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Atorvastatin reduces serum cholesterol and triglycerides with limited improvement in vascular function in adults with sickle cell anemia

Running Title: Atorvastatin in sickle cell disease

Candice Bereal-Williams¹, Roberto F. Machado², Vicki McGowan II¹, Amy Chi¹, Christian J. Hunter¹, and Gregory J. Kato¹

¹Hematology Branch, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA, and ²Division of Pulmonary, Critical Care, Sleep and Allergy, University of Illinois College of Medicine at Chicago, Chicago, IL 60612, USA

Correspondence
Gregory J. Kato, M.D., 9000 Rockville Pike, MSC 1476 Building 10CRC, Room 5-5140 Bethesda, MD 20892-1476. Phone: international +301.4518497. Fax: international +301.4517091. E-mail: gkato@mail.nih.gov
Although red cell rigidity is clearly the principal pathophysiological component of sickle cell anemia (SCA), substantial evidence also implicates disordered blood vessel wall function. Many of these abnormalities, such as vascular dysfunction and associated biomarkers are also seen in atherosclerosis and other vasculopathies, and improve with statin therapy. Besides lowering serum cholesterol, statins indirectly act as Rho kinase inhibitors, which activate endothelial nitric oxide synthase (NOS). NOS-related blood flow increases within one month in hypercholesterolemic patients treated with a low dose of atorvastatin (10 mg daily),¹ and within 24 hours in healthy subjects treated with high dose atorvastatin (80 mg daily).² Statins have shown promise for SCA in the sickle cell mouse,³ in human neutrophils ex vivo,⁴ and in children with sickle cell disease.⁵

SCA subjects were selected from the NIH sickle cell cohort to enrich for vascular dysfunction evidenced by any of the following three characteristics: a higher than median plasma level of soluble vascular cell adhesion molecule (sVCAM-1)(>795 ng/mL; 14 subjects),⁶ or a tricuspid regurgitant velocity (TRV) ≥2.5 m/s by Doppler echocardiography (11 subjects; 2 of the high sVCAM-1 group also had TRV ≥2.5 m/s).⁷ Other inclusion and exclusion criteria were similar to a previous study.⁸ African-American adults without sickle cell trait served as controls only for characterization of baseline blood flow, and did not participate in the treatment study. All subjects signed informed consent forms for a research protocol approved by the institutional review board of the National Heart, Lung and Blood Institute (ClinicalTrials.gov identifier NCT00072826). Data were analyzed with the Prism 4.0 statistical package (GraphPad Software, La Jolla, CA, USA), with Spearman correlations, paired t-test, and one-way or
two-way analysis of variance of the mean (ANOVA) with repeated measures as appropriate, statistical significance assumed at p<0.05.

Twenty-five subjects with SCA (11 male, 14 female; median age 36 years) and 10 healthy subjects (6 male, 4 female; median age 37 years) were enrolled. Detailed laboratory characteristics are provided in Supplemental Table 1. The subjects with SCA demonstrated baseline forearm blood flow (FBF) characteristics that confirmed the previously reported defect in nitric oxide-dependent blood flow (Supplemental Figure 1). Our recruitment strategy successfully enriched for SCA subjects with vascular dysfunction. All enrolled SCA subjects had a blunted vasodilatory response to a nitric oxide (NO) donor compared to the healthy subjects that recapitulates that previously reported in SCA mice, and in about three-quarters of sickle cell anemia patients.

