Anti-proliferative Effects of Benidine Hydrochloride in Porcine Cultured Vascular Smooth Muscle Cells and in Rats Subjected to Balloon Catheter-Induced Endothelial Denudation

Shin-ichi Ide,* Mari KondoH, Hiroyuiki Satoh, and Akira Karasawa

Department of Pharmacology, Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 1188 Shimbogori, Nagatsumi-cho, Sanjo-gun, Shizuoka 411, Japan.
Received September 21, 1993; accepted January 12, 1994

Using the [3H]thymidine incorporation technique, the anti-proliferative effects of benidine, a long-acting calcium antagonist, on porcine cultured vascular smooth muscle cells (VSMCs) were determined and compared with those of other calcium antagonists. Benidine inhibited serum-stimulated [3H]thymidine incorporation into VSMCs (IC50, 0.2 μM), and this inhibitory effect was significantly more potent than that of nitrendipine, felodipine, nisoldipine, manidipine, amlodipine, nifedipine, verapamil and diltiazem. When growth-arrested cells were stimulated with platelet-derived growth factor followed by insulin, benidine, administered with either stimulation, inhibited [3H]thymidine incorporation into VSMCs. This suggests that it acts in both the G0/G1 and G1/S phases. In another series of experiments, the anti-proliferative effect in vivo was investigated using rats subjected to balloon catheter-induced endothelial denudation of the aorta. Benidine (5 mg/kg, p.o., b.i.d.) significantly reduced the incorporation of [3H]thymidine into aortic DNA 48 h after balloon injury, whereas it did not affect incorporation into bone marrow, suggesting that it inhibits arterial DNA synthesis. From our results, benidine was shown to exert anti-proliferative effects on VSMCs in vivo as well as in vitro. The drug may be useful for the treatment of vascular proliferative diseases such as restenosis following percutaneous transluminal angioplasty and atherosclerosis.

Keywords benidine; vascular smooth muscle cell; anti-proliferative effect; calcium antagonist; atherosclerosis

Migration of vascular smooth muscle cells (VSMCs) from the media to the intima of the arterial wall and the proliferation of intimal VSMCs are critical events in the formation of atherosclerotic lesions.1,2) Migration and proliferation of VSMCs are induced by a variety of endogenous substances, such as platelet-derived growth factor (PDGF)3,4) and 12-hydroxyeicosatetraenoic acids.5) PDGF stimulates the turnover of phosphatidyl inositol, which involves the formation of inositol 1,4,5-trisphosphate and diacylglycerol, associated with a rise in the cytoplasmic concentration of free Ca2+.6) In addition, PDGF not only mobilizes Ca2+ from intracellular stores but also causes an influx of Ca2+ into VSMCs.7) Recently, in vitro studies have shown that calcium antagonists inhibit the migration8,9) and proliferation10,11) of VSMCs. In addition, in vivo experiments have demonstrated the antithrombotic effects of the calcium antagonist in various animal models.10,12)

Benidine is a 1,4-dihydropyridine Ca2+ antagonist, which has long-lasting antihypertensive13) and anti-anginal activities.14) Moreover, it has been reported that benidine suppresses vitamin D2-induced aortic calcification in rats.15) In the present study, we have studied the anti-proliferative effects of benidine in both in vitro and in vivo experiments. The cell-cycle dependency of the effect of benidine was also examined using synchronized VSMCs.

MATERIALS AND METHODS

In Vitro Experiments

Cell Culture Porcine aortic smooth muscle cells were prepared from medial explants according to the method described by Ross.16) The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1-glutamine (2 mm), penicillin (50 u/ml) and streptomycin (50 μg/ml). The cells were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The cells were subcultured at the split ratio of 1:3 and used at the third to tenth passages.

Assay of DNA Synthesis When the cells were grown to confluency on 24-well plates, the medium was replaced with DMEM supplemented with 0.1% FCS. Thereafter, the cells were incubated for at least 3 d, when they were considered to be in the quiescent state.17) These arrested cells were incubated in DMEM containing 5% FCS and a calcium antagonist at an appropriate concentration. After 20 h, [3H]thymidine (1 μCi/well) was added and the incubation was continued for an additional 3 h. At the end of this period, the medium was aspirated and the cells were washed twice with 1.0 ml ice-cold phosphate-buffered saline (PBS). They were then allowed to stand in 1.0 ml 10% trichloroacetic acid (TCA) on ice for 10 min. After removing the TCA, the cells were solubilized with 1.0 ml 0.5 N NaOH and the radioactivity of an aliquot was measured using a liquid scintillation counter (Packard Tri-Carb 2200CA).

