Nifedipine improves endothelial function in hypercholesterolemia, independently of an effect on blood pressure or plasma lipids

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Abstract

Objective: Dihydropyridine calcium antagonists have been shown to retard atherogenesis in animal models and to prevent the development of early angiographic lesions in human coronary arteries. Endothelial dysfunction is an early event in the pathogenesis of cardiovascular disease. We investigated whether nifedipine could improve endothelial function in hypercholesterolemia, independently of changes in blood pressure or plasma lipids. Methods: First, we compared in vivo forearm vascular responses to the endothelium-dependent and independent vasodilators serotonin (5-HT) and sodium nitroprusside (SNP) in 11 patients with familial hypercholesterolemia before and after 6-weeks treatment with nifedipine GITS (60 mg, OD) and in 12 matched controls. In a subgroup of six control subjects forearm vascular function was also assessed before and after 6-weeks nifedipine GITS treatment. In vitro, we subsequently explored possible mechanisms underlying the effect of nifedipine on endothelial function. We investigated the effects of nifedipine on both NO production by recombinant endothelial NO synthase (eNOS) and endothelial cells, using L-arginine conversion, as well as on superoxide generation by endothelial cell lysates, using lucigenin enhanced chemiluminescence. Results: In hypercholesterolemia 5-HT-induced vasodilation was impaired (47±9% increase in forearm blood flow vs. 99±8% in controls). Treatment with nifedipine completely restored 5-HT-induced vasodilation (113±13%), whereas it did not influence basal forearm vasomotion or SNP-induced vasodilation. Nifedipine did not alter forearm vascular responses in control subjects and did not alter blood pressure or plasma lipids. In vitro, we found no direct effect of nifedipine on NO production by recombinant eNOS or endothelial cells. However, we did observe a reduction in endothelial superoxide generation. Conclusions: Our data show that nifedipine improves endothelial function in hypercholesterolemia. It is suggested from our in vitro experiments that this effect is due to reduced NO degradation.

Keywords: Endothelium; Nitric oxide; Familial hypercholesterolemia; Human; Forearm; Calcium antagonist

1. Introduction

Dihydropyridine calcium antagonists are widely prescribed in the treatment of hypertension and angina pectoris. Several studies have suggested an additional role for these drugs as antiatherogenic agents. Calcium antagonists have been shown to retard atherogenesis in cholesterol-fed rabbits [1,2] and monkeys [3], independently of reduction of blood pressure or plasma lipids [4]. Furthermore, calcium antagonist drugs prevented the development of de novo angiographic lesions in human coronary arteries [5,6]. Both animal and human studies indicate that calcium antagonists may be effective in early atherogenesis. Indeed, in vitro experiments demonstrated that calcium antagonists interfere with early events in the development of atherosclerosis, such as platelet aggregation [7], leucocyte–endothelial cell adhesion [8], monocyte infiltration of the subendothelium [9], permeability of the endothelium [10] and/or media [11] and smooth muscle cell (SMC) migration [12,13] and proliferation [14–16].
estingly, calcium antagonist drugs have been reported to reverse the impairment in endothelium-dependent, nitric oxide (NO)-mediated vasorelaxation in hypercholesterolemic rabbits [4,17]. Such an increase in NO availability may also attenuate the aforementioned early events in atherogenesis [18].

Atherosclerotic vascular disease, but also major risk factors for atherosclerosis, including hypercholesterolemia, have been associated with endothelial dysfunction, characterized by impaired endothelium-dependent, NO-mediated vasorelaxation [19,20]. In hypercholesterolemia impaired NO availability could be improved by lipid lowering and/or antioxidant therapy [20–23]. We hypothesized that treatment with the dihydropyridine calcium antagonist nifedipine may improve NO availability in normotensive hypercholesterolemic patients with no overt macrovascular disease, without affecting plasma lipid levels or blood pressure. To test our hypothesis we studied endothelium-dependent and independent vasodilation in normotensive hypercholesterolemic patients and in healthy control subjects before and after 6 weeks treatment with nifedipine GITS (slow release preparation). In addition, to explore possible mechanisms underlying an effect on NO availability, we investigated the effect of nifedipine on NO production by endothelial nitric oxide synthase (eNOS) and endothelial cells and on NO degradation due to superoxide generation in endothelial cell lysates in vitro.

