, is used in humans to decrease circulating lipids. Simvastatin also improves cardiovascular end points (reduces the incidence of stroke and myocardial infarction) by inhibition of the Rho/Rho kinase signaling pathway (32, 36). Treatment with statins decreases the synthesis of the isoprenoid geranylgeranyl pyrophosphate, which becomes covalently bound to RhoA and is necessary for the translocation of RhoA to the plasma membrane and its activation (23). Thus, when RhoA activation is inhibited, the activity of its downstream target, Rho kinase, should also be reduced.

In the present study, we tested the hypothesis that treatment with simvastatin would increase both erythrocyte deformability and low O₂ tension-induced ATP release by inhibiting the activation of RhoA and its downstream target, Rho kinase.

METHODS

Collection of rat and healthy human erythrocytes. Rat blood was obtained from male Sprague-Dawley rats (176–200 g, 46–50 days old) fed either standard chow or simvastatin-supplemented standard chow (0.02% simvastatin, equivalent to ~20 mg·kg⁻¹·day⁻¹) for 4 wk before experimentation (14 rats/group). Rats were anesthetized with pentobarbital sodium (65 mg/kg), heparin was administered via the tail vein, and, after 10 min, animals were exsanguinated via the
abdominal aorta. Blood was collected into a heparinized syringe. Human blood was obtained by venipuncture and collected in a syringe containing heparin (500 U/30 ml). Human blood was collected from 8 women and 8 men with an average age of 40 ± 4 yr (range: 23–62 yr), none of whom were taking lipid-lowering drugs. Erythrocytes were obtained from donors on the day of use.

Isolation of erythrocytes. After collection, rat or healthy human blood was centrifuged at 500 g at 4°C for 10 min. The plasma, buffy coat, and uppermost erythrocyte layers of human blood were removed by aspiration. The plasma of rat blood was saved for the determination of cholesterol levels, and the buffy coat and uppermost erythrocyte layer were removed by aspiration. Packed erythrocytes were resuspended and washed three times in wash buffer [containing (in mM) 21.0 tris(hydroxymethyl)aminomethane, 4.7 KCl, 2.0 CaCl₂, 140.5 NaCl, 1.2 MgSO₄, and 5.5 glucose, with 0.5% BSA fraction V; pH adjusted to 7.4].

Measurement of total cholesterol levels in rat plasma. Total cholesterol levels in rat plasma were determined using an assay kit (Pointe Scientific). Briefly, plasma samples were incubated with a reagent mixture (0.25 mM 4-aminonaphtpyrine, 150 U/1 cholesterol esterase, 150 U/1 cholesterol oxidase, 1,500 U/l peroxidase, 15 mM phenol, and phosphate buffer; pH 6.8). After a 5-min incubation, absorbance measurements at 500 nm were recorded for serum samples and cholesterol standards using a spectrophotometer. Plasma cholesterol levels were determined by comparison with cholesterol standards.

Identification of increased endothelial nitric oxide synthase expression with simvastatin treatment. Statin drugs increase the expression of endothelial nitric oxide synthase (eNOS) by inhibiting Rho activity as a result of decreased Rho geranylgeranylation (15, 44). Therefore, a statin-induced increase in eNOS expression can be used to indicate inhibition of Rho activity in an animal by measuring eNOS expression in highly vascularized tissues, such as those of the kidney (19). Increased expression and activity of eNOS and improved endothelial function associated with HMG-CoA reductase inhibitors are mediated through inhibition of the Rho/Rho kinase pathway (36) and occur before any significant changes in serum cholesterol levels (32). To assess the effectiveness of simvastatin in our rat model, femoral arteries and kidneys were isolated from control and simvastatin-treated rats. Isometric tension of femoral arteries was measured as previously described (9). Rat kidneys were isolated and prepared for Western blot analysis of eNOS expression as previously described (19, 28) using a mouse monoclonal primary antibody for eNOS.

