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The effects of membrane cholesterol and simvastatin on red blood cell deformability and ATP release

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ABSTRACT

It is known that deformation of red blood cells (RBCs) is linked to ATP release from the cells. Further, membrane cholesterol has been shown to alter properties of the cell membrane such as fluidity and bending stiffness. Membrane cholesterol content is increased in some cardiovascular diseases, for example, in individuals with acute coronary syndromes and chronic stable angina, and therefore, because of the potential clinical relevance, we investigated the influence of altered RBC membrane cholesterol levels on ATP release. Because of the correlation between statins and reduced membrane cholesterol *in vivo*, we also investigated the effects of simvastatin on RBC deformation and ATP release. We found that reducing membrane cholesterol increases cell deformability and ATP release. We also found that simvastatin increases deformability by acting directly on the membrane in the absence of the liver, and that ATP release was increased for cells with enriched cholesterol after treatment with simvastatin.

Introduction

Cholesterol is lipophilic and plays a crucial role in the regulation of the properties of lipid membrane as it reduces permeability barriers, regulates membrane fluidity, and affects membrane bending stiffness (Chabanel et al., 1983; Meleard et al., 1997; Mouritsen and Zuckermann, 2004). In addition, the cholesterol content of biomembranes has been shown to affect membrane trafficking, cellular signal transduction, enzyme activity, and the phase transitions (Maxfield and Tabas, 2005). It was hypothesized that such phenomena are related to lateral phase separation in biomembranes by forming microdomains enriched in cholesterol, which are also known as lipid rafts (Simons and Ikonen, 1997). Also, changes in membrane cholesterol content are widespread in microvascular diseases such as atherosclerosis, hypertension, and hypercholesterolemia (Kolodgie et al., 2007; Vaya et al., 2008). For example, the internal viscosity is increased in cholesterol-enriched red blood cells (RBCs) in individuals with hypertension, diabetes mellitus, or patients who have had a myocardial infarction (Banerjee et al., 1998).

Cholesterol in the cell membrane not only affects transport processes, but it also alters deformability of the cell. For example, the deformability of RBCs is reduced in patients with hypercholesterolemia, and was shown to improve with pravastatin therapy designed to lower serum cholesterol (Kohno et al., 1997). Recently, Tziakas et al. (2007) identified increased cholesterol content in RBC membranes as a potential marker for clinical instability of patients with acute coronary syndrome. It was also reported that patients with acute coronary syndromes and chronic stable angina have a marked increase in the cholesterol levels in their RBC membranes, which was reversed with statin therapy, including simvastatin (Tziakas et al., 2009). Simvastatin is a lipophilic molecule and a member of the 3-hydroxy-3-methylglutaryl coenzyme reductase inhibitor class of drugs. Its main function is to lower total and low-density lipoprotein (LDL) cholesterol, but it has multiple pleiotropic effects such as increased smooth muscle and endothelial cell function, improved vascular wall function, LDL oxidation, and reduced inflammation (Kolovou et al., 2008).

It is also known that RBCs release ATP when they are deformed or under hypoxic conditions (Ellsworth et al., 2009; Forsyth et al., 2011; Sprague et al., 1998; Wan et al., 2008). RBCs have been shown to reduce pulmonary vascular resistance and trigger the production of nitric oxide in rabbits (Sprague et al., 1995). In addition, coronary blood flow and oxygen consumption have been shown to increase with increased venous ATP levels in dogs during exercise (Farias et al., 2005). These results have led to the hypothesis that RBCs act as a modulator of localized vascular tone. With respect to pathological conditions, altered antioxidant status and reduced ATP release from RBCs have been associated with diabetes mellitus (Subasinghe and Spence, 2008).

