Antioxidant Vitamins C and E Improve Endothelial Function in Children With Hyperlipidemia
Endothelial Assessment of Risk from Lipids in Youth (EARLY) Trial
Marguerite M. Engler, PhD; Mary B. Engler, PhD; Mary J. Malloy, MD; Elisa Y. Chiu, RN, MS; Monique C. Schloetter, RD, MS; Steven M. Paul, PhD; Markus Stuehlinger, MD; Ken Y. Lin; John P. Cooke, MD, PhD; Jason D. Morrow, MD; Paul M Ridker, MD; Nader Rifai, PhD; Elizabeth Miller; Joseph L. Witztum, MD; Michele Mietus-Snyder, MD

**Background**—Hyperlipidemia is associated with endothelial dysfunction, an early event in atherosclerosis and predictor of risk for future coronary artery disease. Epidemiological studies suggest that increased dietary intake of antioxidants reduces the risk of coronary artery disease. The purpose of this study was to determine whether antioxidant vitamin therapy improves endothelial function and affects surrogate biomarkers for oxidative stress and inflammation in hyperlipidemic children.

**Methods and Results**—In a randomized, double-blind, placebo-controlled trial, the effects of antioxidant vitamins C (500 mg/d) and E (400 IU/d) for 6 weeks and the National Cholesterol Education Program Step II (NCEP-II) diet for 6 months on endothelium-dependent flow-mediated dilation (FMD) of the brachial artery were examined in 15 children with familial hypercholesterolemia (FH) or the phenotype of familial combined hyperlipidemia (FCH). Antioxidant vitamin therapy improved FMD of the brachial artery compared with baseline ($P<0.001$) without an effect on biomarkers for oxidative stress (autoantibodies to epitopes of oxidized LDL, F$_2$-isoprostanes, 8-hydroxy-2'-deoxyguanosine), inflammation (C-reactive protein), or levels of asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide.

**Conclusions**—Antioxidant therapy with vitamins C and E restores endothelial function in hyperlipidemic children. Early detection and treatment of endothelial dysfunction in high-risk children may retard the progression of atherosclerosis. (**Circulation. 2003;108:1059-1063.**)

**Key Words:** lipids ■ nutrition ■ pediatrics ■ prevention

Hypercholesterolemia is associated with endothelial dysfunction, an early manifestation of subclinical atherosclerotic disease and predictor of risk for coronary heart disease (CHD) in adults. Endothelial dysfunction is evident in children and adults with familial hypercholesterolemia (FH) and the familial combined hyperlipidemia phenotype (FCH).

The endothelium preserves vascular integrity and prevents atherosclerosis by modulating vasomotor tone, platelet activity, thrombosis, and inflammation. Increased vascular oxidative stress in hypercholesterolemia contributes to impaired endothelial function and atherogenesis. Endothelial dysfunction is characterized by reduced bioavailability of nitric oxide (NO) through decreased production and/or increased degradation of NO in oxidative stress. Reactive oxygen-derived free radicals may promote LDL oxidation in the vascular wall and attenuate endothelium-dependent vasodilation. Recent studies indicate that endothelial dysfunction of the coronary and brachial arteries is associated with future adverse cardiovascular events. Epidemiological evidence suggests that increased intake of dietary antioxidants reduces the risk for CHD. Antioxidant vitamins may provide vascular defense against oxidative stress by scavenging free radicals and protecting NO from inactivation. Antioxidant administration improves endothelial function of coronary and peripheral vessels in hypercholesterolemia and CHD. A previous study also suggests that antioxidants may improve endothelial health in hyperlipidemic children. Dietary strategies to reduce fat intake with the National Cholesterol Education Program Step II (NCEP-II) diet have effectively lowered LDL cholesterol levels in children. Marked reductions in elevated cholesterol levels have been shown to improve endothelial function in hypercholesterolemic adults. Moreover, the combination of cholesterol-lowering and antioxidant thera-
ties have been shown to be synergistic in improving endothelial function. 18,19