2. Methods

2.1. Effects of nifedipine GITS on endothelial function in vivo

2.1.1. Subjects

Eleven patients with familial hypercholesterolemia (three females, one postmenopausal, two using oral contraceptive medication), ranging in age from 19 to 49 (mean 37) years participated in our study. Familial hypercholesterolemia (FH) was defined as LDL-cholesterol of 4.5 mmol/l or more, VLDL-cholesterol of 2.0 mmol/l or less and a family history of premature atherosclerosis or tendon xanthomata [24]. In all our patients a molecular diagnosis of FH was established [25]. Twelve healthy volunteers, matched for age and sex were included in our study as control group. Each participant in the study was screened by clinical history, physical examination and routine chemical analyses; this revealed no evidence of past or present cardiovascular disease, hypertension or diabetes mellitus. Subjects had not used vasoactive medication in the week before the study.

2.1.2. Study design

Vascular function in the forearm of hypercholesterolemic patients was assessed after at least 2 weeks withdrawal of maintenance lipid-lowering medication and again after 6 weeks treatment with nifedipine GITS, 60 mg once daily (Bayer, Germany). Nifedipine was not given on the day of the study. During the study period (8 weeks) patients received no lipid-lowering medication. In all healthy controls forearm basal assessment of vascular function was performed (without treatment). In a subgroup of six control subjects we also assessed the effects of 6 weeks nifedipine GITS (60 mg once daily) treatment on forearm vascular function. Experiments were performed in a quiet, temperature controlled room (22–24.5°C). All experiments were performed at the same time of the day. At least 12 h before each part of the study all subjects abstained from alcohol, tobacco and caffeine containing drinks. The study protocol was approved by the local research committee of the University Hospital, Utrecht. All subjects gave written informed consent. The investigation conformed with the principles outlined in the Declaration of Helsinki.

2.1.3. Protocol

Subjects were supine with both forearms resting slightly above heart level. After local anaesthesia a 22-g needle was inserted into the brachial artery of the non-dominant arm for blood pressure measurements and drug infusions. Drugs were dissolved in physiological saline (0.9%) and administered at a constant infusion rate of 1.3 ml/min. All solutions were prepared aseptically from sterile stock solutions or ampoules on the day of the study. Forearm blood flow (FBF) was measured in both arms at 15-s intervals by venous occlusion plethysmography using calibrated mercury in silastic strain-gauges, with a micro-computer-based R-wave triggered system for on-line, semi-continuous monitoring [26]. Venous occlusion pressure averaged 40 mmHg. During each FBF determination the hands were excluded from the circulation by inflation of wrist cuffs to suprasystolic pressure. FBF measurements were made at 5- or 6-min intervals. Measurements were started at least 30 min after cannulation of the brachial artery, when FBF had stabilised. Between infusions, a 15–20 min rest was applied to allow FBF to recover.

For assessment of endothelium-dependent vasodilation, serotonin (5-HT, Sigma, St. Louis, MO, USA) was infused into the brachial artery in increasing doses of 0–0.6–1.8–6.0 ng/100 ml forearm volume (FAV)/min. These dosages have previously been shown to cause NO-mediated vasodilation [20,27]. For assessment of endothelium-independent vasodilation, sodium nitroprusside (SNP, Merck, Darmstadt, Germany) was administered intraarterially at incremental doses of 0–6–60–180–600 ng/100 ml FAV/min. Serotonin- and sodium nitroprusside-induced vasodilation were assessed in randomized order to avoid any bias related to the order of drug infusion.