To our knowledge, no one has investigated the links between cholesterol and ATP release from RBCs. Because cholesterol affects deformability of RBCs and deformability alters ATP release, it follows that cholesterol might play a role in ATP release. Furthermore,

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cholesterol influences the fluidity of cell membranes and affects the activation of volume-regulated anion current channels, which are known to be involved in ATP release (Braunstein et al., 2001; Levitan et al., 2000). In this paper we used methyl-β-cyclodextrin (MβCD) to remove or enrich cholesterol in the cell membrane in order to determine the effects of membrane cholesterol on RBC size, deformation, and ATP release. We also investigated the direct influence of simvastatin on the deformability of RBCs and ATP release because it has been reported that, as a lipophilic molecule, simvastatin has a significant fluidizing effect in membranes (Bhandary et al., 2009).

Methods

Solution preparation

To reduce cholesterol levels in RBC membranes ((−) chol), a 2.5 mM MβCD solution was prepared in phosphate-buffered saline (PBS). In order to add cholesterol to the RBC membranes ((+) chol), a saturated cholesterol–MβCD solution was prepared by adding approximately 1 mg of cholesterol to 10 ml of 2.5 mM MβCD solution. The solution was sonicated at 37 °C for 15 min, incubated at 37 °C for 2 h, and then further incubated at room temperature overnight. The solution was then filtered with a 0.45 μm pore size filter before use and had an estimated final cholesterol concentration of 0.25 mM based on the solubilization of cyclodextrin (Christian et al., 1997). A physiological salt solution (PSS) was prepared and 10 mg/ml of bovine serum albumin was added for all experiments except for the ATP measurements (Sprague et al., 1998). A small amount of simvastatin was dissolved in PSS by sonicating the solution at 37 °C for 60 min and the solution was then filtered to obtain a final estimated simvastatin concentration of 0.76 mg/L. The pH of all solutions was adjusted to 7.4 and all reagents were purchased from Sigma-Aldrich.

10 μl of blood was obtained by finger prick from healthy volunteers and washed in PSS. After centrifugation, the supernatant was discarded. Then, 220 μl of PSS and 220 μl of either MβCD or cholesterol-MβCD or PBS only (control) were added. The samples were incubated at room temperature for 10 min on a rotating device fabricated in-house. After incubation the RBCs were again washed, resuspended to 1% hematocrit (Hct), and allowed to equilibrate for 15 min with or without simvastatin. This work was approved by the Princeton University Institutional Biosafety Committee.

Analysis of RBC membrane cholesterol

To determine the cholesterol content of native and MβCD modified RBCs, lipids were extracted from packed RBCs (Rose and Oklander, 1965). The cholesterol content was analyzed using the cholesterol-oxidase method (Amundson and Zhou, 1999). Briefly, 150 μl of packed RBCs obtained from a finger prick was lysed in 180 μl of distilled water. Next, 2.025 ml isopropanol was added and mixed. After 1 h of incubation 1.125 ml chloroform was added, mixed, and incubated for 1 h. To remove the denaturated proteins the suspension was centrifuged for 15 min at 14500 RPM. The organic solvent was evaporated, and the lipids were reconstituted in 1 ml of 10% Triton X-100 in ultrapure water. From this solution 33 μl was incubated in 967 μl of the enzymatic solution. The absorbance of the samples at 500 nm was read in a spectrometer (Varian Cary 50, Agilent Technologies). The total amount of cholesterol in the samples was obtained using a 4-point calibration and is reported below per 100 μl of packed RBCs.

The total membrane cholesterol per milligram of membrane protein was measured by using the Bradford method with albumin as a standard (Bradford, 1976). For membrane protein extraction, ghost cells were prepared from a 150 μl aliquot of packed RBCs. Briefly, RBCs were suspended in 2 ml of lysis buffer containing 7.5 mM sodium phosphate and 1 mM sodium EDTA (pH = 7.5) at 4 °C. After 30 min of incubation time, the solution was centrifuged for 5 min at 2000 RPM. This step was repeated three times until an opaque pellet was obtained containing the RBC ghosts.