Atorvastatin significantly reduced serum cholesterol, low density lipoprotein cholesterol (LDL-C), and triglyceride levels in a dose dependent manner (Figure 1). These changes were equivalent in both male and female subgroups. There was no change in serum high density lipoprotein cholesterol (HDL-C). These observations indicate successful inhibition of HMG CoA reductase, the intended target of statin drugs, and provide functional evidence of subjects' adherence to study treatment. Despite the statistically significant favorable effect of atorvastatin upon lipid profiles, vasodilatory responses to an NO donor and NOS inhibitor were not affected (Supplemental Figure 2). Because change in NOS inhibitor response was the pre-specified primary outcome variable, this is therefore a negative study. However, some of the important pre-specified secondary outcome variables showed statistically significant changes in
vascular function induced by atorvastatin therapy. Following the combined total of four weeks of atorvastatin treatment, a small, but statistically significant increase in ACh responsiveness was observed, suggesting increased function of endothelium (p=0.002, Supplemental Figure 2C). The vasodilatory response to the highest dose of ACh was examined with and without the higher dose of the NOS inhibitor L-NMMA. Not only was the ACh peak vasodilatory response to ACh greater on atorvastatin (mean ± standard error of the mean 23.0 ± 2.6 vs. 28.9 ± 3.1 mL/min/100mL, p<0.05, paired t-test), the decrease in ACh response elicited by NOS blockade was statistically significant on atorvastatin (28.9 ± 3.1 vs. 23.0 ± 2.6 mL/min/100mL, p<0.001), but not prior to atorvastatin (Figure 2A). This observation was also supported by the relative change in FBF under those conditions (323 ± 45 vs. 241 ± 34% FBF increase from baseline, p=0.001; Figure 2B). There was a marked change in the amount of NOS-dependent, ACh-stimulated blood flow following atorvastatin treatment (0.9 ± 1.7 vs. 5.8 ± 1.4 mL/min/100mL, p=0.02; Figure 2C), suggesting improved endothelial NOS function. In sex subgroup analyses, females consistently demonstrated significantly higher vasodilatory responses to ACh than males, but males and females responded equivalently to atorvastatin. The blood flow results are negative for the primary hypothesis, but the secondary outcome blood flow variables, suggest some degree of favorable response to atorvastatin, which must be interpreted with caution.

Several markers of endothelial activation, inflammation and clinical outcome did not change during the four weeks of atorvastatin treatment, including hemoglobin levels, fetal hemoglobin, lactate dehydrogenase, bilirubin, C-reactive protein, plasma levels of sVCAM-1, monocyte chemokines RANTES and MIP-1b (Supplemental Figure
or tricuspid regurgitant velocity. These results also point to the overall lack of effectiveness atorvastatin 20 mg in improving relevant vascular biomarkers in adults with SCA.

During the four weeks of treatment with atorvastatin, no evidence of toxicity was observed. Serum alanine aminotransferase and creatine kinase levels remained unchanged from baseline. Adverse events during the study included four acute pain episodes, three of which required hospitalization, equivalent to 1.59 hospitalizations per patient-year during enrollment on the study, not different from SCA population statistics. One of these episodes involved transient mental status changes, a small pulmonary infiltrate and mild transient renal insufficiency, all of which resolved after transfusion of two units of packed red blood cells. This patient was removed from the study due to an exclusion criterion of transfusion within two weeks of entry or endpoint.

Much has been written about the pleiotropic effects of statins in the general population to improve clinical outcomes related to vasculopathies such as atherosclerosis. Part of this activity derives from statin activation of NOS activity. It has been speculated that these vasculoprotective effects might be useful in SCA. Our SCA clinical trial of atorvastatin at moderate doses with physiological outcome measures is a negative study for its primary purpose. However, there are still several findings of scientific value that emerge from this project: (1) reproducing previous baseline findings of nitric oxide resistance in SCA; (2) developing an enrichment strategy for recruitment that resulted in 100% of the SCA subjects having characteristics of nitric oxide resistance; (3) secondary outcome variables indicating some evidence that endothelial function is being impacted, albeit to a smaller degree than expected.
This last finding raises the question whether higher doses of atorvastatin or earlier intervention for longer duration should be considered for future investigation to delay vascular dysfunction in SCA patients.

Additional Contributing Authors

Lori Hunter, Carole K. Dalby, Kristine Partovi Hauser, Anitaben Tailor, Richard O. Cannon III (National Heart, Lung and Blood Institute, Bethesda, MD, USA).

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Authorship and Disclosures

G.J.K. and R.O.C. designed the research, analyzed the data and wrote the paper;

R.F.M., C.H. and A.C. assisted in carrying out the forearm blood flow studies; L.H. and
C.K.D. enrolled subjects, coordinated clinical research and collected data; V.M., K.P.H.,
A.T. and M.R. processed biospecimens and performed laboratory assays.

C.B.W. assisted in data management and analysis and writing of the paper.