Biomarkers for oxidative stress and inflammation may be helpful indicators of the progression of subclinical atherosclerosis in hyperlipidemia. In vivo oxidation of LDL can be assessed by autoantibodies to model epitopes of oxidized LDL (OxLDL) including malondialdehyde-LDL and copper-OxLDL, 20 but their predictive value is unclear. Elevated plasma levels of OxLDL (eg, LDL particles containing oxidation-specific epitopes) have been directly correlated with the severity of CHD. 21 IgG autoantibody titers to epitopes of OxLDL have also been correlated with disease, 9,22 but titers of IgM autoantibodies have been inversely correlated with severity of carotid disease. 23 The etiology of such minimally oxidized LDL particles in plasma LDL has not been defined but may represent modified LDL released from ruptured plaque, damaged cell membranes, or nonatherosclerotic inflammatory sources. 20 Two other promising biomarkers for oxidative stress are plasma F 2 -isoprostanes, 24 products of lipid peroxidation, and urinary 8-hydroxy-2'-deoxyguanosine (8-OH2'dG), an adduct of oxidatively damaged DNA. 25 High resolution ultrasound assessment of brachial artery reactivity provides a noninvasive technique to evaluate the consequences of vascular oxidative stress on endothelial function. 2

It is increasingly evident that inflammation plays a critical role in atherogenesis and C-reactive protein (CRP) may be a useful marker for individuals at high risk for future CHD. 26 Circulating levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase, may also provide information to assess underlying vascular disease. ADMA levels are elevated in hypercholesterolemia and associated with decreased NO production and endothelial dysfunction. 27

The aim of the study was to determine the effects of antioxidative vitamin therapy and the NCEP-II diet on endothelial function as well as on surrogate biomarkers for oxidative stress and inflammation. Direct noninvasive imaging of the vessel wall and potential biomarkers in asymptomatic high-risk children may provide important information for early diagnosis, prevention, and treatment of atherosclerotic disease. 22

Methods

Subjects

The study consisted of 15 subjects (7 female, 8 male) with hyperlipidemia (6 FH, 9 FCH), between the ages of 9 and 20. Four other subjects were excluded due to noncompliance with vitamin therapy. Inclusion criteria were (1) age 8 to 21 years and (2) FH or FCH. FH was characterized by LDL levels >130 mg/dL and a parent diagnosed with this disorder. FCH was characterized by elevated levels of LDL (>130 mg/dL) or triglycerides (>150 mg/dL) or both, and at least one parent presenting with any of these 3 phenotypes. Exclusion criteria were (1) systemic illness with or without secondary hyperlipidemia and (2) current smoking. This study was reviewed and approved by the institutional review board. Parental informed consent and child assent were obtained.

Study Design

A 6-month randomized, double-blind, placebo-controlled crossover study design was used and supplements included either vitamin C (250 mg) and vitamin E (200 IU) twice daily or placebo (Great Earth Companies, Inc) (Figure 1). Nutritional counseling incorporated dietary recommendations based on the food guide pyramid and NCEP-II guidelines.

Dietary intake was assessed using three nonconsecutive 24-hour dietary recalls at each visit. Nutrient intake was determined by use of Food Processor Plus, Nutrition Analysis and Fitness Software version 7.5 (ESHA Research). Compliance with supplements was assessed by pill counts. Blood and urine samples were obtained at baseline and every 6 weeks after an overnight fast. Blood pressure was also measured after rest in supine position for 5 minutes.

Endothelial Function

Endothelium-dependent FMD of the brachial artery was assessed once every 6 weeks (Figure 1) after 5 to 10 minutes of rest supine in a darkened room. All studies were performed by a single investigator blinded to treatment assignment using a 15-MHz linear array vascular transducer and a Sequoia C256 ultrasound system (Acuson) as previously described. 28 FMD was expressed as the peak change in arterial diameter from baseline within 2 minutes of hyperemia. Accuracy and reproducibility of measurements was verified by repeat baseline assessment by a second sonographer. The correlation between the two measurements was 1.0.