Cell size and deformation experiments

Images of cells at 40× (DFC 360 FX, Leica) were taken on an inverted light microscope (DMIRB, Leica) and their projected areas were measured using ImageJ. The cell size is reported as the radius squared, or projected area, normalized by the area of PBS control cells. For the deformation experiments, RBCs were suspended in a PSS solution in order to increase clarity. The cell suspension was then transferred into a syringe and flowed through a microfluidic flow-focusing device by using a syringe pump at flow rates (Q) between 3 and 9 μl/min. The average shear rate ($\dot{\gamma}$) inside the microfluidic channel was calculated as $\dot{\gamma} = Q/h^2w$ (Fig. 2A), where h and w is the channel height and width respectively. It is a good average shear estimate for pressure-driven flow through a rectangular cross-section in which $h/w<1$ (Stone, 2007). The calculated average shear rate is from 2700 to 7500 s^{-1}, which is physiologically relevant to the shear rates in typical arterioles (Vennemann et al., 2007). Video was acquired at 6000 frames per second with a 10 μs exposure time using a high-speed camera (Phantom V9.1, Vision Research) and no cell lysis was observed during the experiments. Although the RBC is a viscoelastic body, we focus on the elongation of red cells during their passage in microfluidic channels. Image processing was used to measure each cell's initial length L_o, as well as the maximum length L as the cell was deforming (Forsyth et al., 2010). The stretch is reported as L/L_o.

ATP release experiments

Following washing, RBC samples were resuspended to 1% Hct by dilution in PSS solution containing 40 g/l of dextran ($MW = 2\times10^6$) to increase the solution viscosity. A luciferin/luciferase solution was prepared by adding 100 μl of 1 mg/ml luciferase and 1.5 mg D-luciferin sodium salt into 5 mL of PSS, which was then N_2 quenched. An ATP stock solution was prepared for calibrations by adding 3 mg of ATP sodium salt in 10 mL of PSS, and then diluted serially in PSS. Samples were sheared at 1000 s^{-1} on a 25 mm, 0.99° cone and plate rheometer (MCR 301, Anton Paar), and then mixed 50/50 with a luciferase/luciferin solution as an ATP detection method. Photon emission was then counted with a photomultiplier tube (Model R1527P, Hamamatsu) and an ATP stock solution was used to calibrate the results (Forsyth et al., 2011). Five measurements of 20 s duration were performed on each sample. The intensity I of the sheared samples was normalized by the average intensity of two control samples I_o, which underwent the same treatment but were not sheared on the rheometer.

Statistical analysis

One-tailed t-tests were used to compare all results with the control group. Error bars represent +/− one standard error of the mean with the exception of the projected surface area in Fig. 1, in which the error bars represent +/− one standard deviation. Standard error of the mean was not used for the projected surface area results due to the very high sample number, which would result in misleadingly small error bars.

Results

In order to determine the effects of membrane cholesterol content on deformation and ATP release a MβCD solution was used to remove cholesterol from the membranes ((−) chol), and a saturated cholesterol-MβCD solution was used to add cholesterol to the membranes ((+) chol). In order to verify that the membrane cholesterol content was being altered we use the cholesterol-oxidase method as a control,

A

treatment	mg chol/ 100µl packed RBC	µg chol/ mg Protein	% chol
control	0.092	36	100
(+) chol	0.251	98	273
(-) chol	0.037	14	40

B

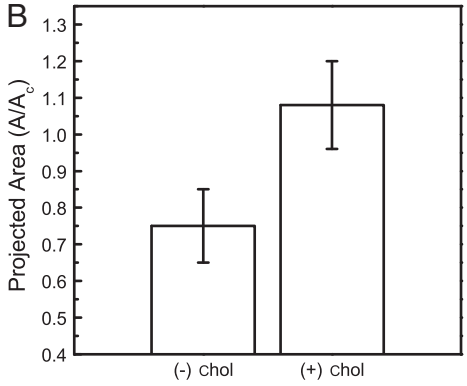

Fig. 1. Verification of altered membrane cholesterol content and the resulting change in cell size. (A) Measurements of the mg cholesterol per 100 µl of packed RBCs and µg cholesterol per mg of protein. Results are shown for PBS controls, and RBCs with cholesterol added and removed. In the third column of the table, the percent cholesterol relative to the PBS control is also reported. (B) The projected cell area of (+) chol and (−) chol cells normalized by the area of control RBCs. N = 104–510; error bars = ± 1 standard deviation.

