NIFEDIPINE AND NITRENDIPINE STIMULATE CHOLESTEROL HYDROXYLATION IN RAT

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Summary

Calcium antagonists nifedipine and nitrendipine inhibit experimentally induced atherosclerosis. Possible antiatherosclerotic mechanisms include alteration of lipid metabolism. 27-Hydroxycholesterol has a number of antiatherogenic effects. The effects of nifedipine and nitrendipine on cholesterol 27-hydroxylation are studied in rats.

The effects of dihydropyridines on 27-hydroxycholesterol production are evaluated in plasma and in liver mitochondria by an HPLC method after lipid extraction. The content of cytochrome P450 is assayed in liver mitochondria. The inhibitory constants of the tested compounds on specific [3H] PK11195 binding in rat liver ex vivo are calculated.

Nitrendipine and nifedipine increase the content of 27-hydroxycholesterol in liver mitochondria by 44.5\% (p < 0.01) and 33.2 \% (p < 0.05) respectively. Both compounds bind to a peripheral benzodiazepine receptor (PBR) – Ki 21.7 \textmu M and 60.9 \textmu M, respectively. The ability of these compounds to stimulate 27-hydroxycholesterol production in liver mitochondria correlated with their affinities for PBR.

Dihydropyridine calcium antagonists nifedipine and nitrendipine stimulate 27-hydroxycholesterol production in liver mitochondria in vivo. We suggest that nifedipine and nitrendipine affect 27-hydroxycholesterol production by a mechanism coupled to PBR and not due to an enzyme induction.

Key words: nifedipine; nitrendipine; 27-hydroxycholesterol; peripheral benzodiazepine receptor; rat liver mitochondria

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Introduction

The role of the enzyme sterol 27-hydroxylase (EC 1.14.13.15) in the elimination of cholesterol was suggested for liver cells, macrophages and vascular endothelial cells. Sterol 27-hydroxylase is believed to play a role in regulating cholesterol homeostasis in peripheral tissues by catalysing the first step in the cholesterol metabolism conversion to 27-hydroxycholesterol. 27-Hydroxycholesterol was found in high concentrations in human atheromas.

Recently, it was found that dihydropyridine calcium antagonists nifedipine (3,5-dimethoxy carbonyl-1,4-dihydro-2,6-dimethyl-4(ortho-nitrophenyl) pyridine) and nitrendipine (3-methoxy5-ethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-(ortonitro-phenyl)pyridine) inhibit experimental atherosclerosis and retard the progression of arterial disease in experimental animals and in humans. Possible antiatherosclerotic mechanisms of calcium antagonists include alteration of lipid metabolism. Nifedipine and nitrendipine are structurally related to the 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC). In a previous study we found that DDC (100 mg/kg i.p.) increased 27-hydroxylation of [14C]-cholesterol in rat liver mitochondria in vitro. The mechanism of DDC-induced effects on cholesterol 27-hydroxylation involves facilitation of cholesterol transport to the enzyme sterol-27-hydroxylase, localised on the inner mitochondrial membranes by a mechanism coupled to peripheral benzodiazepine receptors (PBR). DDC caused an accumulation of large amounts of dicarboxilic porphyrins that are potent endogenous ligands to mitochondrial peripheral benzodiazepine receptors in liver. The PBR is an intracellular protein receptor located mainly on the outer mitochondrial membrane although other localizations have been reported. PBR are expressed in high amounts in atherosclerotic plaques in mice and humans.

It was demonstrated that nifedipine and nitrendipine are exogenous ligands to PBR. Cantor et al. showed that nifedipine displaces Ro 5-4864 from membranes of the heart, kidney, and brain. Nifedipine and nitrendipine bind to the peripheral benzodiazepine receptors with low micromolar affinity.

The aim of this study was to investigate the effect of nifedipine and nitrendipine on cholesterol 27-hydroxylation in rat in vivo.