Plasma total cholesterol, HDL-cholesterol, triglyceride and apo-B were measured using standard laboratory methods.
2.2. Effect of local inhibition of the endogenous NO system on serotonin induced vasodilation

To confirm that serotonin causes NO-mediated vasodilation, we performed an additional, separate series of experiments in eight healthy volunteers. First, serotonin was infused into the brachial artery in increasing doses of 0–0.6–1.8–6.0 ng/100 ml FAV/min. Subsequently, serotonin infusion was repeated during inhibition of the endogenous NO system in the forearm by use of a “NO clamp” [28,29]. This “NO clamp” allowed us to simulate normal basal NO activity during continuous inhibition of endogenous NO synthesis. The “NO clamp” was obtained by continuous infusion of the NO synthase inhibitor L-N^2-monomethylarginine (L-NMMA; Institut fur Pharmazie, Universitat Leipzig, Germany), at a rate of 200 μg/100 ml FAV/min to achieve maximal inhibition of local NO synthase [20,30,31], and subsequent coinfusion of incremental dosages of SNP, an exogenous NO donor until baseline forearm blood flow had been restored. L-NMMA and SNP were then coinjected at these rates for the remainder of the study.

2.3. Effects of nifedipine on NO production by endothelial cells and recombinant endothelial NOS (eNOS)

The effect of nifedipine (Sigma) on endothelial NO production was studied in a human vascular endothelial cell line [32] that expresses eNOS. Cell monolayers were grown to confluence in 175-cm² culture flasks. The cells were subsequently stimulated with the combination of TNFα (200 U/ml), IL-1β (5 U/ml) and IFNγ (200 U/ml) in the absence and presence of nifedipine (0.2 μmol/l, dissolved in dimethylsulfoxide (DMSO)) for 24 h. This nifedipine concentration was similar to the estimated plasma concentrations obtained in vivo [33,34]. Then cells were suspended by brief treatment with trypsin–EDTA and washed with Dulbecco’s phosphate buffered saline. To prepare total cell lysates, cells were resuspended in cold lysis buffer (sucrose 0.3 mol/l, Heps 10 mmol/l, 1% Triton, EDTA 0.1 mmol/l, dithiothreitol 1 mmol/l, leupeptin 10 μg/ml, aprotinin 2 μg/ml, soybean trypsin inhibitor 10 μg/ml, and PMSF 50 μmol/l, pH 7.4), vortexed and kept at 4°C [35]. NOS activity was determined as the formation of L-[2,3,4,5-^3H]-citrulline from L-[2,3,4,5-^3H]-arginine. These experiments were performed in duplicate. Protein concentration of cell lysates was determined with BCA protein assay reagent (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. Additional NOS activity experiments were performed directly on the recombinant endothelial NOS enzyme, derived from a baculovirus/SF9 expression system (Cayman, Ann Arbor, MI, USA), where the effects of incubation with nifedipine (0, 10, 100 and 500 μmol/l) and BH4 (10 μmol/l) were studied. In these experiments we used higher nifedipine concentrations because dihydro-4-pyridines, due to their high lipid bilayer partitioning coefficients, may concentrate more than 1000-fold into biological membranes [36], the site of superoxide and NO production. These experiments were performed in triplicate.

2.4. Effects of nifedipine on superoxide generation by endothelial cells

The effects of nifedipine on superoxide generation by human endothelial cells were investigated. HUVECs were isolated from umbilical cords as described previously [37]. Cell monolayers were grown to confluence in 75-cm² culture flasks. The cells were subsequently cultured for 24 h in absence or presence of nifedipine (0.2 μmol/l). Cells were then washed with Dulbecco’s phosphate-buffered saline and suspended using a rubber policeman. To prepare total cell lysates, cells were homogenized in icecold Krebs–Ringer buffer with a 23-g needle. Superoxide generation was measured using lucigenin-enhanced chemiluminescence, as described previously [38]. In short, scintillation vials containing lucigenin (250 μmol/l) and cell lysates (10–30 μg protein) were placed into a Berthod luminometer (AutoLumat LB 953) at 37°C, in the presence of 0.1 mmol/l NADH. Counts were recorded for 5 min and the respective backgrounds were subtracted. Specificity of the chemiluminescence signal for superoxide was controlled by incubation with superoxide dismutase (SOD, 400 U/ml). All measurements were performed in duplicate.