and we measured (+) chol and (−) chol treated cells to determine the mg cholesterol per 100 µl of packed RBCs (Fig. 1A). All samples were measured in duplicate and the coefficient of variation was not higher than 6%. The (+) chol treated cells had 273% of the cholesterol found in control cells, and the (−) chol treated cells had 40% of the cholesterol found in controls. These results agree closely with other reports that used similar methodology (Cassera et al., 2002; Lange et al., 2004). We note that our (+) chol cells were above the mean value for healthy donors, and above the median value for chronic stable angina patients, but below the median values for patients with acute coronary syndrome (Tziakas et al., 2007).

These changes to the cells were further verified by imaging the cells and measuring their diameter. The reduction of cholesterol drastically reduced the cell size to approximately 75% of the size of control cells (Fig. 1B). The reduction in area made the cells more spherical. To a lesser extent, we were also able to increase the projected surface area of cells in which cholesterol was added.

After experimentally verifying that we had altered cholesterol levels in RBCs, we next investigated the deformability of these cells. We used a microfluidic flow-focusing device that narrows into a constriction while focusing the cells in the center of the channel and increasing the shear rate (Fig. 2A). We found that reducing the cholesterol in the cell membranes ((−) chol) resulted in increased RBC deformability (Fig. 2B). However, deformation measured for (+) chol cells is the same as those on the control samples, which is comparable to the unaltered (+) chol cell size data, despite a large increase in membrane cholesterol (Fig. 1).

Next we sheared the cell solutions on a cone and plate rheometer and used an ATP detection assay to measure extracellular ATP levels. We found that the results for (−) chol cells, normalized by the value for un-sheared (−) chol cells, showed increased ATP release compared with normalized controls and results for the (+) chol cells (Fig. 2C). We performed one-tailed t-tests on all of the results compared to the control, and found that the deformation and ATP release results for the (−) chol samples were significant. The measurements of ATP release from the (+) chol samples did show a slightly reduced, although not significant, result. The increase in ATP levels in the solutions containing cells with reduced cholesterol content, and increased deformability of the same cells, indicates that the membrane cholesterol content plays an important role in ATP release from red blood cells.