The authors declare no competing financial interests.
References


FIGURE LEGENDS

Figure 1. Effect of atorvastatin on serum lipid levels. (A) Administration of atorvastatin to 25 subjects with sickle cell disease at 10 or 20 mg daily yielded a dose-dependent significant reduction of total serum cholesterol, triglycerides, and low-density lipoprotein cholesterol (LDL-C). (B) The specific and percentage changes in lipid level are shown. Data points represent mean values and error bars indicate the standard error of the mean. Significance was tested by analysis of the variance of the mean with repeated measures, and post-test for linear trend.

Figure 2. Atorvastatin use is associated with an increase in agonist-induced, NOS-dependent forearm blood flow. Vasodilatory responses to brachial artery infusions of acetylcholine (ACh, 30 mg/min) and the NOS inhibitor L-NG-monomethylarginine (L-NMMA, 8 mmol/min) were measured by venous occlusion strain gauge plethysmography before and after four weeks of atorvastatin administration. Prior to atorvastatin administration (white bars), the L-NMMA-sensitive fraction of ACh response was negligible, but after atorvastatin treatment was statistically significant, expressed in absolute forearm blood flow (A), or as percentage change in blood flow (B). The amount of acetylcholine-induced, NOS dependent blood flow calculated for each subject is significantly increased after atorvastatin treatment (C). Bars indicate means, and error bars indicate standard error of the mean. Significance was tested by paired t-test.
Figure 1

A

![Graph showing the effect of atorvastatin dose on serum lipids. The x-axis represents atorvastatin dose (0, 10, 20 mg), and the y-axis represents serum lipids (mg/dL). The graph shows the decrease in cholesterol, triglycerides, LDL-C, and HDL-C with increasing atorvastatin dose.]

B

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Mean ± SEM Baseline</th>
<th>Atorvastatin 10mg</th>
<th>Atorvastatin 20mg</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>115 ± 4</td>
<td>103 ± 3</td>
<td>94 ± 2</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>% change from baseline</td>
<td>-13%</td>
<td>-21%</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>111 ± 14</td>
<td>92 ± 8</td>
<td>84 ± 8</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>% change from baseline</td>
<td>-17%</td>
<td>-24%</td>
<td></td>
</tr>
<tr>
<td>LDL-C</td>
<td>63 ± 4</td>
<td>50 ± 3</td>
<td>43 ± 3</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>% change from baseline</td>
<td>-21%</td>
<td>-31%</td>
<td></td>
</tr>
<tr>
<td>HDL-C</td>
<td>38 ± 2</td>
<td>38 ± 2</td>
<td>37 ± 2</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>% change from baseline</td>
<td>+2%</td>
<td>-3%</td>
<td></td>
</tr>
</tbody>
</table>

***p<0.0001, one way ANOVA with repeated measures; p<0.0001, post test for linear trend;

* p<0.05, one way ANOVA with repeated measures; p<0.01, post test for linear trend;
Figure 2

A

FBF (mL/min/100 mL)

p < 0.05

p < 0.001

Pre-Atorvastatin
Post-Atorvastatin

Ach
Ach + L-NMMA
Ach
Ach + L-NMMA

B

FBF (% Change)

p = 0.001

Pre-Atorvastatin
Post-Atorvastatin

Ach
Ach + L-NMMA
Ach
Ach + L-NMMA

C

NOS-Dependent FBF (mL/min/100mL)

p = 0.02

Pre-Atorvastatin
Post-Atorvastatin
SUPPLEMENTARY METHODS AND RESULTS

Venous occlusion plethysmography

Forearm blood flow (FBF) measurements were performed by means of strain gauge venous-occlusion plethysmography. Briefly, a mercury-filled Silastic strain gauge was placed around the widest portion of the forearm, and connected to a plethysmograph calibrated to measure the percent change in volume. The plethysmograph is connected to a computer for FBF measurements following inflation of a wrist cuff to suprasystolic pressure to exclude the hand circulation. A blood pressure cuff on the upper arm was inflated to 50 mmHg for 7 seconds with a rapid cuff inflator in order to occlude venous outflow, but not arterial inflow, into the forearm. This causes indiscernible distention of the forearm at a rate proportionate to arterial inflow. A series of 7 sequential blood flow measurements are averaged for each blood flow value. Blood pressure was recorded directly from the intra-arterial catheter immediately after each series of flow measurements.

