Determinants of Arterial Nitrate-Mediated Dilatation in Children
Role of Oxidized Low-Density Lipoprotein, Endothelial Function, and Carotid Intima-Media Thickness

Mikko J. Järvisalo, MD, PhD; Terho Lehtimäki, MD, PhD; Olli T. Raitakari, MD, PhD

Background—Impaired arterial dilatation response to nitroglycerin has been observed in adults with risk factors for atherosclerosis and in patients with established atherosclerotic disease. This defect parallels changes in vascular endothelial function and may be attributed to increased oxidative stress. Because atherosclerosis begins in childhood, we examined the correlates of nitrate-mediated dilatation (NMD) in children, including brachial artery endothelial function, oxidized LDL, and carotid artery intima-media thickness (IMT).

Methods and Results—Brachial artery flow-mediated endothelium-dependent dilatation (FMD) and nitrate-mediated smooth muscle function, IMT of the carotid bulb, and brachial artery and oxidized LDL were measured in 142 children (mean age, 11 years; range, 8 to 17 years), including 87 healthy children, 41 diabetic children, and 14 children with familial hypercholesterolemia. NMD correlated directly with FMD (r=0.46, P<0.001) and inversely with brachial artery baseline diameter (r=−0.36, P<0.001), age (r=−0.25, P=0.003), body mass index (r=−0.31, P<0.001), diabetes (r=−0.22, P=0.008), oxidized LDL (r=−0.18, P=0.045), and IMT (r=−0.33, P<0.001). In a multivariate regression model, the significant correlates for NMD were FMD response (β=0.003, P<0.001), brachial artery diameter (β=−0.03, P=0.01), oxidized LDL (β=−0.07, P=0.02), and IMT (β=−0.15, P=0.03).

Conclusions—Reduced endothelial function, increased oxidative stress, and preclinical carotid atherosclerosis are independent determinants of impaired NMD in children. These data thus suggest that primary nitrate tolerance occurs in children at risk for atherosclerosis. (Circulation. 2004;109:2885-2889.)

Key Words: arteries ■ diabetes mellitus ■ endothelium ■ nitroglycerin ■ Pediatrics

Endothelial dysfunction is currently considered a key early event in the atherosclerotic process already evident in children with cardiovascular risk factors, such as hypercholesterolemia1 and diabetes.2 The brachial artery ultrasound test for flow-mediated endothelium-dependent vasodilatory function (FMD), described by Celermajer et al,1 includes administration of sublingual nitrates to examine the vasodilating effect of an exogenous source of nitric oxide (NO). NO acts directly at the level of the arterial smooth muscle cells and produces an endothelium-independent dilatation response. Nitrate-mediated dilatation (NMD) has therefore been used as a control test for the FMD measurement to ensure that a decreased FMD capacity observed is truly a consequence of endothelial dysfunction and not a reflection of underlying smooth muscle dysfunction.

Recent evidence indicates that in addition to influencing endothelial function, atherosclerosis may also induce changes in arterial dilatation responses to exogenous NO.3,4 Adult patients with coronary artery disease show impaired brachial NMD compared with healthy control subjects.4 Attenuated NMD is associated with serum cholesterol concentration independently of endothelial function in apparently healthy adults.3 In most previous studies in children, the NMD responses have actually been mildly reduced in high-risk individuals, although the difference has not reached statistical significance, possibly because of limited sample size.1,2,5

Increased oxidative stress and oxidized LDL have been implicated as risk factors for atherosclerosis6 and shown to potentely quench NO7 and decrease its production8 in experimental studies. Recent studies have associated oxidative stress with nitrate tolerance.9 Because the atherosclerotic process begins in childhood,10 we examined the correlates of NMD in children, including brachial artery endothelial function, oxidized LDL, and carotid artery intima-media thickness (IMT).

