Nifedipine Inhibits Accumulation of LDL and Cholesterol in the Aorta of the Normocholesterolemic Rabbit

Peter Görög and Gustav V.R. Born

The effect of 5 days of oral nifedipine treatment (~1 mg/kg per day in drinking water) on low density lipoprotein (LDL) and cholesterol accumulation in rabbit arteries was determined. Compared with control aortas, nifedipine treatment (w=5) significantly reduced homologous 125I-tyramine cellobiose-LDL accumulation (control versus nifedipine: 45.93±4.3 versus 20.14±3.1 ng LDL per milligram dry weight x10^-3, p=0.001) while the reduction of human LDL accumulation (n=5) was not significant (49.1±6.1 versus 35.5±4.1 ng LDL per milligram dry weight x10^-3). Aortic accumulation of orally administered [3H]cholesterol was also inhibited by nifedipine (352±34 versus 257±16 ng cholesterol per milligram dry weight x10^-3, n=10, p=0.022). These findings suggest a possible mechanism for the antiatherosclerotic effect of nifedipine. (Arteriosclerosis and Thrombosis 1993;13:637–639)

KEY WORDS • lipoproteins • cholesterol • calcium channel blockers • atherosclerosis

Increasing evidence shows that Ca^2+ channel blockers, mainly nifedipine, inhibit experimental atherogenesis and exert an antiatherosclerotic effect in humans by retarding the progression of the arterial disease.1-3 The mechanism of this antiatherosclerotic action of Ca^2+ channel blockers is unclear; suggestions include alteration of lipid metabolism in the arterial wall,4,5 inhibition of smooth muscle cell proliferation,6 and antiperoxidative actions.7,8

Lipid accumulation initiates atherosclerosis, and plasma cholesterol, carried mainly by low density lipoprotein (LDL), is the predominant lipid in atherosclerotic lesions. One of the earliest manifestations of atherogenesis is a localized increase of LDL concentration, which develops into the fatty streak lesion.9 The purpose of the present study was to investigate whether subacute nifedipine treatment affects the uptake of plasma cholesterol by the rabbit aorta. All previous studies with nifedipine have been done with cholesterol-fed rabbits. Although this is a traditional and easy way of initiating atherosclerosis in rabbits, the high plasma cholesterol levels produced in this way are even higher than in patients with familial hypercholesterolemia. Thus, this model is relevant to a particularly atherogenesis-prone proportion of the affected human population and differs considerably from native atherosclerosis in humans. For this reason, in this study normocholesterolemic rabbits were used. Two different techniques were employed to measure basically the same biological process. In one technique, 125I-tyramine cellobiose (TC) is attached to LDL (125I-TC) and determination of this radioactivity in arteries is a measure of cumulative LDL uptake.10 In the other technique, orally administered [3H]cholesterol is incorporated physiologically into (endogenous) intact lipoproteins and so provides a measure of arterial lipoprotein accumulation.

Methods

Preparation of LDL

LDL (d=1.019–1.063 g/mL) was prepared from freshly drawn human or rabbit plasma containing Na2EDTA (1.5 mg/mL) by differential ultracentrifugation.11 LDL was dialyzed overnight against 1,000 volumes of 0.15 M NaCl at 4°C before labeling.

Lipoprotein Radiolabeling

LDL preparations were covalently linked to 125I-TC with cyanuric chloride, as described by Pittman et al.10 The labeled preparation was exhaustively dialyzed against 0.15 M NaCl containing 2 mM EDTA, pH 7.4, and sterilized by filtration. Specific activities of the preparations were 416 and 106 counts per minute (cpm) per nanogram protein, and free iodine contents were 1.3% and 1.2% for human and rabbit LDL preparations, respectively. The lack of oxidation during preparation was checked by an iodometric measurement of LDL hydroperoxide content (<10 nmol/mg LDL)12 and by determining plasma clearance rate13 in one separate rabbit (half-life of 6.0 hours).

[7(n)-3H]Cholesterol

[7(n)-3H]Cholesterol in toluene (TRK 122, Amersham, UK; specific activity, 18.3 mCi/mg; total activity, 1 mCi) was diluted with 5.0 mL olive oil, and the solvent toluene was removed by evaporation in a freeze-dryer (Modulyo, Edwards, UK). This cholesterol preparation was kept at 4°C and used within 2 days. Cholesterol

From the Pathopharmacology Unit, The William Harvey Research Institute, St. Bartholomew’s Medical College, London.

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Address for correspondence: Dr. P. Görög, Pathopharmacology, The William Harvey Research Institute, Medical College of St. Bartholomew’s Hospital, London EC1M 6BQ, UK.