Materials and methods

Animals

Adult male Wistar rats (180-220 g) were obtained from Medical Experimental Research Center at the Medical University-Sofia. They were allowed free access to food and water. Nifedipine and nitrendipine were dissolved in polyethylene glycol (PEG 400; Union Carbide Chemicals, Danbury, CT) and were administered by i.p. injection (10 ml/kg) at a dose of 10 mg/kg bw (nifedipine) and 8 mg/kg bw (nitrendipine) for three days. All injected agents were freshly prepared for each injection through the entire study. All solutions were kept away from light. DDC (100 mg/kg) was dissolved in peanut oil (10 ml/kg). Rats, fasted for 24 h were given one i.p. injection. Control animals were given oil alone. After about 24 h after the last dose were given, the animals were sacrificed by decapitation and their livers were removed, rinsed in ice-cold saline and used for preparation of homogenate and mitochondrial fraction.

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (86/609/EEC; National Research Council. The Guide for the Care and Use of Laboratory Animals. Washington, D.C.:National Academy Press, 1996).
Materials
All solvents used were HPLC grade (Merck, Darmstadt, Germany). 27-Hydroxycholesterol was generous gift from Dr. Norman B. Javitt, New York University Medical Center. 3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) was obtained from Eastman Kodak, Rochester, NY, USA and purified before use through recrystallization from an ethanol-water solution. Nitrendipine was obtained from Calbiochem (EMD Biosciences, S. Diego). Nifedipine, cholesterol, cholesterol oxidase, NADPH, Triton X-100, isocitrate dehydrogenase were purchased from (St. Louis). All other materials were obtained from standard sources.

Preparation of rat liver mitochondria
The livers were homogenised in 5mM Tris-chloride buffer, pH 7.2, containing 0.25 M sucrose (1 g liver in 5 ml buffer) using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 800 x g for 10 min, the supernatant was recovered and centrifuged again at 9000 x g for 10 min. The pellets were washed, suspended in 1.0 mM Tris-chloride buffer, pH 7.2, containing 0.28 M sucrose and 0.1 mM EDTA, and spun at 9000 x g for 10 min, yielding an enriched mitochondrial preparation. All preparations were done in ice and solutions were kept at 4°C.

Determination of 27-hydroxycholesterol
The production of 27-hydroxycholesterol was assayed by the method of Petrack and Lotario. Lipid extractions from plasma and liver mitochondria were prepared as described by Folch et al. The organic phase derived from the lipid extractions was evaporated under nitrogen and the residue was dissolved in a final volume of 1ml of methanol/phosphate buffer (100 mM, pH7.5) 10:90 (vol/vol). Cholesterol oxidase (2 units) was added and the tubes were incubated for 20 min at 37°C. The reaction was terminated with 1.5 ml methanol. Testosterone propionate (5 µg/ml hexane) was added as internal standard. The mixture was extracted twice with 3 ml hexane and the organic layer was evaporated under nitrogen. The residue was dissolved in 100 µl of 5% isopropanol in dodecane. Aliquots of 100 µl were analysed via normal phase HPLC on an Alltech spherisorb silica column (4.6 x 250 mm), using an isocratic mobile phase of hexane-isopropanol 95:5 and flow rate of 1 ml per min. Absorbance was monitored at 240 nm.

Radioligand binding assays
3H-1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinoline carboxamide (PK11195) binding assays ex vivo were performed as was previously described. Ro 5-4864 was used as a displacing agent. Ki values were calculated from IC50 values obtained by linear regression analysis according to the Cheng and Prussoff correction.

Other tests
Proteins were measured according to Lowry et al., using BSA as the standard. The cholesterol content was determined after the lipid extraction by the method of Omodeo Sale et al. Mitochondrial cytochrome P450 was assayed as described by Omura and Sato. NADPH cytochrome c reductase activity was measured in liver microsomes and mitochondria according to Nervi et al.