Methods

Subjects
We studied 142 children (83 boys; mean age, 11 years; range, 8 to 17 years), including 41 children with diabetes and 14 children with familial hypercholesterolemia (FH). All subjects were nonsmokers.
Children with diabetes were consecutively recruited from the outpatient clinic of the Department of Pediatrics, Turku University Central Hospital. They met inclusion criteria if they were between 7 and 14 years of age, had a duration of diabetes >6 months, were normotensive, and had no chronic diseases other than type 1 diabetes. The mean duration of diabetes was 4.4 ± 2.9 years. None of the children with diabetes were taking regular medications other than daily insulin. The daily insulin dose was 0.97 ± 0.26 IU/kg (range, 0.62 to 1.53 IU/kg). None of the patients with diabetes had evidence of microvascular complications, such as diabetic retinopathy, nephropathy, or microalbuminuria. In the group with diabetes, the mean glycated hemoglobin (HbA1c) level was 8.9 ± 1.4% (range, 6.2% to 12.8%; reference range, 4.2% to 6.0%) and ratio of urinary albumin to urinary creatinine was 0.80 ± 0.53 mg/mmol. Participants did not differ in any clinical characteristics from the whole eligible diabetic clinical population of the same age.

The children with FH were consecutively recruited from the outpatient clinic of the Department of Pediatrics, Turku University Central Hospital. Every child with FH had a serum total cholesterol level >6.0 mmol/L or LDL cholesterol level >4.5 mmol/L, but none of the children had tendon xanthoma, arcus lipoids, or xanthelasma. FH had been confirmed by lymphocyte testing. Ten of the children with FH were taking lipid-lowering medication, including 7 taking statins (simvastatin 40 mg, 1; simvastatin 5 mg, 1; lovastatin 40 mg, 2; lovastatin 20 mg, 3) and 3 undergoing bile acid sequestrant treatment with cholestyramine 4 to 8 g/d. Four of the children were undergoing diet therapy only.

The healthy control children included in the study were friends of the diabetic children studied, children of Turku University or Turku University Central Hospital staff members, or children participating in an ongoing trial aimed at decreasing children’s exposure to known environmental atherosclerotic risk factors. Written informed consent was acquired from the legal guardians of the children. The study was conducted according to the Declaration of Helsinki, and the Joint Commission on Ethics of Turku University and Turku University Central Hospital approved the study protocol.

Ultrasound Studies

All studies were undertaken in the morning, with fasting subjects, using an Acuson Sequoia 512 mainframe (Acuson) and a 13.0-MHz linear-array transducer.

Brachial artery diameter was measured from ultrasound images, as described earlier. For the measurement of FMD, scans of the left brachial artery 5 cm above the elbow were obtained at rest and during reactive hyperemia induced by inflating and deflating a forearm blood pressure cuff (250 mm Hg, 4.5 minutes). Scans were recorded on super-VHS tapes for offline analysis. Vessel diameter was measured by a blinded reader using ultrasonic calipers at a fixed distance from an anatomic marker. Measurements were taken at end-diastole, coincident with R-wave on ECG, every 10 seconds between 40 and 120 seconds and every 15 seconds between 120 and 180 seconds after cuff release (a total of 13 measurement points). The maximal dilatation from baseline (peak FMD, %) and area under the FMD curve (FMDAUC, percent multiplied by seconds) were calculated. NMD capacity was tested by administering 4 consecutive sublingual 50-µg doses of glyceryl trinitrate (GTN), all 5 minutes apart (cumulative dose, 200 µg). Brachial artery diameter was measured 5 minutes after each dose to acquire a dose-response curve. Maximal proportional increase in diameter from the baseline value during the test was calculated, as well as the area under the dose-response curve. In our laboratory, the interobserver and intraobserver variations of FMD measurements were 8.6% and 6.2%, respectively.

For the measurement of the IMT of the left carotid bulb, the bulb region was first scanned carefully in many interrogation angles to identify the beginning of the bulb and the location of the maximal IMT. The scan was focused on the posterior (far) wall, and resolution box function was used to magnify the arterial far wall. Several images of carotid bulb far wall were acquired. All scans were digitally stored on the ultrasound system internal hard disk for subsequent offline analysis. Two end-diastolic frames with the greatest IMT were selected and analyzed for mean IMT, and the average reading from these 2 frames was calculated. Six to 12 measurements of IMT were taken by 2 independent and experienced readers blinded to the subject’s clinical details. Average values of the 2 readers were used in the analyses.