Measurement of erythrocyte deformability. Erythrocyte deformability was measured using the St. George’s blood filtrometer (Carri-Med) (39–41). This device develops a calibrated pressure gradient across a vertically mounted 13-mm diameter polycarbonate filter with a pore size of 5 nm (Nucleopore) with an exposed surface diameter and average filter pore size relative to the size of the erythrocytes studied. If average filter pore size and hematocrit are kept constant, then RCTT is an index of the degree of deformability of the erythrocytes. Under these conditions, a decrease in RCTT indicates an increase in erythrocyte deformability. The deformability of erythrocytes obtained from rats fed simvastatin-supplemented chow or standard chow was determined after a 30-min incubation with buffer at 37°C. In separate experiments, erythrocyte deformability was measured after a 30-min incubation with a geranylgeranyl transferase inhibitor, GGTTI-2133 (10 μM), or its vehicle, dimethyl formamide (DMF), at 37°C (47). Three to four deformability measurements were taken for each sample and averaged.

ATP measurements. ATP release from erythrocytes was measured using a quantitative luciferin-luciferase assay (2). Briefly, a 200-μl sample of the erythrocyte suspension (0.04% hematocrit) was injected into a cuvette containing 100 μl of firefly tail extract (10 mg/ml, Sigma) and 100 μl of a solution of D-luciferin (0.5 mg/ml, Research Products). The light emitted from the reaction of ATP with the firefly tail extract was measured using a luminometer (TD 20/20, Turner Designs). To determine ATP levels, the peak light emitted was compared with an ATP standard curve generated on the day of the experiment. ATP values were normalized to an erythrocyte count of 4 × 10⁶ cells.

Exposure of erythrocytes to O₂ tension in the presence and absence of pharmacological agents. Washed erythrocytes from simvastatin-fed or control rats were diluted to 20% hematocrit with a bicarbonate-based buffer [containing (in mM) 4.7 KCl, 2.0 CaCl₂, 140.5 NaCl, 1.2 MgSO₄, 11.0 glucose, 21.4 NaHCO₃, and 0.5% BSA; pH 7.4] and equilibrated with 15% O₂-6% CO₂-balance nitrogen in a thin-film tonometer (model 237, Instrumentation Laboratories) at 37°C (6). After 30 min, ATP levels were measured. The erythrocyte suspension was then equilibrated with 0% O₂-6% CO₂-balance nitrogen for 10 min, and ATP release was measured again. In separate experiments, either 10 μM GGTTI-2133 or its vehicle (DMF) was added to a suspension (20% hematocrit) of healthy human erythrocytes 30 min before ATP levels were determined as described above. P0₂, Pco₂, and pH of the erythrocyte suspension were determined at the time of ATP measurement using a blood gas analyzer (Stat Profile pHX, Nova Biomedical).

Exposure of rat erythrocytes to mastoparan 7, a direct activator of Gp. Washed erythrocytes from control or simvastatin-fed rats were diluted to a 20% hematocrit, and, after 30 min, ATP levels were measured. After baseline readings had been obtained, mastoparan 7 (Mas 7; 10 μM, BioMol), a general activator of the heterotrimeric G protein Gg, was added, and ATP release was measured after 5, 10, and 15 min. For each experiment, the maximal response to Mas 7 is reported.

Exposure of healthy human erythrocytes to pharmacological agents that modulate ATP release in the absence and presence of GGTTI-2133. Washed, healthy human erythrocytes were diluted to 20% hematocrit and incubated with either GGTTI-2133 (10 μM) or its vehicle (DMF). After a 30-min incubation with GGTTI-2133, baseline ATP levels were measured. After baseline readings had been obtained, Mas 7 (10 μM, BioMol), a general activator of the heterotrimeric G protein Gg, was added, and ATP release was measured after 5, 10, and 15 min. In separate experiments, an activator of the β-adrenergic receptor, isoproterenol (Iso; 1 μM, Sigma) was added to the erythrocyte suspension, and ATP release was measured 1, 5, and 10 min later. For each experiment, the maximal response to Mas 7 or Iso is reported.

Measurement of total ATP. In all experiments in which ATP was measured, total intracellular levels of ATP were also determined to establish that the intervention did not alter ATP synthesis. A known number of erythrocytes (1% hematocrit) was lysed in distilled water at room temperature. This suspension was diluted 1:400 in wash buffer, and ATP was measured using the luciferin-luciferase assay. ATP values were normalized to ATP concentration per erythrocyte.

