NO activity in familial combined hyperlipidemia: potential role of cholesterol remnants

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Abstract

**Objective:** Patients with familial combined hyperlipidemia (FCH) have an increased cardiovascular mortality despite only moderate elevations of LDL-cholesterol. Since endothelial NO release is intimately involved in the anti-atherosclerotic effects of the endothelium, we studied the effect of short-term lipid-lowering therapy on NO-mediated vasodilatation in patients with FCH. In view of only moderate LDL elevations, we evaluated whether alterations in other lipid fractions upon therapy correlated to changes in NO-mediated vasodilatation.

**Methods:** NO activity was assessed by serotonin-induced, nitric oxide-mediated increase in forearm blood flow (FBF). Measurements were performed 2 weeks off and 4 weeks on lipid-lowering therapy in 12 FCH patients using forearm venous occlusion plethysmography. Control experiments were performed in 12 healthy subjects.

**Results:** Serotonin-induced vasodilatation was impaired in FCH patients (FBF unit ml/100 ml forearm tissue/min from 3.0 (0.3) to 4.8 (0.4)) compared to controls (FBF from 2.9 (0.3) to 6.5 (0.6); \(p < 0.05\) vs. FCH). FBF response to serotonin improved significantly upon lipid-lowering therapy (from 3.0 (0.3) to 5.7 (0.5); \(p < 0.05\) treated vs. untreated). The level of improvement in endothelial function was significantly correlated to the absolute reduction of intermediate density lipoproteins upon lipid-lowering therapy (\(r = -0.64; p < 0.05\)), whereas it did not correlate to changes in VLDL- or LDL-cholesterol, nor to Lp(a).

**Conclusion:** Patients with familial combined hyperlipidemia have impaired NO-mediated vasodilatation, that responds rapidly to lipid lowering medication, and may be related to changes in intermediate density lipoproteins. © 1997 Elsevier Science B.V.

**Keywords:** Endothelium; Nitric oxide; Familial combined hyperlipidemia; Human; Forearm; Lipid-lowering therapy

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1. Introduction

Familial combined hyperlipidemia (FCH) is a multi-gene disorder with an estimated prevalence of 1% [1]. Patients with FCH are characterized by an overproduction of apolipoprotein B and very low density lipoproteins [2] in combination with a relative impairment of lipoprotein catabolism, resulting in a characteristic increase of intermediate density lipoprotein (IDL) or remnant particles [3,4]. LDL-cholesterol, an established independent risk factor for cardiovascular disease, is usually only moderately elevated in FCH [5]. The predisposition for cardiovascular disease in FCH is not restricted to patients with hypercholesterolemia alone, but is also clearly demonstrable in predominantly hypertriglyceridemic FCH patients [6]. Therefore, additional factors contributing to accelerated atherosclerosis in FCH remain to be elucidated.

Endothelium-derived nitric oxide (NO) plays an important protective role during the early phases of atherosclerosis by inhibiting leucocyte adhesion [7]. In the course of atherosclerotic disease progression, NO may play a beneficial role in haemodynamically mediated coronary ischaemia by causing coronary vasodilatation [8] and in
2. Methods

2.1. Subjects

Studies were performed in 12 male patients, mean age 40 (2) years (range 23–47 years), with familial combined hyperlipidemia, characterized by (1) hyperlipidemia, defined as cholesterol and/or triglyceride plasma concentrations > 6.5 and 2.0 mmol/l, respectively; (2) at least one first-degree relative with a different lipoprotein phenotype than the index patient; and (3) at least one first-degree relative with an elevated plasma concentration of apoB and a history of premature myocardial infarction [3,12]. None had clinical signs of atherosclerotic disease on physical examination and ECG. Six were smokers. Forearm volume averaged 1249 (65) ml, and body mass index 25.9 (1.0). Control studies were performed in 12 healthy male subjects, mean age 33 (3) years (range 25–50 years). Six were smokers. Forearm volume averaged 1210 (75) ml, and mean body mass index 24.9 (0.9).