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concentration in plasma was measured by an enzymatic method (CHOD-iodide, Merck, FRG).

**Animal Treatment**

New Zealand White rabbits (n=30) weighing 2.8–3.0 kg and maintained on standard rabbit chow were placed in a room illuminated by a 70-W sodium lamp (daytime only). Animals were allowed to adapt for 2 days before treatment began. Control rabbits had tap water to drink, while the drinking water of the treated animals contained 1 mg % nifedipine (1 mL of a 10 mg/mL nifedipine solution in ethanol was added to 1 L of tap water). The drinking bottles were wrapped in aluminum foil to provide additional protection from light, and the nifedipine dilutions were freshly made each day. Five days after the start of treatment, 125I-TC-LDL (3.5×10^8 cpm; 0.9 or 3.3 mg LDL protein) was injected through the marginal ear vein followed by oral administration of [3H]cholesterol (250 μCi; 11 μg cholesterol in 1.0 mL oil) by an infant-feeding catheter (Portex No. 10).

Twenty-four hours after the administration of labeled LDL or cholesterol, animals were killed by an overdose of pentobarbital, the thoracic cavity was opened, and the entire thoracic aorta was excised. These procedures were approved by the Animal Care and Safety Committee and conformed to guidelines established by the Home Office. The adventitia was removed under a stereomicroscope and the vessel carefully cleaned and opened longitudinally. Tissue samples were washed 10 times for 10 minutes in 50 mL ice-cold saline, followed by washing in 5% KI in 0.9% saline, and rinsed again with saline. Under these conditions, no measurable amount of blood remained in the tissue (as demonstrated earlier by the absence of radioactivity when red blood cells had been labeled with 51Cr). The washed vessels were transferred to counting vials, and 125I activities were counted in a Beckman gamma counter. Vials containing the samples were then kept in an incubator for 48 hours at 60°C and weighed. Aortic segments (=30 mg dry weight) were dissolved in 1 mL tissue solubilizer (Scintran-SolvEase, Merck-BDH, UK) at 40°C for 3–4 days, and then 5.0 mL scintillation cocktail (PCS, Amersham UK) was added and the [3H] activities measured in a liquid scintillation counter (Beckman LS 8100). Results were expressed as nanograms of 125I-TC-LDL per milligram dry weight or nanograms of [3H]cholesterol per milligram dry weight.

**Statistical Analysis**

Results are expressed as mean±SEM. Comparisons of LDL and cholesterol accumulation between aortas of control and nifedipine-treated rabbits were made by the nonparametric Mann-Whitney U test.

**Results**

The average daily drinking water consumptions were 290±15 and 302±11 mL (control and treated rabbits, respectively). Accordingly, there was very little variation between animals in the daily nifedipine dose administered through the drinking water (1.04±0.05, 1.01±0.07, 1.15±0.09, and 0.92±0.07 mg/kg per day for days 1–4 of treatment, respectively). In a separate experiment, rabbits had water with (n=4) or without (n=4) nifedipine to drink for 4 days, and then plasma cholesterol concentrations were measured and LDL was isolated from the individual plasma. Nifedipine treatment caused a slight decrease of plasma total cholesterol concentration (63.6±2.8 versus 74.5±2.2 mg/dL, mean±SEM, nifedipine treated versus control, respectively). Cholesterol contents of plasma LDL fractions (milligrams of total cholesterol per milligram of LDL protein) were 1.30±0.07 and 1.08±0.04 (mean±SEM, control versus nifedipine treated, respectively).

**Discussion**

Administration of nifedipine through drinking water proved to be an easy and convenient way of treatment, with very little variation between animals. When tested under identical conditions, plasma levels of nifedipine showed a strong relation to the dose administered; i.e., 1 mg/kg per day and 10 mg/kg per day p.o. resulted in plasma concentrations of 28 and 280 ng/mL of the drug, respectively (personal communication from Dr. S. Wohlfeil, Bayer AG, FRG). Thus, the daily dose administered in this study resulted in a plasma level of ≈30 ng/mL, much less than that measured in humans (80 ng/mL) after a single 10-mg oral dose of nifedipine. It is assumed that in the present study nifedipine treatment did not affect blood pressure. This assumption is based on an earlier study in which long-term treatment of rabbits with 40 mg/kg per day nifedipine did not cause significant differences in mean arterial blood pressure. In this study, LDL was coupled to the biologically nondegradable marker 125I-TC. When LDL enters the arterial wall, it undergoes slow degradation while the separated marker remains trapped and metabolically inert. Thus, the actual radioactivity of the blood-free