Statistical analysis
Statistical analysis was done using ANOVA test; Dunnett’s test for multiple comparison was used when F was significant. A p value of less than 0.05 was considered significant.
Results

Purity of mitochondrial preparation
To ensure that the mitochondrial preparation was free of microsomal contamination compartmental marker enzymes were determined. The activities of NADPH cytochrome c reductase, a marker enzyme for endoplasmic reticulum and cytochrome c oxidase, a marker enzyme for mitochondrial fraction were measured in liver mitochondria and microsomes. Specific enzyme activities are expressed as µmol/ min/mg protein. As shown in Table 1, the contamination of mitochondria by microsomes was less than 2.3 %.

Table 1 Distribution of marker enzymes in subcellular fractions of rat liver. The specific activities of NADPH cytochrome c reductase, a marker enzyme for endoplasmic reticulum and cytochrome c oxidase, a marker enzyme for inner mitochondrial fraction were measured in liver mitochondria and microsomes.

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>NADPH cytochrome c reductase</th>
<th>Cytochrome c oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity (µmol/min/mgP)</td>
<td>Recovery (%)</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.6 ± 0.4</td>
<td>2.3</td>
</tr>
<tr>
<td>Microsomes</td>
<td>70.2 ± 13.8</td>
<td>97.7</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. of 3 representative separate preparations.

Effect of nifedipine, nitrendipine and DDC on cholesterol content in plasma and liver mitochondria
The effects of dihydropyridines on total cholesterol concentrations in rat liver mitochondria were determined. Nifedipine, nitrendipine and DDC did not change significantly the total cholesterol in liver mitochondria compared to controls (Table 2). DDC decreased total cholesterol by 39.8 % (p<0.01) in plasma whereas nifedipine and nitrendipine showed reduction respectively by 65.4% % (p<0.01) and by 70.7% (p<0.01) vs control animals (Table 2).

Table 2 Effect of DDC, nifedipine and nitrendipine on total cholesterol content in liver mitochondria and plasma. Animals were given i.p. injection (10 ml/ kg) of nifedipine (10 mg/kg), nitrendipine (8 mg/kg) or DDC (100 mg/kg) as described in Materials and Methods. The animals were killed by decapitation, plasma was collected and the livers were removed, rinsed in ice-cold saline and used for preparation of mitochondrial fraction.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma (mmol/l)</td>
</tr>
<tr>
<td>Control</td>
<td>1.33 ± 0.15</td>
</tr>
<tr>
<td>DDC</td>
<td>0.81 ± 0.08**</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>0.46 ± 0.05**</td>
</tr>
<tr>
<td>Nitrendipine</td>
<td>0.39 ± 0.06**</td>
</tr>
</tbody>
</table>

The values are mean ± S.E.M. from four animals per group from two representative experiments. **p < 0.01 vs control
**Effect of nifedipine, nitrendipine and DDC on 27-hydroxycholesterol production**

The content of 27-hydroxycholesterol was measured in liver mitochondria after the treatment and compared with controls. As shown on Table 3, all compounds stimulated significantly mitochondrial 27-hydroxycholesterol production. Nitrendipine showed major effect and increased the content of 27-hydroxycholesterol by 44.5 % (p<0.01), while DDC and nifedipine were less effective (increase by 35.9 % and 33.2 %, respectively, p<0.05).

In rat plasma DDC caused significantly increase of 27-hydroxycholesterol content by 65.3 % (p<0.01), and nifedipine and nitrendipine were also effective (increase respectively by 44.2 % and 49.2 %, p<0.01).

**Table 3** Effect of DDC, nifedipine and nitrendipine on 27-hydroxycholesterol production in plasma and liver mitochondria. Animals were given i.p. injection (10 ml/kg) of nifedipine (10 mg/kg), nitrendipine (8 mg/kg) or DDC (100 mg/kg) as described in Materials and Methods. The animals were killed by decapitation, plasma was collected for 27-hydroxycholesterol determination and the livers were removed, rinsed in ice-cold saline and used for preparation of mitochondrial fraction.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma (µg x 10^{-2}/ml)</th>
<th>Liver mitochondria (µg x 10^{-2}/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.60 ± 0.10</td>
<td>2.20 ± 0.40</td>
</tr>
<tr>
<td>DDC</td>
<td>4.30 ± 0.60**</td>
<td>2.99 ± 0.27*</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>3.75 ± 0.75**</td>
<td>2.93 ± 0.30*</td>
</tr>
<tr>
<td>Nitrendipine</td>
<td>3.88 ± 0.40**</td>
<td>3.18 ± 0.17**</td>
</tr>
</tbody>
</table>

The values are mean ± S.E.M. from four animals per group from two representative experiments. **p < 0.01, *p < 0.05 vs control.