Measurement of hemoglobin. Extracellular hemoglobin was measured at the completion of all ATP experiments to ensure that the levels of ATP measured were not a result of cell lysis. Erythrocyte suspensions were centrifuged at 500 g for 10 min at 4°C. The amount of hemoglobin present in the supernatant was measured using a spectrophotometer (Spectronic 20, Milton Roy) at 405 nm (17). Samples in which an increase in hemoglobin was detected were not included in the results.

Data analysis. Statistical significance among experiments was determined using either ANOVA or a Student’s t-test, as appropriate.
In the case of ANOVA, in the event that the F ratio indicated that a change had occurred, a Fisher's least-significant difference test was performed to identify individual differences between groups. Results are reported as means ± SE.

Institutional approval. The protocols used to obtain blood from rats and all related surgical and experimental procedures involving rats were approved by the Institutional Animal Care and Use Committee of Saint Louis University. The protocol for the collection of human blood for this study was approved by the Institutional Review Board of Saint Louis University.

RESULTS

Effect of simvastatin on rat plasma cholesterol levels. Similar to previous reports (26, 27), simvastatin treatment did not alter plasma cholesterol levels in Sprague-Dawley rats. Values were 51 ± 3 and 57 ± 3 mg/dl for control and simvastatin-treated rats, respectively.

Effect of simvastatin treatment on eNOS expression and activity as a marker of Rho inhibition. Statins have been shown to increase eNOS expression (36, 44, 49) by inhibiting Rho activation (15). As previously reported (19), we observed a significant increase in eNOS expression in the kidneys of simvastatin-treated rats compared with control rats (P < 0.05; data not shown). Simvastatin treatment also increased relaxation to acetylcholine in femoral artery segments compared with responses in control rats (P < 0.05; data not shown).

Effect of simvastatin and GGTL-2133 on low O2 tension-induced ATP release from rat and healthy human erythrocytes, respectively. Erythrocytes from rats fed simvastatin were increased compared with erythrocytes from control rats (n = 6, P < 0.05; Fig. 1). In addition, at a concentration of 10 µM, GGTL-2133, an inhibitor of geranylgeranylation, decreased RCTT of healthy human erythrocytes (n = 9, P < 0.05; Fig. 2), indicating increased deformability. Thus, both simvastatin treatment in intact rats and incubation of isolated human erythrocytes with GGTL-2133 increased erythrocyte deformability, suggesting a common mechanism of action via inhibition of Rho geranylgeranylation.

Effect of simvastatin and GGTL-2133 on low O2 tension-induced ATP release from rat and healthy human erythrocytes. As exposure of erythrocytes to low O2 tension resulting in hemoglobin O2 desaturation is thought to initiate a signaling pathway for ATP release that requires the activation of Gi, we examined the effect of GGTL-2133 on two distinct signaling pathways, Mas 7- and Iso-induced ATP release from healthy human erythrocytes. To investigate whether inhibition of geranylgeranylation can nonspecifically inhibit ATP release from erythrocytes, we examined the effect of GGTL-2133 on two distinct signaling pathways for ATP release from erythrocytes. First, we determined that a direct activator of Gi, Mas 7, increased ATP release from healthy human erythrocytes similarly in the presence and absence of GGTL-2133 (n = 7, P < 0.05; Fig. 3B). Next, we determined that Iso-induced activation of the β-adrenergic receptor, which is coupled to the heterotrimeric G protein Gi, also stimulated ATP release in the absence and presence of GGTL-2133 (n = 5, P < 0.05; Fig. 5B).
DISCUSSION

We investigated the hypothesis that treatment with simvastatin would increase both erythrocyte deformability and low O₂ tension-induced ATP release. In the presence of decreased geranylgeranylation, direct activation of G₁ still resulted in ATP release after simvastatin treatment. Simvastatin treatment did not inhibit Mas 7-induced ATP release. These results demonstrate that in the presence of decreased geranylgeranylation, direct activation of G₁ still resulted in ATP release from erythrocytes, suggesting that simvastatin-induced inhibition of ATP release lies upstream from G₁ activation.