**Table 1. Effect of 5-Day Oral Nifedipine Treatment on LDL and Cholesterol Uptake by Rabbit Aortas**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LDL source</th>
<th>n</th>
<th>125I-TC-LDL</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Human</td>
<td>5</td>
<td>49.08±6.14</td>
<td>...</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>Human</td>
<td>5</td>
<td>35.5±4.11</td>
<td>0.1037</td>
</tr>
<tr>
<td>Control</td>
<td>Rabbit</td>
<td>5</td>
<td>45.93±4.31</td>
<td>...</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>Rabbit</td>
<td>5</td>
<td>20.14±3.12</td>
<td>0.0013</td>
</tr>
<tr>
<td>Arterial</td>
<td>[3H]cholesterol content (ng/mg dry weight x 10^-3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>351.6±34.0</td>
<td>...</td>
<td></td>
</tr>
<tr>
<td>Nifedipine</td>
<td>10</td>
<td>257.4±16.2</td>
<td>0.022</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SEM. LDL, low density lipoprotein; TC, tyramine cellobiose.

The effect of subacute nifedipine treatment of rabbits (n=10) on LDL and cholesterol accumulation in aortas is shown in Table 1. Nifedipine inhibited accumulation of both homologous and heterologous (human) LDL in the aortas. These experiments were performed at different times and in different animals. Inhibition of aortic accumulation of homologous LDL (n=5) by nifedipine was significant, while inhibition of heterologous LDL (n=5) was not.

Cholesterol accumulation in aortas (n=10) was determined again in two separate experiments under identical conditions. In both experiments, nifedipine treatment resulted in a reduction of arterial [3H]cholesterol content (33.3% and 20.3% inhibition); the combined results demonstrated statistically highly significant inhibition.
vascular segment 24 hours after $^{125}$I-TC–LDL administration reflects the sum of internalized intact LDL plus products arising from degraded LDL.\(^{15,16}\)

When isolated LDL is administered to an animal, there is always a possibility that during the preparation, the lipoprotein undergoes changes that may influence its biological properties. Currently, great significance is attributed to “altered” or oxidized LDL.\(^{17}\) Although in this study the lipid peroxide assay of the LDL preparations showed no significant oxidation and plasma clearance rates were in the normal range, minimal oxidation or change in physical characteristics of both LDL and cholesterol cannot be ruled out by these tests.\(^{18,19}\)

Oral administration of radioactive cholesterol allows its physiological incorporation into intact (endogenous) cholesterol of lipoproteins.\(^{20}\) There is evidence that cholesterol is then transferred into the arterial wall in the proportion in which it is present in the lipoprotein fraction.\(^{21}\) In $[^{3}H]$cholesterol-fed rabbits, approximately 40% of cholesterol was present in lipoproteins of 1.006 $< r_f < 1.019$ g/mL and 20% in lipoproteins of 1.019 $< r_f < 1.063$ g/mL. Only a small proportion of the vessel wall radioactivity was derived from the exchange of free cholesterol between plasma and tissues.\(^{21}\)

The similarities and differences between the two in vivo techniques employed in this study may explain the differences observed in the effect of nifedipine on the uptake of exogenous LDL and endogenous cholesterol. Nifedipine had the most pronounced inhibitory effect on the accumulation of homologous LDL. The smaller inhibition of endogenous cholesterol accumulation was, however, consistent and statistically significant. Although nifedipine treatment slightly reduced plasma cholesterol, this is unlikely to be the (sole) reason for the observed inhibition of aortic LDL accumulation. Because of the very low rate of uptake of LDL cholesterol by arterial tissues, even slight inhibition of the process is biologically significant.

Whether the effect of nifedipine’s inhibition of uptake of plasma lipoproteins by the arterial tissue either involves voltage-dependent (L-type) calcium channels or is independent of them is unclear. In large arteries susceptible to atherosclerosis, uptake of LDL from the plasma occurs almost entirely by endocytosis, and high-affinity receptors play a very minor role in the process.\(^{22,23}\) It is the crossing of the endothelial barrier that is the main determinant of LDL uptake, and intracellular Ca\(^{2+}\) has been shown to play an important role in modulating endocytosis in endothelial cells and in vesicular transendothelial transport.\(^{24,25}\)

In conclusion, our observation that subacute oral nifedipine significantly inhibited LDL cholesterol accumulation in aortas provides a potential mechanism for the drug’s antiatherosclerotic effect in experimental animals and humans.

**Acknowledgments**

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