Subjects with sickle cell disease underwent catheterization of the brachial artery with baseline blood sampling. FBF was measured following 20 minutes of normal saline infused at 1 mL/min into the brachial artery. The endothelium-independent vasodilator sodium nitroprusside was infused at 0.8, 1.6, and 3.2 µg/min respectively, in order to test the vascular responsiveness to an NO donor. After three minutes of each infusion, forearm flows were measured. After a 20-minute rest period, another baseline measurement was obtained and the endothelium-dependent vasodilator acetylcholine was infused at 7.5, 15, and 30 µg/min respectively, to test for endothelial release of relaxant factors including NO. After three minutes of each infusion, forearm flows were measured. Following a 30-minute rest period, the NOS inhibitor L-NMMA was infused at 4 and 8 µmol/min, to test basal production of NO. After three minutes of each infusion, FBF was measured. At the conclusion of the measurements on L-NMMA at 8 µmol/min, combine infusion of L-NMMA 8 µmol/min with acetylcholine at 30 µg/min for 3 minutes was administered, and then FBF was measured. The extent to which L-NMMA reduced acetylcholine induced vasodilation indicate the degree of agonist-stimulated NOS-dependent FBF.
Online Supplementary Table 1. Characteristics of sickle cell anemia (SCA) and control subjects.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Units</th>
<th>SCA (n=25) Mean</th>
<th>SD</th>
<th>Control (n=10) Mean</th>
<th>SD</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>years</td>
<td>36.7</td>
<td>9.6</td>
<td>35.9</td>
<td>8.5</td>
<td>0.82</td>
</tr>
<tr>
<td>Females</td>
<td>fraction</td>
<td>0.56</td>
<td></td>
<td>0.40</td>
<td></td>
<td>0.47</td>
</tr>
<tr>
<td>Hydroxyurea treatment</td>
<td>fraction</td>
<td>0.68</td>
<td></td>
<td>n/a</td>
<td></td>
<td>n/a</td>
</tr>
<tr>
<td>Hydroxyurea dose</td>
<td>mg</td>
<td>1395</td>
<td>773</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Tricuspid regurgitant velocity</td>
<td>m/s</td>
<td>2.5</td>
<td>0.3</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>NT-proBNP</td>
<td>pg/mL</td>
<td>98</td>
<td>116</td>
<td>29</td>
<td>33</td>
<td>0.09</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>mg/L</td>
<td>0.83</td>
<td>0.89</td>
<td>0.37</td>
<td>0.38</td>
<td>0.14</td>
</tr>
<tr>
<td>Creatinine</td>
<td>mmol/L</td>
<td>58</td>
<td>24</td>
<td>78</td>
<td>13</td>
<td>0.02</td>
</tr>
<tr>
<td>Urea</td>
<td>mmol/L</td>
<td>2.5</td>
<td>1.6</td>
<td>3.2</td>
<td>0.9</td>
<td>0.18</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>IU/L</td>
<td>90</td>
<td>44</td>
<td>2</td>
<td>0</td>
<td>0.04</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>IU/L</td>
<td>24</td>
<td>9</td>
<td>23</td>
<td>4</td>
<td>0.55</td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>IU/L</td>
<td>43</td>
<td>18</td>
<td>1</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>IU/L</td>
<td>357</td>
<td>137</td>
<td>147</td>
<td>24</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bilirubin, total</td>
<td>mmol/L</td>
<td>60</td>
<td>38</td>
<td>11</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bilirubin, direct</td>
<td>mmol/L</td>
<td>8.2</td>
<td>5.8</td>
<td>2.4</td>
<td>0.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>10^9/L</td>
<td>9.5</td>
<td>3.9</td>
<td>4.9</td>
<td>1.4</td>
<td>0.008</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>g/L</td>
<td>84</td>
<td>16</td>
<td>132</td>
<td>16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin</td>
<td>fL</td>
<td>99</td>
<td>15</td>
<td>87</td>
<td>8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platelets</td>
<td>10^9/L</td>
<td>308</td>
<td>162</td>
<td>251</td>
<td>69</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>10^9/L</td>
<td>233</td>
<td>117</td>
<td>54</td>
<td>15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fetal hemoglobin</td>
<td>fraction</td>
<td>0.106</td>
<td>0.069</td>
<td>0.003</td>
<td>0.005</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sickle hemoglobin</td>
<td>fraction</td>
<td>0.803</td>
<td>0.078</td>
<td>0.000</td>
<td>0.000</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hemoglobin A</td>
<td>fraction</td>
<td>0.036</td>
<td>0.056</td>
<td>0.967</td>
<td>0.006</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