Laboratory Measurements

Blood was drawn into EDTA tubes and immediately stored on ice until centrifugation at 2000 rpm for 20 minutes at 4°C. Cholesterol was determined in plasma and lipoprotein fractions by an enzymatic technique and triglycerides were determined by a glycerokinase reaction as described previously. 29 The lipoprotein (a) content in plasma samples was determined by ELISA. 30 Plasma titers of IgM and IgG autoantibodies binding to MDA-LDL and Cu-OxLDL were determined by chemiluminescence ELISA as previously described. 31 Plasma levels of OxLDL-E06 (a measure of minimally oxidized LDL, determined as the content of oxidized phospholipids measured by monoclonal antibody E06 per apoB-100), were measured as previously described. 32

Plasma concentrations of ADMA were determined by high-performance liquid chromatography (HPLC) using a modification of a previously described method. 33

Figure 1. Outline of study design.
Clinical Characteristics and Biochemical Parameters

<table>
<thead>
<tr>
<th>Variables</th>
<th>Baseline</th>
<th>NCEP-II Diet</th>
<th>Placebo + NCEP-II Diet</th>
<th>Antioxidants + NCEP-II Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>112±12</td>
<td>114±11</td>
<td>116±12</td>
<td>117±11</td>
</tr>
<tr>
<td>Diastolic</td>
<td>59±9</td>
<td>58±9</td>
<td>58±8</td>
<td>58±7</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25±8</td>
<td>25±8</td>
<td>25±8</td>
<td>25±8</td>
</tr>
<tr>
<td>Brachial artery baseline diameter, mm</td>
<td>3.6±0.6</td>
<td>3.6±0.6</td>
<td>3.6±0.5</td>
<td>3.6±0.6</td>
</tr>
<tr>
<td>Flow-mediated dilation, %</td>
<td>5.7±2.9</td>
<td>6.1±1.4</td>
<td>5.9±2.0</td>
<td>9.5±4.2*</td>
</tr>
<tr>
<td>Cholesterol, mg/dL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>242±51</td>
<td>231±52</td>
<td>235±58</td>
<td>237±58</td>
</tr>
<tr>
<td>LDL</td>
<td>187±49</td>
<td>172±48†</td>
<td>173±53†</td>
<td>176±54</td>
</tr>
<tr>
<td>VLDL</td>
<td>20±11</td>
<td>18±11</td>
<td>18±11</td>
<td>20±18</td>
</tr>
<tr>
<td>HDL</td>
<td>37±10</td>
<td>40±10</td>
<td>42±9</td>
<td>41±9</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>145±65</td>
<td>137±65</td>
<td>143±67</td>
<td>148±110</td>
</tr>
<tr>
<td>Lipoprotein (a), nmol/L</td>
<td>185±140</td>
<td>190±154</td>
<td>196±148</td>
<td>208±151</td>
</tr>
<tr>
<td>OxLDL autoantibody titer†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA-LDL IgG</td>
<td>4945±3393</td>
<td>4620±2872</td>
<td>5213±3795</td>
<td>5385±3972</td>
</tr>
<tr>
<td>MDA LDL IgM</td>
<td>20 008±7236</td>
<td>17 842±5448</td>
<td>18 336±5894</td>
<td>19 623±7197</td>
</tr>
<tr>
<td>Cu-OxLDL IgG</td>
<td>4059±3001</td>
<td>4028±2748</td>
<td>4272±3089</td>
<td>4324±2874</td>
</tr>
<tr>
<td>Cu-OxLDL IgM</td>
<td>11 516±5781</td>
<td>10 524±5250</td>
<td>10 822±5243</td>
<td>11 181±5663</td>
</tr>
<tr>
<td>OxLDL-E06 levels</td>
<td>10 123±9528</td>
<td>9536±10021</td>
<td>11 560±12345</td>
<td>12 145±12279</td>
</tr>
<tr>
<td>ADMA, μmol/L</td>
<td>1.00±0.38</td>
<td>0.91±0.37</td>
<td>0.87±0.40</td>
<td>1.10±0.85</td>
</tr>
<tr>
<td>F₂-isoprostanes, ng/mL§</td>
<td>0.073±0.026</td>
<td>0.060±0.015</td>
<td>0.068±0.023</td>
<td>0.064±0.017</td>
</tr>
<tr>
<td>C-reactive protein, mg/dL§</td>
<td>0.17±0.27</td>
<td>0.22±0.33</td>
<td>0.31±0.42</td>
<td>0.21±0.29</td>
</tr>
<tr>
<td>Urinary 8-OH-2’-dG, μg/g creatinine</td>
<td>3.0±1.1</td>
<td>2.6±0.84</td>
<td>2.7±0.95</td>
<td>3.1±1.3</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; MDA, malondialdehyde; Cu, copper; OxLDL, oxidized LDL; ADMA, asymmetric dimethylarginine.