Analysis of Cell-Cycle Dependency Analysis of the cell-cycle dependency was carried out according to the method of Shirotani et al.18) PDGF works as a competence factor, which transfers cells from the G0 to the G1 (G0/G1) phase and insulin works as a progression factor, which transfers cells from the G1 to the S (G1/S) phase. Growth arrested VSMCs in 96-well plates were stimulated with PDGF-BB, 10 ng/ml for 2 h and the cells were then washed twice with serum-free DMEM containing 0.2% bovine serum albumin, followed by stimulation with insulin, 30 μg/ml for 22 h. At each stimulation, a calcium antagonist
or its vehicle was added. [3H]Thymidine (1 μCi/well) was added for the last 17 h and at the end of this period the medium was aspirated. Thereafter, the cells were washed twice with ice-cold PBS and the precipitate of ice-cold TCA-insoluble material was obtained. The radioactivity of the precipitate was measured using a liquid scintillation counter (Packard Tri-Carb 2200CA).

**Drug Cytotoxicity**  The cytotoxic action of the calcium antagonists was determined according to the method of Saeki et al. Growth-arrested VSMCs in 24-well plates were incubated in DMEM containing 0.1% FCS and [3H]adenine (0.5 μCi/ml) for 2 h. The incubation medium was then aspirated and the cells were washed twice with ice-cold PBS. The cells were then incubated in DMEM containing 5% FCS and a calcium antagonist. After 20 h, the culture media were collected and the cells were lysed by adding 1.0 ml Triton X-100. The radioactivity due to [3H]adenine in the medium and cell lysate was measured. The percentage of [3H]adenine release, an index of cytotoxicity, was calculated according to the following formula:

\[
\%{[3H] adenine \ release} = \frac{[3H] adenine \ in \ medium}{[3H] adenine \ in \ both \ medium \ and \ cell \ lysate} \times 100
\]

**In Vivo Experiments**

**Balloon Catheterisation** Male Sprague-Dawley rats weighing approximately 200 g (Japan SLC, Inc.) were used. Balloon catheterisation was performed using a slight modification of the method described by McCaffrey et al. In brief, rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and then subjected to an arterial injury using a 2F Fogarty balloon catheter passed 4 times at 1.5 atm across the aorta via the left carotid artery. After this procedure, the catheter was withdrawn and the left carotid artery was ligated. A drug or its vehicle (0.3% sodium carboxymethyl cellulose; CMC) was orally administered 2 h before and 10, 22, 34 and 46 h after the balloon catheterisation. For oral administration, benidipine and nifedipine were suspended in 0.3% CMC.

**[3H]Thymidine Incorporation** The incorporation of [3H]thymidine into the DNA of the aorta and bone marrow was measured 48 h after balloon catheterisation. Rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and then given [3H]thymidine solution (0.5 mCi/kg) intravenously. After 30 min, the animals were sacrificed by administering an overdose of anaesthesia. Thereafter, the thoracic aorta and right femur were removed and rapidly frozen using dry ice for subsequent analysis. The preparation of media from the whole aorta was carried out by scraping with blunt forceps. The specific activity of DNA in DNA was determined following extraction of the DNA from the washed tissue homogenerate using hot dilute perchloric acid. DNA was determined by the diphenylamine method and the radioactivity was measured using a liquid scintillation counter (Packard Tri-Carb 2200CA).

**Drugs** Benidipine (hydrochloride), nilvadipine, nisoldipine, nicardipine (hydrochloride), felodipine, manidipine (dihydrochloride), amlodipine and nitrendipine were synthesized in our laboratories. Nifedipine and verapamil hydrochloride were purchased from Sigma Chemical Co. Diltiazem was extracted from Herbesser® tablets. The other chemicals and reagents were obtained as follows: Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical Co., Ltd.), 1-glutamine, penicillin G, sodium-streptomycin sulfate (Gibco), trypsin (Flow laboratories), EDTA (Nakarai Tesuk), Thymidine-H3- (NEN) and [2-3H]adenine (Amersham).

**Statistical Analysis** Values are expressed as means ± S.E. Differences were examined using Steel’s test, following the Kruskal-Wallis test, by Student’s t-test, or by the Wilcoxon rank sum test. p values of 0.05 or less were considered to be statistically significant.

**RESULTS**

Effects of benidipine and some other calcium antagonists on serum-stimulated [3H]thymidine incorporation into VSMCs.

Figure 1. shows the concentration–response curves for the inhibitory effects of benidipine and some other calcium antagonists on serum-stimulated [3H]thymidine incorporation into VSMCs. Table I summarizes the corresponding IC50 values. Benidipine was significantly more potent in inhibiting [3H]thymidine incorporation than nitrendipine, felodipine, nisoldipine, manidipine, amlodipine, nifedipine, verapamil and diltiazem. At concentrations effective in inhibiting [3H]thymidine incorporation, benidipine and the other calcium antagonists examined were free from any cytotoxic effects (Table II).