SCA, sickle cell anemia; NT-proBNP, amino terminal pro-brain natriuretic hormone; SD, standard deviation; n/a, not applicable; nd, not done. Statistical significance calculated by unpaired t-test or Fisher’s exact test as appropriate.
**Online Supplementary Table 2. Selected laboratory markers before and following atorvastatin therapy.**
Values indicate median and interquartile ratios, with significance tested by Kruskal-Wallis test.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Baseline</th>
<th>Atorvastatin 10 mg</th>
<th>Atorvastatin 20 mg</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tricuspid regurgitant velocity (m/sec)</td>
<td>2.4 (2.3, 2.7)</td>
<td>2.5 (2.3, 2.7)</td>
<td>2.5 (2.3, 2.7)</td>
<td>0.8</td>
</tr>
<tr>
<td>Urinary microalbumin (mg/L)</td>
<td>18 (7, 122)</td>
<td>13 (4, 72)</td>
<td>9 (3, 45)</td>
<td>0.6</td>
</tr>
<tr>
<td>NT-proBNP (pg/mL)</td>
<td>60 (15, 95)</td>
<td>76 (28, 129)</td>
<td>64 (38, 110)</td>
<td>0.8</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>6 (4, 9)</td>
<td>5 (2, 8)</td>
<td>5 (2, 9)</td>
<td>0.7</td>
</tr>
</tbody>
</table>
Online Supplementary Figure 1. Altered vascular responsiveness in subjects with sickle cell disease. Prior to initiation of atorvastatin therapy, vasodilatory responses to brachial artery infusions of sodium nitroprusside (SNP), acetylcholine (ACh) and L-NG-monomethylarginine (L-NMMA) were measured by venous occlusion strain gauge plethysmography. Results are presented both in terms of absolute blood flow (A, C, E) and percentage change from baseline (B, D, F). Sickle cell subjects (n=25) showed lower relative responsiveness than African-American controls of comparable age (n=10) to the NO donor SNP (B), although on average hyperresponsiveness to ACh (C, D). The control group included three subjects with atypically low responsiveness to L-NMMA (F). Data points represent mean values and error bars indicate the standard error of the mean. Significance was tested by two-way analysis of the variance of the mean with repeated measures (A-E), or paired t-test (F).
Online Supplementary Figure 2. Effect of atorvastatin on vascular function in sickle cell anemia. Vasodilatory responses to brachial artery infusions of sodium nitroprusside (SNP), acetylcholine (ACh) and L-NG-monomethylarginine (L-NMMA) were measured by venous occlusion strain gauge plethysmography before and after four weeks of atorvastatin administration. Results are presented both in terms of absolute blood flow (A, C, E) and percentage change from baseline (B, D, F). Compared to the pre-study baseline forearm blood flow values, vascular responsiveness to SNP was unchanged by atorvastatin (A, B), but absolute blood flow response to ACh increased by a modest but significant degree, an effect that persisted during L-NMMA infusion (C). Data points represent mean values and error bars indicate the standard error of the mean. Significance was tested by two-way analysis of the variance of the mean with repeated measures (A-E), or paired t-test (F).
Online Supplementary Figure 3. Markers of endothelial and monocyte activation in SCD and controls. Plasma levels were significantly higher than healthy African-American control subjects (n=9) in SCD subjects (n=22) at baseline for (A) soluble vascular cell adhesion molecule-1 (sVCAM-1, \( p<0.001 \), unpaired t-test); (B) monocyte chemokines RANTES (\( p=0.002 \), unpaired t-test); and (C) macrophage inflammatory protein 1β (MIP-1β, \( p<0.001 \), Mann-Whitney test). Following the four week course of atorvastatin in the SCD subjects, no significant changes were observed in these three variables (\( p>0.05 \), Wilcoxon matched-pairs signed rank test or unpaired t-test).