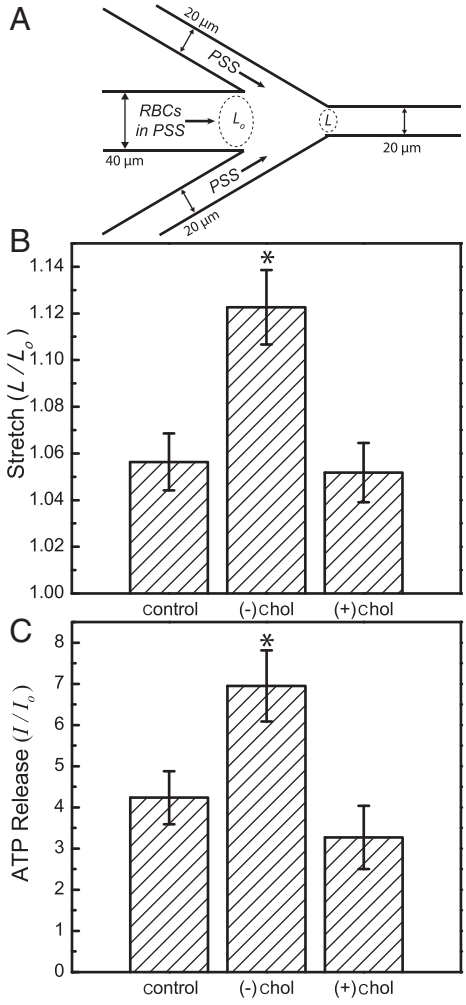

Fig. 2. The effects of cholesterol on RBC deformation and ATP release. (A) A schematic of the microfluidic flow-focusing device. Cells in PSS solution were flowed through the central inlet, and PSS in the side inlets acts to focus the cells in the center of the channel leading to the constriction. The regions are marked where the initial cell length (L_o), and the maximum cell length (L) were measured. (B) Stretch, defined as the maximum cell length divided by the initial cell length, in a microfluidic flow-focusing device, is reported for samples with control, (−) chol, and (+) chol RBCs. N = 20–24. (C) ATP release, following 30 s of shearing on the cone and plate rheometer at 1000 s^{-1}, is reported, relative to unsheared samples, for solutions with control, (−) chol, and (+) chol RBCs. N = 7–8. *p value < 0.05 relative to the control in a one-tailed t-test. Error bars = ± 1 s.e.m.

Next we investigated the effects of simvastatin on RBC deformability and ATP release. We treated normal cells and (+) chol cells with simvastatin (+SS). We found that cells in both samples exhibited increased deformability (Fig. 3A). Also, we found that the (+) chol cells, after treatment with simvastatin, released more ATP than the control sample or normal cells treated with simvastatin (Fig. 3B). Finally, (+) chol cells incubated with simvastatin had a similar response to (−) chol cell, *i.e.* increased deformation and ATP release.

Discussion

It has been reported that deformation of RBCs induces ATP release (Sprague et al., 1998; Wan et al., 2008) and that the deformation is reduced by increased levels of membrane cholesterol (Banerjee et al., 1998; Tziakas et al., 2009). However to our knowledge, no one has linked these two concepts and shown that ATP release from RBCs is also affected by membrane cholesterol. Here we have shown that changes in membrane cholesterol alter cell deformation and ATP release (Fig. 2).

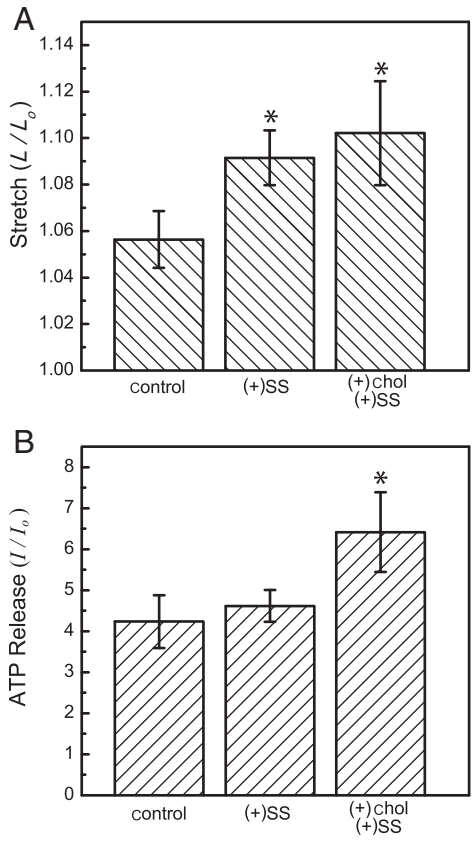

Fig. 3. The effects of simvastatin on RBC deformation and ATP release. (A) Stretch in a microfluidic constriction is reported for solutions with control, (+) simvastatin (SS), and (+) chol (+) SS RBCs. N = 20–28. (B) ATP release for solutions with control, (+) SS, and (+) chol (+) SSRBCs. N = 5–7. *p value < 0.05.Error bars = ± 1 s.e.m.

Moreover, research has associated statin therapy with an increase in RBC deformability. For example, it has been reported that statin therapy reduces cholesterol content of RBC membranes and increases deformability of the RBC (Caliskan et al., 2000; Kohno et al., 1997; Tziakas et al., 2009). Our findings that simvastatin not only increases deformability in the (+) chol cells, but also increases ATP release (Fig. 3), support the hypothesis that *in vivo* statins have an additional pleiotropic effect by causing increased ATP release from RBCs. However, in rats Caliskan et al. (2000) found that simvastatin lowered blood ATP levels, so more work into the *in vivo* implications of altered ATP release from RBCs is still needed.