In the present study, simvastatin did not alter plasma cholesterol levels, a result consistent with previous studies in rats fed

Fig. 3. Inhibition of low O₂ tension-induced ATP release from erythrocytes after treatment with simvastatin and GGTI-2133. A: control (n = 10) and simvastatin-treated (n = 11) rat erythrocytes were equilibrated with 15% O₂-6% CO₂-balance nitrogen (normoxia; Po₂: 107 ± 2 mmHg, Pco₂: 38 ± 0.4 mmHg, pH 7.37 ± 0.02). Baseline ATP values obtained for control rats and simvastatin-treated rats were 23.5 ± 4.7 and 21.7 ± 3.0 nmol/10⁸ erythrocytes, respectively. Erythrocytes were then exposed to 0% O₂-6% CO₂-balance nitrogen (reduced O₂; Po₂: 17 ± 1 mmHg, Pco₂: 38 ± 0.5 mmHg, pH 7.4 ± 0.02). ATP values for control and simvastatin-treated rats were 35.8 ± 7.0 and 23.8 ± 3.7 nmol/10⁸ erythrocytes, respectively. The percent change in ATP release in response to low O₂ was significantly increased for erythrocytes from control rats compared with those obtained from simvastatin-treated rats. Values are reported as percent changes in ATP release from normoxia ± SE; n = 9 for the control group and 8 for the simvastatin-treated group. *P < 0.05, significantly different from the simvastatin-treated group. 

Fig. 4. Simvastatin does not inhibit mastoparan 7 (Mas 7)-induced ATP release from erythrocytes. Baseline erythrocyte ATP release was measured from control and simvastatin-treated rats and was not different between the two groups. Mas 7 stimulated ATP release similarly from both control and simvastatin-treated rat erythrocytes. Values are reported as means ± SE; n = 6 for the simvastatin-treated group and 5 for the control group. *P < 0.05, significantly different from baseline.
normal chow (19, 24, 27). Since rats lack cholesterol ester transfer protein (35), which transfers cholesteryl esters to very-low-density lipoprotein and low-density lipoprotein in humans, a greater relative portion of plasma cholesterol is transported by high-density lipoprotein in the rat compared with humans. This deficiency of cholesterol ester transfer protein makes rat plasma cholesterol levels less affected by statins, making the rat an ideal subject in which to study effects of statins that are independent of their cholesterol-lowering properties.

The high concentration of hemoglobin present in erythrocytes interferes with quantitative measurements of Rho activity in these cells. Therefore, we examined inhibition of Rho activity by measuring the most commonly described downstream effect of in vivo Rho inhibition, that of increased eNOS expression. We measured eNOS expression in kidney tissue, a source previously reported to exhibit increased eNOS expression after statin treatment (18, 19). We identified a significant increase in eNOS expression in the kidneys of simvastatin-treated rats compared with rats fed the control diet. Simvastatin treatment also increased acetylcholine-induced relaxation of isolated rat femoral arteries compared with control rats, consistent with increased eNOS expression. Thus, the dose of simvastatin administered to the rats was sufficient to inhibit Rho activation.

Forsyth et al. (11) recently examined the effects of acute (15 min) in vitro incubation of erythrocytes with simvastatin. They measured shear-induced erythrocyte deformability and ATP release (11). In contrast to our findings, they reported increased deformability and increased ATP release with shear in simvastatin-treated erythrocytes. They attributed their results to increased membrane fluidity resulting from incorporation of the lipophilic simvastatin molecule into the erythrocyte membrane, as had been previously reported when simvastatin was acutely incubated with the lipid membranes of liposomes (3). However, when administered to rats in vivo, as was done in the present study, Caliskan et al. (5) reported that simvastatin lowered blood ATP levels, a finding consistent with simvastatin-induced depletion of the geranylgeranyl moiety and consistent with the findings of the present study.