2.2. Study design

First, forearm vascular function was studied in the absence of lipid-lowering medication. For this purpose lipid-lowering medication had been stopped 2 weeks prior to the investigation in nine cases, while three patients were not using maintenance lipid-lowering medication beforehand. Measurements were subsequently repeated after 4 weeks reinstitution of lipid-lowering therapy with simvastatin 40 mg/day. Out of twelve patients, six agreed to a third study, after cessation of lipid-lowering medication for 4 weeks. This final study was added to evaluate the reproducibility of the first measurement without medication and to evaluate a possible additional effect of 4 weeks compared to the two weeks period of cessation of medication. Control subjects were studied once. All studies were performed after an overnight fast of twelve hours. During this period all subjects also refrained from smoking and drinking alcohol or caffeine-containing beverages. The study protocol was approved by the Utrecht University Hospital Ethics Committee for study in human beings. Patients and control subjects gave written informed consent after explanation of the protocol. The investigation conforms with the principles outlined in the Declaration of Helsinki.

2.3. Infusion protocol

The protocol was performed as described previously [10]. In short, forearm blood flow (FBF) was measured in both arms at 15-second intervals by venous occlusion plethysmography with mercury-in-silastic strain-gauges using a microcomputer-based, R-wave triggered system for on-line, semi-continuous monitoring [10]. After cannulation of the brachial artery of the measurement arm serotonin (5-HT, Sigma Chemical), sodium nitroprusside (SNP, Merck) and L-arginine hydrochloride (L-arg, Bufa) were infused intra-arterially. The drugs were dissolved in 0.9% saline. All solutions were prepared aseptically from sterile stock solutions or ampoules on the day of the study and stored at 4°C until use.

5-HT was administered in a cumulative dose infusion of 0.1, 0.3, and 1.0 ng/kg/min. Each dose-step was infused for 5 minutes. 5-HT induced a rapid and transient vasodilatation, followed by a persistent vasodilatation. The latter is completely abolished by L-NMMA infusion, which indicates that NO is the sole agent responsible for its vasodilator effect [10,13]. Endothelium-independent vasodilatation was studied by infusing SNP in a cumulative dose of 1, 10, 30 and 100 ng/kg/min [10]. Each dose-step was infused for 3 minutes. Calculations were based on measurements made during the final 1.5 min of each infusion step. Afterwards, the same infusions were repeated during co-infusion of L-arginine in a dose of 0.2 mg/kg/min [10].

2.4. Biochemical analysis

Plasma lipoproteins were separated by density-gradient ultracentrifugation at 4°C for 20 h, 40,000 rotations per minute, in a 50.3 Ti-rotor (Beckman Instruments, Palo Alto, Ca, USA) into five fractions (very low density lipoprotein, VLDL, d < 1.006 g/ml; intermediate density lipoprotein, IDL, d 1.006–1.019 g/ml; low density lipoprotein, LDL, d 1.019–1.063 g/ml; high density lipoprotein, HDL, d 1.063–1.21 g/ml and an infranatant with d > 1.21 g/ml) [14]. Cholesterol concentrations were assayed enzymatically, using a commercially available kit (Boehringer GmbH, Mannheim, Germany). Triglyceride was measured automatically with a fully automated Hi-
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Fig. 1. Endothelium-dependent and -independent vasodilatation. The ratios of forearm blood flow between measurement and control arm (M/C ratio) for nitroprusside (left panel) were not different between groups. Serotonin-mediated response (right panel) was impaired in patients (*p < 0.05 untreated patients vs. controls), whereas lipid-lowering therapy significantly improved this response (**p < 0.05 treated vs. untreated patients).

tachi 717 analyzer, using enzymatic applications with reagents obtained from Boehringer Mannheim, Germany. Small dense LDL were not specifically measured. These poly-disperse lipoproteins are invariably present at LDL density after equilibrium ultracentrifugation. Lp(a) was determined with an ELISA (Apo-Tek, Organon Teknika,

Fig. 2. Effect of L-arginine on endothelium-dependent vasodilatation. Impaired serotonin response (*p < 0.05 patients vs. controls) was improved by L-arginine (** p < 0.05 saline vs. L-arginine) (left panel). L-arginine no longer exerted an ameliorative effect after lipid-lowering therapy in patients (right panel).
Fig. 3. Reversibility of changes in endothelium-dependent vasodilatation. Lipid-lowering therapy had no effect on nitroprusside-induced vasodilatation (left panel). In contrast, after four weeks of lipid-lowering therapy serotonergic, endothelium-dependent response was significantly enhanced in FCH patients (*p < 0.05 treated vs. untreated) (right panel). This amelioration disappeared after cessation of lipid-lowering therapy for another four weeks (right panel).