It has been known that the cytoskeleton plays a key role in RBC's deformation (Evans, 1989; Hochmuth and Waugh, 1987). However, we note that in our experiments deformation of red cells happens on timescales on the order of milliseconds, while early work by Evans and Hochmuth using micropipette aspiration and exposing cells temporarily attached to a substrate to shear flow are performed at timescales several orders of magnitude longer than our experiments. As the cell is viscoelastic, the relevance of the viscosity plays a more important role at shorter timescales. Therefore, it is possible that the viscosity of the membrane plays a more important role in cell deformation.

The (−) chol values in our ATP release and deformation measurements were significantly increased when compared to controls, while the values for (+) chol cells were similar to controls, despite having increased membrane cholesterol content (Figs. 1A and 2). These findings might be explained by the hypothesis that cholesterol forms complexes of varied strength and stoichiometry with different phospholipids of the RBC membrane (Lange et al., 2004; Lange et al., 2007). Therefore, it is likely that membrane stiffness does not increase monotonically as more cholesterol is added, and that there might be a threshold beyond which membrane stiffness does not increase. This threshold is presumably set by the availability of phospholipids forming stable complexes with cholesterol leading to the formation of liquid-ordered domains (lipid rafts) (Maxfield and Tabas, 2005). Thus we assume that for our experiments increased cholesterol in the membrane ((+) chol) does not significantly contribute to the formation or expansion of liquid-ordered domains due to lack of adequate lipid partners for complex formation. Finally, in our experiments, removing membrane cholesterol ((−) chol) decreased the total amount of cholesterol-phospholipid complexes, *i.e.* the total area of liquid-ordered domains, and thus increased membrane fluidity and deformability (Munro, 2003).

It is known that transport properties of volume-regulated anion current channels are modulated by the fluidity of their lipid environment, and thus by the membrane cholesterol content (Levitan et al., 2000). In fact, cholesterol content plays a vital role in the control of the phase transition of membranes by segregation in liquid-ordered and liquid-disordered phase domains (Maxfield and Tabas, 2005). In addition, recently it was reported that simvastatin and other statins have a significant fluidizing effect on lipid membranes in liposomes (Bhandary et al., 2009), and may thus act directly on the RBC membrane. Since we observed that simvastatin increased deformation and ATP release in the (+) chol samples, we suggest that these results are consistent with increased RBC membrane fluidity.

Finally, we hypothesize that in our experiments the simvastatin may be directly altering the fluidity of the membrane, presumably because the bulky, lipophilic simvastatin molecule increases membrane disorder (and thus fluidity) by disrupting cholesterol–phospholipid interactions. This affect may alter the volume-regulated channels, which are involved in the ATP release cascade (Braunstein et al., 2001). It should also be noted that treatment with simvastatin only increased ATP release for the (+) chol cells. It has been shown that uncomplexed cholesterol increased chemical activity with increased potential to diffuse and distribute laterally or across both leaflets of the bilayer (Lange et al., 2004, 2007; Radhakrishnan et al., 2000). We hypothesize that the added cholesterol ((+) chol) is mostly uncomplexed, and chemically active, which increased the disorder of the membrane, resulting in an increased effect by the statin.

Our finding that RBC membrane cholesterol content effects ATP release suggests that individuals with pathological levels of RBC membrane cholesterol may also have altered release of ATP from RBCs. However, further work is needed in order to establish the significance of altered ATP release from RBCs *in vivo*. Our work also suggests that simvastatin can act directly on RBC membranes to change the ATP release cascade. The prevalent use of statins to lower LDL cholesterol and improve cardiovascular health highlights the need for further investigation into the effects of these drugs on the RBC membrane.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.mvr.2012.02.004.

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