Preservation of Endothelium-Dependent Vascular Relaxation in Cholesterol-Fed Rabbit by Treatment with the Calcium Blocker PN 200110

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SUMMARY. We tested the effects of low doses of a dihydropyridine calcium antagonist, PN 200110, on endothelium-dependent vascular relaxation in rabbits fed a 1% cholesterol diet. The drug was given orally, 1 mg/day, and control rabbits received placebo. Plasma total cholesterol after 10 weeks, was similar in the placebo- and PN 200110-treated groups. The respective values averaged 2140 ± 116 (n = 14; mean ± SEM) and 2012 ± 115 mg/dl (n = 13). In placebo-treated rabbits, sudanophilic aortic lesions covered 52 ± 5% of the intimal surface, and the aortic cholesterol concentration was 72 ± 6 mg/g protein. Corresponding values in aortas from PN 200110-treated rabbits were significantly lower [36 ± 5% (P < 0.03) and 52 ± 3 mg/g protein (P < 0.03)]. Maximal endothelium-dependent cholinergic relaxation of aortic strips in untreated (n = 14) and treated cholesterol-fed rabbits (n = 13) differed significantly (P < 0.01) and averaged 31 ± 4% and 61 ± 7% of the value in normocholesterolemic controls (n = 13). We conclude that cholesterol feeding suppresses endothelium-dependent relaxation evoked by acetylcholine, and that PN 200110 reduces the severity of atherosclerosis and impairment of endothelium-dependent relaxation. (Circ Res 58: 305-309, 1986)

RECENT studies indicate that calcium antagonists such as nifedipine (Henry and Bentley, 1981; Panagotopoulios and Naylor, 1984; Willis et al., 1985; Miyazaki et al., 1985), verapamil (Rouleau et al., 1983; Blumlein et al., 1984), nicardipine (Willis et al., 1985), diltiazem (Ginsburg et al., 1983), and flunarizine (Ginsburg et al., 1983) may suppress atherosogenesis in cholesterol-fed rabbits. In addition, experiments with arterial smooth muscle cells in culture suggest that calcium antagonists may inhibit cell migration (Nakao et al., 1983), influence the uptake and catabolism of lipoproteins (Stein et al., 1985), and promote the depletion of intracellular cholesterol ester stores (Etingin and Hajjar, 1985). Anti-atherosclerotic effects of calcium antagonists may be related to those of LaCl₃ and calcium-chelating agents (Kramsch et al., 1981), since these agents are known to inhibit calcium-dependent reactions. Organic or inorganic calcium antagonists and chelating agents appear to exert their antiatherogenic effects without altering circulating lipoproteins, but detailed analyses of circulating lipids and lipoproteins in animals treated with these drugs have not been reported (Henry, 1985).

One important question is whether calcium antagonists protect against the effects of hypercholesterolemia by lowering arterial pressure (Henry and Bentley, 1981). Therefore, in this study, we treated cholesterol-fed rabbits with a calcium antagonist in a dosage that exerted no hypotensive effect. In addition, since endothelial injury may play an important role in atherogenesis, we addressed the question whether atherogenesis in cholesterol-fed rabbits is associated with impaired endothelial function. Recent studies have demonstrated that cholinergic relaxation of isolated arteries depends upon an intact endothelium (Furchgott et al., 1984). Accordingly, we have evaluated the functional integrity of endothelium by measuring the relaxant effects of acetylcholine on aortas isolated from cholesterol-fed rabbits.

Methods

Animals

Forty-five male New Zealand white rabbits weighing between 3.1 and 3.9 kg were housed individually in wire-bottomed cages in an air-conditioned room at 20°C and 50% humidity with 12 hr light/12 hr dark cycles. After an adaptation period of 1 week, the animals were separated into three equal groups which were randomly assigned to one of three dietary and therapeutic regimens: (1) standard chow and placebo, (2) chow containing 1% cholesterol and placebo, and (3) chow containing 1% cholesterol and PN 200110. Standard and cholesterol chows were purchased from ICN Nutritional Biochemicals. Rabbits of groups 2 and 3 received daily, at 7 a.m. and 7 p.m., a placebo capsule or an identical capsule containing 0.5 mg of PN 200110 (1 mg/rabbit per day or <0.3 mg/kg per 24 hours). The capsules were placed by hand into the pharynx of the rabbits, which were maintained on their re-
spective regimen for a period of 10 weeks. At the beginning and end of this period, blood samples from the central ear artery were collected into tubes containing Na2EDTA (1.5 mg/ml blood) after a 14-hour fast. To determine the effects of oral PN 200110 (0.5 mg) on hemodynamics in the conscious rabbit, we measured arterial pressure by the central ear artery technique as previously described (Henry and Bentley, 1981). Six animals from each group were chosen at random for these experiments, and measurements were performed during the 1st, 3rd, 6th, 8th, and 10th weeks of the diet period. A no. 21 pediatric butterfly needle was inserted into the proximal middle ear artery and taped to the ear. The needle was connected to a Gould P23Db pressure transducer which was placed at mid-chest level and attached to a Gould amplifier (model 11-4123-01) and recorder (model 2200 S). Measurements were performed in the early morning 1 hour after drug administration. In some rabbits, we measured arterial pressure at different times of the day to characterize diurnal pressure profiles.