**Effect of DDC, nifedipine and nitrendipine on cytochrome P450 in liver mitochondria**

To examine whether dihydropyridines directly affects the enzyme cholesterol 27-hydroxylase (cytochrome P450 dependent), the activity of cytochrome P450 was assayed in liver mitochondria. It seems unlikely that this effect was due to DDC-induced effects on mitochondrial cytochrome P450, since absorbance of the Fe^{2+} - CO complex of mitochondrial cytochrome P450 was similar in controls and DDC-treated animals (0.156±0.08 and 0.1845±0.11 nmol cytochrome P450/mgP, respectively). Nifedipine and nitrendipine have no effect on mitochondrial cytochrome P450 content.

**Effect of DDC, nifedipine and nitrendipine treatment on [3H] PK11195 binding to PBR in rat liver**

The inhibitory constants were determined by a competition of specific [3H] PK11195 binding in liver from rats treated with DDC, nifedipine and nitrendipine. The affinity (Ki) of Ro 5-4864 (PBR agonist) for [3H] PK11195 binding sites was significantly reduced in livers from animals treated with DDC (73.1± 31.6 µM, p<0.05 ). The estimated Ki values of Ro 5-4864 for [3H] PK11195 binding sites in livers from animals treated with nitrendipine and nifedipine were 21.7 ± 2.9 µM and 60.9 ± 15.7 µM, respectively. Values are mean ± SEM of six animals per group.
Discussion

Several animal studies have suggested that 1,4-dihydropyridine calcium antagonists can inhibit the development of experimentally induced atherosclerosis. In the present work we found that 1,4-dihydropyridines nifedipine and nitrendipine decreased the total cholesterol content in rat plasma in vivo. This finding is in agreement with previous results by Gorog and Born who demonstrated a decrease in plasma total cholesterol concentration in nifedipine treated animals. Chronic treatment with 1,4-dihydropyridines lowered plasma cholesterol in different animal species and humans. Although the biological process underlying this phenomenon has not been fully elucidated, several mechanisms have been proposed, including alteration of lipid metabolism.