**Analysis of Cell Cycle Dependency** Benidipine inhibited [3H]thymidine incorporation into VSMCs in both

<table>
<thead>
<tr>
<th>Table I. IC50 of Benidipine and Some Other Calcium Antagonists for [3H]Thymidine Incorporation into VSMCs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agents</strong></td>
</tr>
<tr>
<td>Benidipine</td>
</tr>
<tr>
<td>Nilvadipine</td>
</tr>
<tr>
<td>Nicardipine</td>
</tr>
<tr>
<td>Nitrendipine</td>
</tr>
<tr>
<td>Felodipine</td>
</tr>
<tr>
<td>Nisoldipine</td>
</tr>
</tbody>
</table>

Values are means ± S.E. of 6 experiments. a) p < 0.05 vs. benidipine (Wilcoxon rank sum test).

<table>
<thead>
<tr>
<th>Table II. Effects of Benidipine and Some Other Calcium Antagonists on [3H]Adenine Release from VSMCs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agents</strong></td>
</tr>
<tr>
<td>Benidipine (10 μM)</td>
</tr>
<tr>
<td>Verapamil (10 μM)</td>
</tr>
<tr>
<td>Diltiazem (100 μM)</td>
</tr>
<tr>
<td>Nifedipine (10 μM)</td>
</tr>
<tr>
<td>Nilvadipine (10 μM)</td>
</tr>
<tr>
<td>Nisoldipine (10 μM)</td>
</tr>
<tr>
<td>Nicardipine (10 μM)</td>
</tr>
<tr>
<td>Felodipine (10 μM)</td>
</tr>
<tr>
<td>Manidipine (10 μM)</td>
</tr>
<tr>
<td>Nitrendipine (10 μM)</td>
</tr>
<tr>
<td>Amlodipine (10 μM)</td>
</tr>
</tbody>
</table>

Values are means ± S.E. of 6 experiments. Figures in brackets are means ± S.E. of [3H] adenine release (%) from the corresponding vehicle-treated VSMCs.
Fig. 1. Effects of Benidipine and Other Calcium Antagonists on $[^3]$HThymidine Incorporation into VSMCs
Each column represents the mean ± S.E. of 6 experiments.

Fig. 2. Effects of Benidipine and Nifedipine, Added at the Time of PDGF ($G_0/G_1$, A) or Insulin ($G_1/S$, B) Stimulation, on $[^3]$HThymidine Incorporation into VSMCs
-■- benidipine, -□- nifedipine. Results are means ± S.E. of 12 experiments. a) $p < 0.05$ vs. control.
TABLE III. Effects of Benidine and Nifedipine on [3H]Thymidine Incorporation in the Balloon Catheterised Rat

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg p.o., b.i.d.)</th>
<th>No. of animals</th>
<th>[3H]thymidine incorporation (% of control animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Media</td>
</tr>
<tr>
<td>Benidine</td>
<td>2.5</td>
<td>6</td>
<td>113.8 ± 16.8</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>9</td>
<td>73.9 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>9</td>
<td>80.7 ± 6.4</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>10.0</td>
<td>6</td>
<td>159.1 ± 18.5</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>8</td>
<td>73.6 ± 12.5</td>
</tr>
</tbody>
</table>

Figures in brackets are mean ± S.E. of [3H] in tissue DNA (expressed as dpm/μg DNA) samples of control (balloon-injured vehicle-treated) animals. Animals were intravenously injected with [3H]thymidine and sacrificed at 30 min later. This was 48 h after balloon catheterisation. a Not tested; b p < 0.05, vs (Student’s t-test).

the G0/G1 and G1/S phases in a concentration-dependent manner. The inhibitory effect of benidine tended to be greater in the G1/S phase than in the G0/G1 phase. Nifedipine (10 μM) inhibited [3H]thymidine incorporation into VSMCs in the G1/S phase, but did not significantly inhibit incorporation in the G0/G1 phase (Fig. 2).

In Vivo Experiments The [3H]thymidine incorporation into the thoracic aorta of sham-operated rats and rats subjected to balloon catheterisation was 18.7 ± 3.0 (n = 4) and 64.2 ± 7.5 (n = 6) dpm/μg DNA, respectively. Thus, [3H]thymidine incorporation into aorta was significantly (p < 0.01) increased by balloon catheterisation. Benidine (5 mg/kg, b.i.d.) significantly inhibited [3H]thymidine incorporation into the balloon catheterised aorta. Nifedipine (20 mg/kg, b.i.d.) appeared to reduce [3H]thymidine incorporation into the injured aorta, although this effect was not statistically significant. Neither benidine nor nifedipine affected [3H]thymidine incorporation into bone marrow (Table III).