**Pharmacological Procedures**

At the end of the diet period, and after a 24-hour fast, the rabbits were anesthetized with pentobarbital (30 mg/kg, iv), and the entire aorta from the aortic valve to the iliac bifurcation was excised and placed immediately in oxygenated Krebs-Henseleit buffer at 21°C. The isolated aorta was cleaned of perivascular tissue, and two 2-mm-wide rings were cut from the mid-thoracic aorta. These rings were opened and mounted as transverse strips in an organ bath filled with oxygenated Krebs-Henseleit solution (pH 7.4). One end of the strip was attached to a no. 21 bottom of the chamber, the other to a Statham model 4C-2 force transducer which was connected to a Gould recorder/amplifier system. The relationship between preload and developed force in response to 1 μM phentolamine was determined in 39 preparations. In untreated (n = 20) and treated rabbits (n = 19), a preload of 2 g produced maximal contractions of 2.4 ± 0.1 and 2.5 ± 0.2 g. After an equilibration period of 30 minutes, the preparations were contracted with 1 μM phenylephrine and subsequently relaxed by the cumulative addition of acetylcholine, 1 nM–10 μM. In some experiments, the dose-response experiments were repeated with or without prior removal of endothelium from the strips. The endothelium was removed mechanically by rubbing the intimal surface of the arteries with a wooden stick, as described by Furchgott et al. (1984).

**Morphometric Procedures**

At the end of the experiment and without dismounting the strips, the arteries were fixed in 0.1 M sodium phosphate buffer (pH 7.4) containing 1% glutaraldehyde, and postfixed with osmium tetroxide in phosphate buffer for 30 minutes. Subsequently, the samples were dehydrated in a graded series of ethanol, and dried in a Sorvall critical point drier. The specimens were mounted on viewing stubs, sputter-coated with gold-palladium (60:40), and examined at 20 kV in a JEOL 100C scanning electron microscope. The average density of endothelial cells visualized on the electronmicrographs (magnification 600X) was estimated by counting the number of cells in an area 300 μm × 300 μm. The total number of cells counted per strip ranged between 130 and 160.

The aorta (minus the two rings used for the pharmacological procedure) was halved with longitudinal cuts through the ventral and dorsal walls of the vessel. At the level of the arch, the cuts were performed along the small and large curvatures. The symmetrical half-aortas were blotted and weighed, and one-half was immediately frozen in liquid nitrogen and stored at −70°C. The other half was laid flat, intimal side up, and pinned on a cork board. The pinned preparations were photographed under water to reduce specular reflectance. An MF3 Land Polaroid camera with a 127-mm lens and a blue filter, and Polacolor 669 color film (ASA 80-20 DIN) were used. The preparations were subsequently stained with Sudan IV (Henry and Bentley, 1981) and rephotographed. Plaque areas of the unstained and stained aortas visualized on the Polaroid pictures (magnification 0.8X) were delineated on translucent paper. The outlined lesions were planimetered with a model M-200 Digisonix computerized planimeter. Outlining the lesions and planimetry was performed independently by two blinded observers who were not directly involved in the project. Plaque areas were expressed as a percent of the total intimal surface area of the aorta.

**Biochemical Procedures**

Plasma of blood samples anticoagulated with ethylenediamine tetraacetic acid (EDTA) was promptly separated by centrifugation for the measurement of plasma lipids. Plasma total cholesterol and triglycerides were measured on an automatic analyzer (model II, Technicon Technical Instruments), and phospholipids were assayed enzymatically by the method of McGowan et al. (1982). The phospholipid assay was calibrated on the basis of phospholipid phosphorus measurements by the method of Bartlett (1979). To measure tissue lipids, the frozen half-aortas were pulverized at liquid nitrogen temperature and homogenized in 10 ml of chloroform-methanol (2:1, vol/vol) in a Duall homogenizer (Kontes Company). The homogenate was extracted by the method described by Chan et al. (1978). Total cholesterol and cholesteryl esters were measured by the cholesterol oxidase method, as described by Allain et al. (1974), and triglycerides were quantified with a Boehringer triglyceride assay kit (catalog number GP701912). Enzymatic measurements of cholesterol and triglycerides were calibrated according to Standard Lipid Research Clinic procedures (Lipid Research Clinics Program, 1975). We estimated protein in whole homogenates by the method of Lowry et al., using bovine serum albumin as the standard, as previously described (Henry and Bentley, 1981).