There are different pathways for cholesterol elimination from the cells. An important role of the mitochondrial enzyme sterol 27-hydroxylase in the elimination of cholesterol was suggested in peripheral tissues. The enzyme sterol 27-hydroxylase might be involved in the removal of excess cholesterol from peripheral tissues, since this enzyme is present in liver, kidney, cultured fibroblasts, and mRNA for it’s synthesis was found in many tissues and cells: macrophages, endothelium, ovary. The intramembrane transport of cholesterol to the enzyme is a limiting factor for its further metabolic transformation in mitochondria. It was demonstrated that a mitochondrial membrane protein, called peripheral benzodiazepine receptor (PBR) facilitates the transport of cholesterol from outer to the inner mitochondrial membranes in liver mitochondria, where the enzyme sterol 27-hydroxylase is localised. In a previous work we showed that exogenous (PK11195 and Ro 5-4864) and endogenous PBR ligands (porphyrins) increased significantly the production of [14-C] 27-hydroxycholesterol in isolated rat liver mitochondria in vitro. The availability of [14-C]Cholesterol to the enzyme sterol 27-hydroxylase was a limiting factor for its further metabolism to [14-C]27-hydroxycholesterol in vitro. With the present work we have demonstrated that nifedipine, nitrendipine and DDC increase significantly the amount of 27-hydroxycholesterol in rat liver mitochondria in vivo. The enzyme markers were measured to check the purity of mitochondrial preparation. The degree of possible contamination of mitochondria with microsomes was less than 2.3 %. This contamination was considered not to interfere with the results obtained, because the enzyme sterol 27-hydroxylase is located predominantly in mitochondria and its activity in microsomes is extremely low. To investigate whether the stimulation of 27-HC production was mediated by PBR function, the respective inhibitory constants to PBR were determined in rat liver mitochondria ex vivo. The radio labeled ligand PK11195 binds with high affinity and selectivity to peripheral benzodiazepine receptor. We found that the binding of [3H]PK11195 in rat hepatic PBR was reduced after treatment with nifedipine and nitrendipine. In other studies was shown that 1, 4 dihydropyridines inhibit the binding of [3H]Ro5-4864 (a specific ligand to PBR), but not the binding of [3H]Flunitrazepam or [3H]Clonazepam in rat kidney, heart and brain. The inhibition was competitive, with Ki values in micromolar range. Most probably, the reduction in the binding of [3H]PK11195 to hepatic PBR in liver of rats, treated with DDC is attributable to specific DDC-induced formation of protoporphyrins. In animals, DDC caused conversion of the heme of Cytochrome P-450 into N-methylprotoporphyrin IX (NMePP). NMePP is a powerful inhibitor of the mitochondrial enzyme ferrochelatase and it caused a10- fold increase in accumulation of protoporphyrine IX (PPIX) in liver. NMePP and PPIX are potent endogenous ligands of mitochondrial PBR.
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In a previous work we have shown that NMePP and PPIX facilitated the incorporation and the membrane translocation of cholesterol in rat liver mitochondria in vitro.[11] NMePP and PPIX stimulated the mitochondrial intramembrane transport of cholesterol to the enzyme sterol 27-hydroxylase by a mechanism coupled to PBR.[10] We suppose that the mechanism of the observed stimulation on 27-hydroxycholesterol production by DDC is probably coupled to the accumulation of porphyrins in liver mitochondria.

Further studies were aimed at characterising more thoroughly the mechanism of stimulation of 27-hydroxycholesterol production by DDC, nifedipine and nitrendipine. We examined if nifedipine, nitrendipine and DDC stimulated 27-hydroxycholesterol production by a mechanism coupled to the enzyme induction. The cloning, structure and expression of the mitochondrial enzyme sterol 27-hydroxylase showed that it is a member of the cytochrome P450 superfamily of mixed function oxygenases.[2, 40] From our experiments it seems unlikely that the effect of DDC, nifedipine and nitrendipine may act on 27-hydroxylation of cholesterol through mitochondrial cytochrome P450 induction, since absorbance of the Fe$^{2+}$-CO complex of mitochondrial cytochrome P450 was similar in controls and treated animals.

Conclusions

We found that dihydropyridine calcium antagonists nifedipine and nitrendipine increased 27-hydroxycholesterol production in liver mitochondria in vivo. The mechanism of the observed effects of nifedipine and nitrendipine on the production of 27-hydroxycholesterol is not known, but it is likely that they act by a mechanism coupled to PBR. Oxysterols have been suggested to be physiological regulators of the enzyme HMG-CoA reductase and therefore intracellular regulators of cholesterol production. 27-Hydroxycholesterol is one of the most potent naturally occurring oxysterol inhibitors of HMG-CoA reductase.[2] The conversion of cholesterol to 27-hydroxycholesterol might be involved in a general defence mechanism for many peripheral cells exposed to cholesterol. It was shown that diazepam (PBR ligand) given chronically, lowered plasma cholesterol in a model of atherogenesis in different animal species and inhibits cholesterol synthesis in normal liver.[40,41] PK 11195 increases total plasma cholesterol and triglycerides in rats and antagonised the antiatherogenic effect of diazepam.[42] More studies are required to elucidate the precise mechanism of the stimulation of 27-hydroxycholesterol production and cholesterol elimination from the cells by different ligands to PBR.

References


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