2.5. Analysis

Average values of FBF (expressed as ml/100 ml forearm volume/min) in the infused and non-infused arm were obtained from the last five or six consecutive recordings of each measurement period. The ratio of flows in the infused and non-infused arms (M/C-ratio [39]) was calculated for each time point and expressed as percentage change from baseline. Results of in vivo studies are expressed as mean±S.E.M. Differences in forearm vascular reactivity induced by nifedipine were examined by repeated measures analysis of variance (ANOVA), where the interaction variance ratios indicate differences between the curves (Jandel Scientific, USA). Group comparisons with respect to clinical characteristics were made with
unpaired and two-tailed t tests with Bonferroni correction or analysis of variance for vascular function measurements. Results of in vitro experiments are presented as mean±S.E.M. of two or three experiments. These data were examined by analysis of variance. If variance ratios reached statistical significance, differences between the means were analyzed with the Student–Newman–Keuls test for P<0.05 and P<0.01.

### 3. Results

There were no significant differences between hypercholesterolemic patients and controls with respect to age, sex, body mass index and smoking habit. Mean arterial pressure was not significantly different between groups and did not change significantly during drug infusions. Plasma total cholesterol levels were significantly higher in patients than in controls. A 6-week treatment with nifedipine GITS did not significantly alter mean arterial pressure, total cholesterol, triglycerides or basal FBF in hypercholesterolemic patients (Table 1). There was also no significant effect of 6 weeks nifedipine GITS treatment on these parameters in the nifedipine-treated control subgroup (data not shown).

#### 3.1. Effects of nifedipine GITS on endothelium-dependent vasorelaxation in vivo

Serotonin-induced vasodilation was significantly impaired in hypercholesterolemic patients compared to controls; the highest dose of serotonin increased FBF (M/C ratio) by 47±9% (FBFi: 3.2±0.4; 4.1±0.4; 6.4±0.8; 9.2±0.8; 12.8±1.5] vs. 364±52% [3.1±0.4; 3.9±0.3; 7.5±0.8; 11.2±1.4; 16.0±2.3], respectively; ns). Nifedipine GITS treatment did not significantly alter endothelium-independent vasodilation in hypercholesterolemic patients (367±43% increase in FBF [3.3±0.3; 3.5±0.2; 7.3±0.7; 11.0±0.8; 16.4±1.5]; ns vs. untreated) (Fig. 1a). Nifedipine GITS treatment also did not significantly alter endothelium-independent vasorelaxation in control subjects (Fig. 2b).

#### 3.2. Effects of nifedipine GITS on endothelium-independent vasorelaxation in vivo

Nitroprusside-infusion caused similar increases in FBF in hypercholesterolemic patients and controls (278±40% [FBFi: 3.2±0.4; 4.1±0.4; 6.4±0.8; 9.2±0.8; 12.8±1.5] vs. 364±52% [3.1±0.4; 3.9±0.3; 7.5±0.8; 11.2±1.4; 16.0±2.3], respectively; ns). Nifedipine GITS treatment did not significantly alter endothelium-independent vasodilation in hypercholesterolemic patients (367±43% increase in FBF [3.3±0.3; 3.5±0.2; 7.3±0.7; 11.0±0.8; 16.4±1.5]; ns vs. untreated) (Fig. 1b). Nifedipine GITS treatment also did not significantly alter endothelium-independent vasorelaxation in control subjects (Fig. 2b).

#### 3.3. Effect of local inhibition of the endogenous NO system on serotonin induced vasodilation

Infusion of serotonin during saline coinfusion caused significant vasodilation; the highest dose of serotonin increased FBF (M/C ratio) by 77±17% (FBFi: 2.6±0.5; 3.0±0.6; 4.2±1.0; 4.6±1.0), P<0.05. During the “NO clamp” baseline forearm blood flow was restored (basal FBF in the infused arm during saline coinfusion: 2.6±0.5; during basal “NO clamp”: 2.4±0.4; P=0.5). During the “NO clamp” serotonin infusion caused no significant vasodilation, an increase in FBF (M/C ratio) of 3±8% (FBFi: 2.4±0.4; 2.3±0.3; 2.5±0.4; 2.6±0.4), P=0.9, indicating that serotonin-induced vasodilation is NO mediated (Fig. 3).