To evaluate whether direct inhibition of protein geranylgeranylation would replicate the results in which simvastatin was used to deplete the geranylgeranyl moiety, we examined the effects of a selective geranylgeranyl transferase inhibitor, GGTI-2133, on the deformability of healthy human erythrocytes and low O₂ tension-induced ATP release. GGTI-2133 directly inhibits the geranylgeranylation of proteins by inhibiting the transferase that attaches the geranylgeranyl moiety to the protein. Incubation of human erythrocytes with GGTI-2133 increased their deformability, and, as was observed with simvastatin, GGTI-2133 also inhibited low O₂ tension-induced ATP release. Moreover, GGTI-2133 did not inhibit Mas 7-induced ATP release. These findings are consistent with the hypothesis that the effects of simvastatin on erythrocyte deformability and low O₂ tension-induced ATP release are due to decreased geranylgeranylation of RhoA.

Receptor-mediated activation of the heterotrimeric G protein Gₛ can also result in ATP release from erythrocytes (34). To further investigate the selectivity of geranylgeranylation on low O₂ tension-induced ATP release from erythrocytes, a Gₛ-dependent mechanism (33, 34), we examined whether geranylgeranylation is also important for Gₛ-mediated ATP release. ATP release in response to Iso, a β-adrenergic receptor agonist, was not inhibited by GGTI-2133, suggesting that geranylgeranylation is not necessary for Gₛ-mediated ATP release from erythrocytes. This result, coupled with the observation that geranylgeranylation is not necessary for ATP release when Gₛ is directly stimulated with Mas 7, suggests that the requirement for geranylgeranylation is specific to ATP release in response to low O₂ tension.

More detailed investigation of the mechanisms that account for the divergent effects of Y-27632 and simvastatin and GGTI-2133 on deformability and low O₂ tension-induced ATP release from erythrocytes are beyond the scope of the present study. However, the finding that Mas 7-induced ATP release is unaffected by simvastatin or GGTI-2133 suggests that the requirement for geranylgeranylation is selective for low O₂ tension-induced ATP release and must be upstream of Gₛ in this signaling pathway. Recently, Stefanovic, et al. (43) demonstrated that the O₂ saturation state of hemoglobin modulates the primary bridge linking the erythrocyte membrane to its cytoskeleton. Deoxyhemoglobin but not oxyhemoglobin competes with the cytoskeletal protein ankyrin for binding to band 3 on the erythrocyte membrane (43) as a result of deoxyhemo-
globin’s much higher affinity for band 3 than that of oxyhemoglobin (7). When hemoglobin displaces cytoskeletal ankyrin from the membrane, the less-constrained band 3 diffuses faster and farther in the membrane than it does under oxygenated conditions (43). Thus, when erythrocyte oxyhemoglobin desaturates in a low O2 tension environment, the weakened membrane/cytoskeleton interaction resulting from ankyrin displacement may promote Gi activation, initiating the signaling pathway toward ATP release. How inhibition of geranylgeranylation with either statin treatment or a geranylgeranyl transferase inhibitor interferes specifically with the low O2 tension-induced ATP release component of this mechanism is not known. However, another ankyrin-binding protein abundantly expressed in erythrocyte is CD44, a transmembrane protein that in nonerythroid cells complexes with RhoA (4). When RhoA is activated, ankyrin attachment to the membrane increases via CD44 binding, strengthening the membrane/cytoskeleton interaction. Although this particular interaction may not specifically influence low O2 tension-induced ATP release from erythrocytes, it illustrates a scenario in which RhoA activity can regulate ankyrin membrane binding, a potentially important component of low O2 tension-induced ATP release.

In summary, treatment of rats with simvastatin or incubation of healthy human erythrocytes with the geranylgeranyl transferase inhibitor GGTT-2133 increased the deformability of erythrocytes but impaired low O2 tension-induced ATP release. These findings suggest that unlike the Rho kinase inhibitor Y-27632, which increases erythrocyte deformability and ATP release. These findings suggest that unlike the Rho kinase inhibitor Y-27632, which increases erythrocyte deformability and ATP release, it illustrates a scenario in which RhoA activity can regulate ankyrin membrane binding, a potentially important component of low O2 tension-induced ATP release.

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