**Statistical Analysis**

Statistical evaluations were performed at the Baylor Computer Center. Differences between group means were evaluated by t-tests for unpaired comparisons or, in the absence of a normal distribution, by Wilcoxon's rank sum tests. The mean interobserver variability for planimetric measurements was calculated as the mean of the absolute differences between measurements of the same areas planimetered by two independent blinded observers. Correlations between morphometric measurements and tissue lipids and correlations between planimetric measurements performed by two independent observers were evaluated by linear regression analyses. All results are expressed as means ± 1 SEM.
Results

Response of the Rabbits to the Diet and Drug Treatment

During the first 4 weeks, there were two deaths in the standard diet group, and one death each in the cholesterol-placebo and cholesterol-drug groups. Although autopsies were performed on these four rabbits, the cause of death could not be determined. One rabbit in the cholesterol-drug group developed an ear infection and was discarded. The weight gain at the end of the 10-week feeding period averaged 620 ± 48, 600 ± 20, and 650 ± 55 g in the standard diet group (n = 13), cholesterol-placebo group (n = 14), and cholesterol-drug group (n = 13), respectively.

Hemodynamic Measurements

Results of the hemodynamic measurements are described in Table 1. Orally administered PN 200110 had no effect on mean arterial pressure during the course of the study. Also, the drug did not alter the diurnal pressure profiles at the beginning and end of the diet period.

Biochemical Measurements

Results of the measurements of the lipids in plasma and aortic tissue are summarized in Table 2. There was no statistically significant difference between treated and untreated cholesterol-fed rabbits with respect to plasma total cholesterol, triglycerides, and phospholipids. In contrast, aortic tissue from treated animals exhibited significantly lower levels of total cholesterol, cholesteryl esters, and triglycerides, compared with untreated rabbits.

Table 1: Hemodynamic Measurements

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean arterial pressure (mm Hg) during diet period, at 0.5 Hr</th>
<th>1.0 Hr</th>
<th>6.0 Hr</th>
<th>12.0 Hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wk 1</td>
<td>83 ± 2</td>
<td>88 ± 7</td>
<td>79 ± 6</td>
<td>81 ± 5</td>
</tr>
<tr>
<td>Wk 3</td>
<td>80 ± 4</td>
<td>80 ± 6</td>
<td>86 ± 8</td>
<td>79 ± 8</td>
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Table 2: Lipids in Plasma and Aortic Wall

<table>
<thead>
<tr>
<th>Group</th>
<th>Total cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
<th>Phospholipids (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>Group I (n = 13)</td>
<td>57 ± 5</td>
<td>81 ± 8</td>
</tr>
<tr>
<td>Group II (n = 14)</td>
<td>2140 ± 116</td>
<td>122 ± 15</td>
<td>792 ± 49</td>
</tr>
<tr>
<td>Group III (n = 13)*</td>
<td>2012 ± 115</td>
<td>103 ± 16</td>
<td>683 ± 43</td>
</tr>
</tbody>
</table>

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Table 3: Atherosclerotic Plaque Areas

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<td>52 ± 5%</td>
<td>36 ± 5%</td>
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Aortas of rabbits maintained on a regular diet had no atherosclerotic plaques. Stained and unstained aortas exhibited fewer lesions in the treated group compared to the placebo group (Table 3). Unstained plaque areas (U) correlated well with sudanophilic plaque areas (S) (U = 1.06(S) - 10.22; r = 0.96; P < 0.001; n = 27). Similarly, there was a good correlation between tissue total cholesterol (C) and sudanophilic plaque areas (U) with regression analyses between results by observer I and those by observer II yielding correlation coefficients >0.98 with significance levels of <0.0001.

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The integrity of the endothelial cell lining was evaluated by scanning electron microscopy. The number of endothelial cells in an area 300 μm x 300 μm in strips from control rabbits (n = 5), treated cholesterol-fed rabbits (n = 6), and untreated cholesterol-fed rabbits (n = 6) averaged 138 ± 24, 145 ± 28, and 155 ± 29, (P between groups > 0.05). The maximum number of damaged cells in an area 300 μm x 300 μm was three, i.e., the endothelial cell lining was intact in all groups. In mechanically denuded strips, cell counts per 300 μm x 300 μm area in strips from control (n = 6) and untreated hypercholesterolemic rabbits (n = 5) were 10 ± 2 and 13 ± 3. Almost all residual cells appeared distorted or damaged.