### Table 1

Clinical characteristics, haemodynamic and laboratory data

<table>
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<th>Familial hypercholesterolaemia Untreated</th>
<th>Familial hypercholesterolaemia Treated</th>
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<td>Triglycerides (mmol/l)</td>
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<td>Apo-B (g/l)</td>
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<td>1.83±0.13*</td>
<td>1.80±0.11*</td>
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</table>

Data are expressed as mean±S.E.M.

* P<0.05 vs. controls (t-test). There were no significant differences between hypercholesterolaemic patients before and after treatment (paired t-test).
Fig. 1. Percentual change in forearm blood flow after stimulation of endothelium-dependent (a) and endothelium-independent (b) vasodilation with respectively serotonin (5-HT) and sodium nitroprusside (SNP) in patients with familial hypercholesterolemia ($n=11$) before and after 6 weeks treatment with nifedipine GITS (60 mg OD) and in healthy control subjects, matched for age and sex ($n=12$).

Fig. 2. Percentual change in forearm blood flow after stimulation of endothelium-dependent, 5-HT induced vasodilation (a) and endothelium-independent, SNP induced vasodilation (b) in healthy control subjects ($n=6$), before and after 6 weeks treatment with nifedipine GITS (60 mg OD).
The present study demonstrates that treatment with the dihydropyridine calcium antagonist nifedipine GITS restores endothelial function in hypercholesterolemic patients without overt vascular damage. The impaired serotonin-induced, endothelium-dependent vasorelaxation in our group of hypercholesterolemic patients could be completely restored by nifedipine treatment, whereas nifedipine had no significant effect on the serotonin response in healthy control subjects. Nifedipine treatment also did not influence basal forearm vasomotion or nitroprusside-induced, endothelium-independent vasorelaxation. Importantly, the beneficial effect on endothelial function occurred without any changes in plasma lipid levels or blood pressure.

In the present study we used serotonin to assess endothelium-dependent vasodilation. Previous studies in hypercholesterolemia have shown impaired endothelium-dependent relaxation in response to acetylcholine, serotonin, substance P, but not to bradykinin [20,40,41]. The differences in response may be due to differences in signal transduction pathways activated by the agonists. It has been suggested that hypercholesterolemia (in early stages) might act by specifically inhibiting the Gi protein-dependent signal transduction pathway [42]. Alternatively, different endothelial mediators may be involved in the vasodilator responses. Serotonin acts through activation of endothelial Gi proteins [42]. The present study confirms previous observations [20,27] that the vasodilator response to serotonin is fully NO mediated. During “NO clamp” or L-NMMA co-infusion serotonin-induced vasodilation is completely abolished. In contrast, acetylcholine induced vasodilation is only partially inhibited by L-NMMA co-infusion, suggesting that this response is not only mediated by NO, but is also due to release of other vascular factors, such as prostaglandins and endothelium derived hyperpolarizing factor. Therefore our data suggest a specific improvement of impaired NO bioavailability.