Values represent mean±SD; n=15.

*P<0.001 vs baseline.
†P<0.01 vs baseline.
‡Autoantibody titers and OxLDL-E06 levels are expressed as relative light units/100 ms.
§In addition to mean±SD, the median and interquartile range values are presented.

Sample Size Calculation and Statistical Analysis

A sample size of 15 was sufficient to detect a difference of 3.2% in FMD, with a power of 80% using a paired t test with a 0.017 two-sided significance level. Statistical analysis was performed using SPSS 11.0 for Windows (SPSS Inc). Phases were compared by one-way repeated measures analysis of variance. If the overall F statistic was significant, pairwise within-subject contrasts comparing each phase to baseline were performed using the Bonferroni procedure. If results of the Mauchly test of sphericity were significant, then the Greenhouse-Geisser corrected tests were used.

Results

The average daily intake of total and saturated fats at baseline exceeded the NCEP-II guidelines at 32±9% and 11±4%, respectively. Intake of monounsaturated and polyunsaturated fat, cholesterol, carbohydrates, and protein were within the NCEP-II guidelines. After the dietary intervention, intake of total fat was reduced to 28±6%, saturated fat to 8±3%, monounsaturated fat to 7±2%, and cholesterol to 168±94 mg/d (NS). The average reported daily intake of calories also decreased from 1966±938 at baseline to 1729±685 with diet (NS). Nutrient intakes of polyunsaturated fat, carbohydrates, and protein were unchanged from baseline.

Clinical characteristics and biochemical parameters of hyperlipidemic subjects were similar at each phase of the study (Table). The primary effect of the NCEP-II diet was an 8% reduction in LDL cholesterol (P<0.01), which was generally sustained throughout the intervention. There were no significant effects of diet or antioxidants on other lipid levels or biomarkers of oxidative stress, inflammation, or ADMA.

Baseline diameter and changes in FMD of the brachial artery in response to the diet and antioxidant interventions are summarized in the Table. There was no significant change in FMD as a result of diet alone. However, FMD increased significantly from baseline (5.7±2.9%) in response to antioxidants and diet (9.5±4.2%; P<0.001) (Figure 2).
Discussion

The results of our study indicate that supplementation with antioxidant vitamins C and E restores endothelial function in hyperlipidemic children. Normal FMD of the brachial artery in children is reportedly between 8% and 12%. We detected diminished FMD in our young subjects at baseline, suggestive of endothelial dysfunction and consistent with our previous work and the findings of others. These findings have important implications for vascular health because attenuated FMD of the brachial artery is predictive of endothelial dysfunction in the coronary arteries and presents decades before the onset of symptomatic CHD. Moreover, impairment of endothelial vasodilator responses assessed by ultrasound precedes the appearance of atherosclerotic intimal lesions. It was recently shown that statin therapy can improve endothelial function in hypercholesterolemic children, but our report is the first randomized clinical trial to show that moderate doses of antioxidant vitamins C and E can restore abnormal FMD in such children as well. Our results are consistent with previous studies, in which higher doses of antioxidants improved endothelial function in both hyperlipidemic children and adults. The dose of vitamin C used in the present study is readily achievable with whole foods, but only 20% of children consume the recommended 5 or more servings of fruits and vegetables daily. Rich sources of vitamin E such as whole grains and nuts are also deficient in the typical American child’s diet. Whether the vitamin intake is achieved by improved dietary strategies or by vitamin supplementation, our study suggests a novel and simple strategy to improve the long-term cardiovascular health of hyperlipidemic children.

The reduction in LDL levels after an educational and behavioral intervention is concordant with the results of the DISC trial in hyperlipidemic children. LDL reductions alone confer benefits in decreasing the risk of future CHD but no improvement in endothelial function was observed with our modest 8% reduction in LDL achieved by diet.