Rockville, MD). ApoB was measured using a nephelometric method with antiserum obtained from Behringwerke (standardization with International Federation of Clinical Chemistry Reference Preparation SP3-07).

2.5. Calculations and statistics

During each infusion step the final six values of FBF from both measurement and control arm were used to calculate the mean FBF and the mean ratio of FBF between measurement and control arm (M/C ratio) [15]. From the M/C ratios curves were constructed (Figs. 1–3) [15]. The area under the M/C curve was calculated using a curve-fitting computer program. The (change in) area under the M/C curve was used as an index of (change in) endothelium-dependent vasodilatation (Fig. 4). Correlations were tested with the Pearson-product moment test.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Hemodynamic and laboratory data</th>
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<td></td>
<td>Control group</td>
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<tr>
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<td>Mean arterial pressure (mmHg)</td>
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<td>Baseline forearm blood flow (ml/100 ml forearm tissue/min)</td>
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<td>Total cholesterol (mmol/l)</td>
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<tr>
<td>HDL-cholesterol (mmol/l)</td>
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<td>LDL-cholesterol (mmol/l)</td>
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<td>VLDL-cholesterol (mmol/l)</td>
<td>1.97 [1.15–3.64]</td>
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<tr>
<td>Apo-B (g/l)</td>
<td>1.42 [0.95–1.76]</td>
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<tr>
<td>Apo-A (mg/l)</td>
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<tr>
<td>Triglycerides (g/l)</td>
<td>1.2 [0.5–2.1]</td>
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Data are expressed as mean (SE) or median [range].

*p < 0.05 vs. control group.

*p < 0.05 untreated vs. treated FCH patients.
Results are expressed as means (SE). Results of parameters with a skewed distribution are reported as medians [range]. Statistical analysis of differences in forearm blood flows and M/C ratios was performed using either repeated measures ANOVA when comparing data within the patient group, or ANOVA when comparing patients with controls. In case of an uneven distribution, ANOVA was performed after log-transformation of the data. If variance ratios reached statistical significance, differences between the means were analyzed with the Student–Newman–Keuls test for $p < 0.05$.

### 3. Results

There were no significant differences between patient and control group in age, body mass index, smoking habit or forearm volume. Mean arterial pressure and basal forearm blood flow were not significantly different (Table 1). Plasma total cholesterol, VLDL- and IDL-cholesterol and apoB concentrations were significantly higher, and HDL-cholesterol significantly lower in the patients (Table 1). Plasma LDL-cholesterol level was in the upper normal range, but not significantly different compared to the control subjects. Lp(a) was also comparable (Table 1). Patients demonstrated variability of hyperlipidemic phenotypes as found in FCH (Table 1). Four weeks of treatment with simvastatin 40 mg/day lowered total cholesterol, VLDL-, IDL- and LDL-cholesterol in the patients by, respectively, 30 (4), 36 (5), 31 (11) and 21 (8)%. HDL and Lp(a) were not significantly altered by lipid-lowering therapy.