Brachial IMT was measured offline from digitized B-mode ultrasound images acquired 5 cm proximal to the antecubital fossa. Resolution box function was used to magnify a brachial artery far-wall segment of 1 cm in width. The images were captured at end-diastole, incident with the R-wave on a continuously recorded ECG, and digitally stored for offline analyses. Six to 8 measurements were taken, covering the entire segment of arterial wall, by a single blinded observer. The average of these measurements was used as a measure of mean brachial IMT in the analyses.

Serum Lipids and Oxidized LDL

Venous blood samples were taken in the morning, after an overnight fast (10 to 12 hours). Serum total cholesterol, HDL cholesterol, and triglyceride concentrations were measured using standard enzymatic methods with the use of Boehringer Mannheim GmbH reagents, with a fully automated analyzer (Hitachi 917; Hitachi Ltd). LDL cholesterol concentration was calculated using the Friedewald equation. Serum apolipoprotein B was measured using immunophelometry (Behring Nephelometer II, Dade Behring Inc). HbA1c was measured with high-performance liquid chromatography (Variant Analyser, Bio-Rad). Thawed samples were used for the determination of oxidized LDL by a capture ELISA (also known as a sandwich ELISA) according to manufacturer’s instructions (Mercodia AB). Shortly thereafter, microtiter plates were coated with the specific murine monoclonal antibody, mAb-4E6. During incubation, oxidized LDL in the sample reacted with the mAb-4E6 antibodies bound to the wells. After washing steps, a peroxidase-conjugated antiapolipoprotein B antibody was used to recognize the oxidized LDL. Finally, the bound conjugate was detected by reaction with 3,3’,5,5’-tetramethylbenzidine, and the reaction was stopped by adding acid to give a colorimetric end point that was read spectrophotometrically at 450 nm. The detection limit of the method is <1 µg/L according to the manufacturer.

Statistical Methods

Results are expressed as mean ± SD. Univariate associations between the study variables were analyzed by calculating the Spearman’s correlation coefficients. Multivariate analyses were done using the linear regression technique. To examine whether disease group (healthy versus diabetes mellitus versus FH) modifies the associations between risk factors and NMD, we included group x risk factor interaction terms in the regression models. The associations between risk factors and NMD were not different between the groups (all interaction terms, P > 0.4). All statistical analyses were performed using the statistical analysis system SAS.
were excluded from the analyses, the association between NMD and age ($r = -0.35$, $P = 0.001$), body mass index ($r = -0.32$, $P < 0.001$), and IMT ($r = -0.24$, $P = 0.028$) persisted, but the association between NMD and oxidized LDL was abolished ($r = -0.17$, $P = 0.16$). In all subjects pooled together, FMD and NMD were directly correlated (Table 2, Figure).

In the multivariate analysis including all study subjects, the independent predictors of a reduced NMD response included low FMD, oxidized LDL, and increased IMT. Similar results were obtained when girls and boys were examined separately (data not shown).

Discussion

The present study demonstrates that NMD, the vasodilatory response to an exogenous source of NO, is closely associated with endothelial function in children. The independent determinants of impaired NMD response included low FMD, increased oxidized LDL, and higher IMT. These novel findings suggest that decreased dilatation response to exogenous NO donors occurs in children at increased risk for atherosclerosis.