Pharmacological Measurements

Phenylephrine (1 μM) evoked in nonatherosclerotic control rabbits (n = 13), untreated cholesterol-fed rabbits (n = 14), and treated cholesterol-fed rabbits (n = 13) contractions of 2.5 ± 0.2, 2.6 ± 0.3, and 2.1 ± 0.2 g, values that did not differ significantly by t-tests for unpaired observations (P > 0.05). Acetylcholine relaxed phenylephrine-contracted strips in a concentration-dependent manner in all rabbits (Fig. 1). However, atherosclerotic arteries compared to nonatherosclerotic control arteries exhibited a significant suppression of the responses to acetylcholine. The suppression was less marked in treated versus untreated cholesterol-fed rabbits (Fig. 1). In some experiments, we repeated the dose-response experiments in the same strips with or without prior deendothelialization. Without deendothelialization, the second contractions with 1 μM phenylephrine averaged 2.4 ± 0.3 g in control strips (n = 8) and 2.2 ± 0.4 g in strips from untreated hypercholesterolemic rabbits (n = 5). Corresponding maximal relaxations with acetylcholine were 1.2 ± 0.2 g and 0.65 ± 0.09 g. These repeat contractions and relaxations did not differ significantly (P > 0.05) from the responses of the initial experiments. Deendothelialization did not alter the contractions in response to phenylephrine in either control (n = 13) or untreated atherosclerotic strips (n = 12). However, 1 μM acetylcholine no longer elicited relaxations and produced instead weak contractions of 0.12 ± 0.02 g in control strips and 0.15 ± 0.02 g in atherosclerotic strips.

Discussion

This study demonstrates for the first time that atherosclerosis in the cholesterol-fed rabbit produces a suppression of endothelium-dependent cholinergic relaxation. It has been shown that several endogenous vasorelaxing agents relax vessels only in the presence of an intact endothelium (Furchgott et al., 1984). The endothelium acts by releasing a diffusible unstable factor, the chemical nature of which remains to be elucidated (Furchgott et al., 1984). Defective endothelium-mediated vasorelaxation in atherosclerosis may explain the fact that atherosclerotic coronary arteries from patients undergoing cardiac transplantation exhibit no relaxation in response to carbachol (Ginsburg et al. 1984). Although the physiological significance of endothelium-dependent relaxation remains to be delineated, the fact that atherosclerotic arteries may lack a vasodilator principle appears potentially important for the pathophysiology of ischemia due to atherosclerosis.

One important observation was that a dihydropyridine calcium antagonist administered in a dosage that corresponds to that used in clinical therapeutics is capable of inhibiting atherosclerosis in cholesterol-fed rabbits. Although earlier studies have demonstrated that the dihydropyridines, nifedipine and nicardipine, may exert anti-atherosclerotic effects in cholesterol-fed rabbits, the dosages expressed in mg/kg body weight were approximately 40–120 times higher than those used in this study. Rabbits tolerate high doses of dihydropyridine calcium antagonists without apparent ill effects, but it is questionable whether such doses could be used in humans. Cell culture experiments demonstrating an influence of calcium antagonists on lipid metabolism have likewise involved rather high drug concentrations. In the study of Stein et al. (1985), in which verapamil influenced the receptor-dependent uptake of low density lipoproteins, verapamil concentrations as high as 100 μM were used. This concentration is approximately 1000 times higher than therapeutic drug levels. In the study of Etingin and Hajjar (1985), in which nifedipine promoted the hydrolysis of cholesterol esters in cultured smooth muscle cells, the drug concentrations were 10–100
times higher than therapeutic drug levels. Therefore, it was important to demonstrate that antiatherosclerotic effects of dihydropyridines in vivo do not depend upon high drug concentrations which are known to exert effects unrelated to calcium channel blockade (Henry, 1983). Also, it was important to show that PN 200110 did not reduce arterial pressure, since the antiatherosclerotic effects of the drug might be ascribed to arterial hypotension (Henry and Bentley, 1981).

In summary, this study demonstrates that endothelium-dependent cholinergic relaxation is impaired in the cholesterol-fed rabbit. Treatment with the calcium antagonist PN 200110 partly prevented the functional impairment and, at the same time, reduced structural and biochemical changes of atherosclerosis. Although the drug did not exert hypolipidemic effects, it should be stressed that this does not rule out the possibility that PN 200110 affected intracellular lipid metabolism or some circulating lipoprotein not characterized in this study.

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