The highest dosage of serotonin increased FBF in the control group from 2.9 (0.3) to 6.5 (0.6) ml/100 ml/min ($p < 0.05$ compared to baseline). In the FCH patients, taking no lipid lowering medication, FBF increased from 3.0 (0.3) to 4.8 (0.4) ml/100 ml/min ($p < 0.05$ compared to baseline; $p < 0.05$ patients vs. controls). Serotonin response significantly improved during lipid lowering therapy (FBF from 3.0 (0.3) to 5.7 (0.5) ml/100 ml/min; $p < 0.05$ treated vs. untreated). Accordingly, ratios of forearm blood flow between measurement and control arm (M/C ratio) reached significantly higher values after therapy (Fig. 1). L-arginine co-infusion increased M/C ratios upon serotonin only in untreated patients, not in patients treated with lipid-lowering therapy (Fig. 2). Sodium nitroprusside in the highest dosage used, increased FBF from 2.7 (0.3) to 15.9 (1.0) ml/100 ml/min in the control group, from 3.1 (0.5) to 14.4 (1.2) ml/100 ml/min in the FCH patients off therapy, and from 2.9 (0.3) to 14.1 (1.2) ml/100 ml/min in the same FCH patients on therapy ($p < 0.05$ vs. baseline in all groups). Both the absolute FBF as well as the M/C ratio curves for nitroprusside were not significantly different between controls and untreated or treated patients (Fig. 1, left panel), nor did it change during co-infusion of L-arginine (data not shown).

Six out of twelve patients consented to a third experiment after a second (4 week) period of discontinuation of the medication. Plasma lipid and plethysmographic data were representative for the whole group, as were the changes induced by lipid-lowering therapy. Levels of total cholesterol, LDL-, IDL- and VLDL-cholesterol were significantly lowered during lipid-lowering therapy, and not different between first and second off-therapy periods (respective findings at baseline 6.8 [5.6–7.9], 2.65 [1.34–5.28], 1.00 [0.40–1.41] and 2.14 [0.23–3.4] mmol/l; after 4 weeks on therapy 4.7 [3.4–5.9], 1.99 [0.75–4.05], 0.43 [0.23–0.98] and 1.23 [0.13–1.73] mmol/l (all $p < 0.05$ vs. untreated baseline); and after 4 weeks untreated 6.7

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**Fig. 4.** Relation between endothelium-dependent vasodilatation and IDL-cholesterol. (A) Relation between IDL-cholesterol at baseline and endothelium-dependent vasodilatation, assessed as area under the M/C curve for serotonin. ($r = −0.56; p = 0.056$). (B) Relation between changes in IDL-cholesterol and change in endothelium-dependent vasodilatation, assessed as change in area under the M/C curve for serotonin before and after therapy. Changes in IDL-cholesterol significantly correlated to change in endothelium-dependent vasodilatation ($r = −0.64, p < 0.05$).
Finally, we evaluated correlations between lipid fractions and forearm vascular function in all 12 FCH patients. Endothelium-dependent vasomotion was expressed as area under the curve (AUC) of the ratio of forearm blood flow between measurement and control arm (M/C ratio) upon serotonin stimulation (AUCserotonin). There was no correlation between plasma VLDL- or LDL-cholesterol at baseline and endothelium-dependent vasodilatation ($r = -0.14$ and $-0.16$ respectively). Also, correlation testing between endothelium-dependent vasodilatation and IDL-cholesterol level at baseline did not reach statistical significance ($r = -0.56; p = 0.056$) (Fig. 4). In contrast, the larger the reduction in IDL-cholesterol upon lipid-lowering therapy, the higher the increase in endothelium-dependent vasomotion ($r = -0.64; p < 0.05$) (Fig. 4). There were no significant correlations between change in endothelium-dependent vasomotion and changes in VLDL- ($r = 0.09; ns$) or LDL-cholesterol ($r = -0.17; ns$). Endothelium-dependent vasodilatation was also not correlated to respectively HDL-cholesterol, age, blood pressure, baseline forearm blood flow or Lp(a).

4. Discussion

Over the last years it has become clear that endothelial NO release is a first line defense mechanism against the development of atherosclerosis [16]. Major cardiovascular risk factors such as elevated LDL-cholesterol levels, diabetes and smoking have now been associated with impaired NO-dependent vasorelaxation in vivo [10,11,17,18]. The present study expands our previous finding of impaired NO-mediated vasodilatation in patients with increased LDL-cholesterol [10] to patients with combined hyperlipidemia, in whom LDL-cholesterol was not significantly elevated compared to controls. This impairment in NO-mediated vasodilatation ameliorated after short-term lipid-lowering therapy with simvastatin and recurred after cessation of therapy. The latter not only underscores the validity of the observation, but also demonstrates that modulation of NO activity by lipid lowering therapy is a rapid process. This is in accordance with recent data, describing amelioration of endothelial function upon 4–12 weeks of lipid-lowering therapy [10,19–21] and even instantaneous improvement after a single session of LDL-apheresis [22].