Increasing evidence suggests an association between hypercholesterolemia, increased oxidative stress, and endothelial dysfunction. Putative mechanisms for endothelial dysfunction include inefficient utilization of L-arginine substrate, impaired signal transduction of membrane receptors that enhance endothelial NO production, and increased ADMA concentrations that inhibit NO synthase and subsequently decrease NO synthesis. Under conditions of oxidative stress, reactive oxygen species (ROS) involved in oxidative modification of LDL also degrade NO.

The precise mechanisms by which antioxidants modulate endothelial function are unclear. Antioxidants may be protective against oxidative stress and preserve NO by scavenging ROS and inhibiting OxLDL formation. Vitamin E alone decreases ROS and apoptosis in endothelial cells induced by OxLDL. Moreover, the combination of vitamins C and E has been shown to protect endothelial cells from the cytotoxic effects of Ox-LDL. Antioxidants may also enhance endothelial NO synthase (eNOS) activity. A previous study showed that eNOS activity is impaired in hypercholesterolemia. Loss of eNOS in the endothelium coupled with impaired NO production in hypercholesterolemic vessels may potentiate endothelial dysfunction.

Recent evidence suggests that antioxidants decrease activation of redox-transcription factors and increase eNOS expression in human endothelial cells and atherosclerotic-prone areas in hypercholesterolemic LDL receptor knockout mice. Antioxidants may alter redox genes and reduce free radical-induced inflammatory responses in the endothelium. Vitamin E (400 IU daily for 8 weeks) reportedly increases NO concentrations in hypercholesterolemic adults. Moreover, vitamin C may stabilize the NOS cofactor tetrahydrobiopterin, which is associated with an increase in endothelial NO synthesis.

Strengths of the present study include the randomized, placebo-controlled study design and investigation of biomarkers in hyperlipidemic children. A potential limitation of the study is the small sample size, which warrants careful extrapolation of our results to other populations.

In conclusion, we have demonstrated that moderate daily doses of vitamins C and E restore endothelial function in hyperlipidemic children. This effect was most likely mediated at the level of the vascular wall and in our short term study was not associated with changes in plasma biomarkers of oxidative stress, inflammation, or ADMA. Antioxidant vitamins C and E may improve endothelial function by increasing local NO bioavailability and may therefore retard the progression of atherosclerosis in high-risk children. The intervention is economical and well-tolerated, and if confirmed in further studies, could have important long-term beneficial effects to prevent cardiovascular complications in children with hyperlipidemias.

Acknowledgments

This research was supported by NIH grants NR 04902, GM 15431, and DK 48831 (to Dr Morrow) and by gifts from the Valentine Foundation, and Judge and Mrs. Mana. Dr Morrow is the recipient of a Burroughs Wellcome Fund Clinical Scientist Award in Translational Research. The studies were also performed in the Pediatric Clinical Research Center funded by grant M01 RR-01271 and in the General Clinical Research Center, University of California, San Francisco, funded by grant M01-00079, National Center for Research Resources, USPHS. We gratefully thank the nurses and laboratory staff in the PCRC and the dietetic staff of the GCRC at UCSF Medical Center for their exceptional assistance with this study. We give special thanks to Great Earth Vitamins for kindly providing the antioxidant vitamin supplements.
Antioxidants Improve Endothelial Function

References


Antioxidant Vitamins C and E Improve Endothelial Function in Children With Hyperlipidemia: Endothelial Assessment of Risk from Lipids in Youth (EARLY) Trial
Marguerite M. Engler, Mary B. Engler, Mary J. Malloy, Elisa Y. Chiu, Monique C. Schloetter, Steven M. Paul, Markus Stuehlinger, Ken Y. Lin, John P. Cooke, Jason D. Morrow, Paul M Ridker, Nader Rifai, Elizabeth Miller, Joseph L. Witztum and Michele Mietus-Snyder

_Circulation_. 2003;108:1059-1063; originally published online August 11, 2003; doi: 10.1161/01.CIR.0000086345.09861.A0
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/108/9/1059

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/