Endothelial dysfunction is an early event in atherosclerosis. It is generally thought that in the early stages of atherosclerosis, the endothelium-independent NMD responses remain unaltered. In most previous small-scale studies in children and in adults, however, the NMD responses have actually been mildly reduced in high-risk individuals, although this result has not always reached statistical significance. Adams et al have previously shown in a large population-based sample of adults that an attenuated response to GTN associates with cardiovascular risk factors independently of endothelial dysfunction. In addition, Raitakari et al have recently demonstrated that patients with coronary artery disease have impaired NMD response compared with healthy controls. In that study, the difference in NMD between the groups was evident with subplateau doses of GTN. Therefore, it is likely that small sample sizes and the use of full antianginal doses of nitrates that induce a plateau arterial dilatation have led to the misinterpretation that NMD would not be compromised in individuals at increased risk for atherosclerosis. In the present study, we administered 4 consecutive 50-$\mu$g doses of GTN to acquire a dose-response

<table>
<thead>
<tr>
<th>Variable</th>
<th>Maximal NMD</th>
<th>AUC</th>
<th>FMADAU C</th>
</tr>
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<tbody>
<tr>
<td>FMDAUC</td>
<td>0.46</td>
<td>&lt;0.001</td>
<td>0.45</td>
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<tr>
<td>Oxidized LDL</td>
<td>−0.18</td>
<td>0.045</td>
<td>−0.14</td>
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<tr>
<td>Total cholesterol</td>
<td>−0.15</td>
<td>0.070</td>
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<td>Diabetes</td>
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<td>−0.25</td>
</tr>
<tr>
<td>Age</td>
<td>−0.25</td>
<td>0.003</td>
<td>−0.22</td>
</tr>
<tr>
<td>Body mass index</td>
<td>−0.31</td>
<td>&lt;0.001</td>
<td>−0.27</td>
</tr>
<tr>
<td>IMT</td>
<td>−0.33</td>
<td>&lt;0.001</td>
<td>−0.33</td>
</tr>
<tr>
<td>Brachial IMT</td>
<td>−0.19</td>
<td>0.022</td>
<td>−0.17</td>
</tr>
<tr>
<td>Vessel size</td>
<td>−0.36</td>
<td>&lt;0.001</td>
<td>−0.32</td>
</tr>
</tbody>
</table>

AUC indicates area under the NMD curve.
able to similar risk factor influences on these measures of arterial function.

Our present results demonstrate that NMD is inversely associated with oxidized LDL, a marker of increased oxidative stress. Under physiological conditions, there is a balance between endothelial release of NO and vascular oxygen-derived free radicals. Various conditions associated with atherosclerosis and endothelial dysfunction (eg, hypertension, hypercholesterolemia, diabetes, and smoking) are characterized by increased oxidative stress and the formation of O₂⁻ and oxidized LDL.²¹–²³ O₂⁻ and oxidized LDL potently quench NO, decrease its bioavailability, and therefore inhibit vasodilatation by both exogenous and endogenous sources of NO as they parallel a common final pathway during vasodilatation.²⁴ In line with the hypothesis that increased oxidative stress is causally associated with decreased NMD, vitamin C has been shown to protect against nitrate tolerance, possibly by decreasing O₂⁻ formation.²⁵ Oxidized LDL was not associated with FMD in the present study, although one would assume that if NO produced by GTN bioconversion is inactivated by oxidized LDL, the same would occur when NO is released from the endothelium. The reason for this controversy remains unclear, although it may be thought that other risk factors than oxidized LDL might be more potent in the development of endothelial dysfunction in children.

Our study has limitations. We examined the relationships between NMD, FMD, arterial IMT, and risk factors using a cross-sectional setting. Additional experimental studies are required to determine a causal relationship between impaired NMD, increased oxidative stress, and atherosclerosis. The study included a relatively small number of participants, which may have increased the risk of selection bias. However, the children with diabetes and the children with FH participating in the present study were representative of the total eligible clinic population of children with diabetes and children with FH, respectively. Increased oxidative stress was assessed by measuring oxidized LDL, which reflects only one aspect of increased oxidative burden.

In conclusion, the attenuated vasodilatation response to exogenous NO donor GTN is associated with endothelial dysfunction, oxidative stress, and increased arterial IMT in children. Thus, these data suggest primary nitrate tolerance in children at risk for atherosclerosis. Potential causes for this early impairment of NMD may include accelerated inactivation of NO by increased oxidative stress or a defect in smooth muscle function.

Acknowledgments

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References


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