The spectrum of FCH comprises a wide array of lipid abnormalities. In the present study, this group was characterized by increased fasting IDL-levels and variably increased levels of LDL- and/or VLDL-cholesterol. We found that the severity of impairment in NO-mediated vasodilatation varied individually, whereas no correlation could be demonstrated with either VLDL-, IDL- or LDL-cholesterol level at baseline. However, the improvement in NO-mediated vasodilatation following lipid-lowering therapy correlated to the decrease in IDL-cholesterol plasma concentrations, not to changes in LDL- or VLDL-cholesterol levels. An atherogenic potential of IDL-cholesterol and remnant particles is supported by several lines of evidence. First, epidemiological studies have observed that both presence [23–25] as well as progression [26–28] of vascular disease can also be associated with increased IDL-cholesterol. In accordance recent data from the MARS study have demonstrated an association between progression of atherosclerosis and IDL-cholesterol, whereas LDL- and VLDL-cholesterol were not correlated [29]. Second, the smaller remnant particles are known to penetrate more easily into the arterial wall [30] and, due to the presence of apoE, bind to receptors on arterial-wall macrophages, which may promote formation of foam cells [31,32]. Third, triglyceride-rich particles may promote atherogenesis through the metabolic consequences of hypertriglyceridemia per se, such as low HDL levels and induction of a procoagulant state [33]. Finally, several studies have observed that postprandial triglyceride concentrations were highly predictive of coronary artery disease [34], whereas delayed remnant clearance is a hallmark of FCH [3]. Our present observation suggests that impaired NO activity may be an additional mechanism, which potentially contributes to the atherogenic potential of IDL-cholesterol. We previously found that lowering LDL-cholesterol concentrations in patients with familial hypercholesterolemia improved NO-mediated vasodilatation [10]. Thus, absence of a correlation with LDL-cholesterol in the present FCH patients does not necessarily preclude a role in endothelial dysfunction for LDL-cholesterol. Notably, in the present study mean LDL-cholesterol level in patients with FCH was not significantly elevated compared to controls.

From the present data it cannot be excluded that HMG-CoA reductase inhibitors influence NO activity independent of the lipid-lowering effects of these drugs [35]. For example, statin therapy has been associated with reduction of ex-vivo platelet aggregation and production of thromboxane B$_2$ and A$_2$ [36], while atherosclerotic disease has been associated with increased production of constrictor prostanooids [37]. Consequently, NO activity could be facilitated by decreased vasoconstrictor tone. However, the observation that lipid-lowering therapy with other compounds, such as fibrates [38] and cholesteramine [39], also has beneficial effects on endothelium-dependent vasodilation suggests that lipid-lowering per se may be the main factor causing improved NO activity.

Decreased bio-availability of NO by increased LDL-cholesterol may be the consequence of either decreased availability and/or affinity of the substrate for NOS or a decreased availability of cofactors of NOS [40,41], which can in part be restored by L-arginine infusion [10,42].
the present study we extend this observation to combined hyperlipidemia, since infusion of l-arginine could ameliorate the impaired response in the present FCH patients. Further studies are required to determine whether other mechanisms contributing to impaired NO-mediated vasodilatation, such as tetrahydrobiopterin deficiency [42], are different during respectively increased LDL- and/or increased IDL-cholesterol levels.

The clinical relevance of the present observation is twofold: First, impaired NO activity during not significantly elevated LDL-cholesterol levels and the correlation of reduction in IDL-cholesterol with improvement in NO activity upon lipid-lowering therapy reinforces the potential relevance of IDL-cholesterol as a cardiovascular risk factor in FCH. Second, the observed rapid modulation of NO-mediated vasodilatation in forearm vessels by lowering cholesterol, which is correlated to reduction in IDL-cholesterol, implies that this anti-atherogenic potential of the endothelium can be improved by short-term lipid-lowering therapy.

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