Our observations are in agreement with other preliminary reports. Kiowski et al. demonstrated that isradipine, also a dihydropyridine calcium antagonist, could improve NO dependent vasodilation in patients with coronary artery disease and hyperlipidemia [43]. These results seem to indicate that the observed beneficial effects on endothelial function may be a class effect for the dihydropyridine calcium antagonists. Recent data from Taddei et al. also suggest a beneficial effect of nifedipine on endothelium-dependent vasodilation in hypertensive patients [44]. Frielingsdorf et al. [45] and Kaufmann et al. [46] demonstrated an improvement of the impaired exercise-induced vasodilation in hypertensive patients with coronary atherosclerosis as well as in hypercholesterolemic patients after acute intracoronary calcium antagonist administration. However, these results may have been due to an effect on vascular smooth muscle, resulting in facilitation of the vasodilator response, rather than a beneficial effect on NO availability.
It is suggested that a beneficial effect on endothelial function or NO availability may be translated to a decrease in cardiovascular risk. NO has important antiatherogenic properties and it has been shown that improvement of endothelial function precedes structural regression of atherosclerosis [47,48]. Furthermore, similar functional improvement upon treatment with lipid lowering drugs was paralleled by the outcome of clinical trials showing reductions in acute cardiovascular events [20,21,49–55]. Indeed, both the INTACT and Montreal Heart study demonstrated a reduction in the number of new coronary lesions on angiography during calcium antagonist treatment [5,6]. In the REGRESS trial a synergistic effect of calcium antagonists with lipid-lowering therapy in retarding the progression of coronary atherosclerosis has been observed [56]. So far, several studies have shown a beneficial effect of long-acting dihydropyridine calcium antagonists on cardiovascular morbidity and mortality [57,58], whereas others reported an increase in mortality in patients with coronary heart disease [59]. Currently ongoing studies, like ALLHAT [60] and INSIGHT [61] will determine whether treatment with long-acting calcium antagonists has a beneficial effect on cardiovascular morbidity and mortality compared to other antihypertensive agents.

Calcium antagonists inhibit voltage-gated L-type calcium channels in various cell types, thereby attenuating the contractile effects of vasoconstrictors and facilitating the actions of endothelium-derived relaxant factors, like NO. The inhibition of calcium influx in SMCs is the principal mechanism responsible for the therapeutic vasodilator effects of calcium antagonists in hypertension and angina pectoris [62]. However, such an effect on vascular smooth muscle does not seem to be involved in our (normotensive) subjects as in our study no changes in blood pressure, heart rate or basal forearm bloodflow were induced by nifedipine GITS treatment, in agreement with previous observations [63]. Furthermore, nifedipine did not cause a significant increase in the vasodilator response to nitroprusside, a phenomenon that did occur in the aforementioned study by Frielingdorf et al. [45]. This excludes the possibility that our results can be attributed to facilitation of vasorelaxation at the level of vascular SMCs.

Impaired NO bioavailability in hypercholesterolemia is caused by reduced NO production, enhanced NO degradation or a combination of both. Our in vitro experiments demonstrate that nifedipine exerts no direct effect on NO production by endothelial cells or recombinant eNOS. These results are consistent with a recent report from Zhang and Hintze who found that nifedipine caused no significant increase in NO production, measured as nitrite production, in canine coronary microvessels or large coronary arteries in vitro [64]. These observations suggest that the improved NO bioavailability in hypercholesterolemic patients during nifedipine treatment is most likely due to reduced catabolism of NO. In support of this hypothesis we found that nifedipine reduced superoxide generation in endothelial cells (lysates), a major determinant of impaired NO availability in hypercholesterolemia [23,65,66]. These observed antioxidant properties of dihydropyridine calcium antagonists are consistent with their chemical structure: they possess aromatic resonance rings, a feature common to most classic chain-breaking antioxidants [67] and have high membrane partition coefficients, which may cause accumulation in membrane lipid bilayers, the main targets for oxidative damage [68]. In agreement, calcium antagonists have previously been shown to reduce lipid peroxidation, including formation of oxidised LDL, in vitro [67,69–72] and to protect against free radical induced injury in cultured endothelial cells [70] and isolated rat hearts [73]. Mechanisms such as a modulatory role on endothelial protein kinase C activity or improvement of the cellular redox state by intracellular enzymatic induction may also play a role in the antioxidant capacity of calcium antagonists.

A limitation of the present study is that it was not a randomized, placebo-controlled design. However, a non-specific time effect is highly unlikely as endothelial dysfunction assessed with our method has previously been shown to be a reproducible and consistent observation in time in FH patients [20]. Furthermore, while we observed a substantial improvement in endothelial function in our patient group, we did not find any alterations in the response in healthy controls.

In conclusion, our data demonstrate that nifedipine improves endothelial function in hypercholesterolemia, independently of blood pressure or plasma lipid lowering effects. It is suggested that this beneficial effect of nifedipine is due to reduced NO catabolism.

Acknowledgements

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References