DISCUSSION

In the present study, we have demonstrated the anti-proliferative effects of benidine on VSMCs both in vitro and in vivo. In the in vitro studies, benidine inhibited serum-stimulated [3H]thymidine incorporation into VSMCs, as did the other calcium antagonists. However, the inhibition of [3H]thymidine incorporation by benidine was statistically greater than that produced by nitrendipine, felodipine, nisoldipine, manidipine, amldipine, nifedipine, verapamil and diltiazem. The potency of its anti-proliferative action seems to agree well with its action as a calcium antagonist.24 It is well known that nifedipine, diltiazem and verapamil inhibit the PDGF-induced proliferation of rat VSMCs and PDGF-induced rise in intracellular calcium.7 In the present study, benidine was also shown to inhibit PDGF-stimulated [3H]thymidine incorporation into VSMCs (Fig. 2). Therefore, the anti-proliferative effect of benidine may be due to inhibition of PDGF-induced phenomena, which are, in part, mediated by an increase in intracellular calcium.7

Recently, it has been reported that the inhibition of [3H]thymidine incorporation by dihydropyridine calcium antagonists is partly due to their ability to inhibit the salvage pathway (e.g.; nucleoside transport).25 A number of transport processes mediate the entry of purine and pyrimidine nucleosides and various nucleoside analogues into mammalian cells.26 Subtypes of these nucleoside transporters can be distinguished by their sensitivity to nitrobenzyl-thionosine (NBI) or nitrobenzyl-thioquinoine.27 Verna and Marangos28 have reported that nimodipine inhibits the binding of [3H]NBI to cerebral cortical membrane preparations. Furthermore, Striessing et al.29 have demonstrated that nimodipine inhibits [14C]adenosine uptake into human erythrocytes. In our experiments, benidine had no effect on either the binding of [3H]NBI to the cerebral cortical membrane of the guinea-pig or [3H]adenosine uptake into human erythrocytes, even at 1 μM (unpublished observation). Therefore, we can exclude the possibility that the inhibitory effect of benidine on [3H]thymidine incorporation into VSMCs was mediated via inhibition of NBI-sensitive nucleoside transport.

In cell proliferation, there are two critical steps: competent factors (e.g.; PDGF) are involved in the first step, where cells leave the G0 phase to enter the G1 phase, while progression factors (e.g.; insulin) are involved in the onset of DNA synthesis regulating the progression of the competent cells from the G1 to the S phase.30–31 In the present study, benidine exerted its inhibitory effects in both the G0/G1 and G1/S phases. Nifedipine also inhibits [3H]thymidine incorporation into VSMCs at both phases, although its inhibitory effect was weaker than that of benidine. An increase in intracellular calcium is important for cell proliferation in both the G0/G132 and G1/S33 phases. Thus, it might be assumed that benidine exhibits its calcium antagonistic action throughout the cell cycle, resulting in the anti-proliferative effect. In the present study, however, it was not clear whether benidine reduced the proportion of DNA synthesizing cells or the activity of DNA synthesis in each cell. Further studies are required to clarify this point.

In the in vivo study in rats, benidine (5 mg/kg) significantly inhibited [3H]thymidine incorporation into the balloon catheterised aorta. The effect of nifedipine (20 mg/kg), although somewhat similar to that of benidine, failed to reach statistical significance. There are several possible mechanisms by which benidine might be able to reduce arterial DNA synthesis. Direct inhibition by benidine of DNA metabolism can be ruled out since [3H]thymidine incorporation into bone marrow, a proliferating tissue, was not affected by a similar dose of this drug. One explanation suggested by the present in vitro study, is that benidine inhibits PDGF-induced phenomena, such as proliferation and migration of VSMCs. In addition, it is known that benidine, at 3 mg/kg, p.o., reduces arterial blood pressure in rats,34 and hence the reduced vascular tone might also contribute to the anti-proliferative effect of benidine seen in the in vivo experiments. We do not know why benidine, at 10 mg/kg, did not significantly inhibit [3H]thymidine incorporation into the balloon catheterised aorta. At this
high dose, the antiproliferative effect of benidipine might be mitigated by its adverse effects such as severe hypotension and neurohumoral activation.

In the present study, the effect of nifedipine in reducing $[^3]$H]thymidine incorporation into the balloon catheterised aorta did not reach statistical significance, even at 20 mg/kg (p.o., b.i.d.). However, Jackson et al. 12) have reported that nifedipine, at 1 mg/kg twice daily, was effective in a similar model to ours. The body weights of their rats were 400–450 g, whereas ours were approximately 200 g. In addition, we subjected our animals to an arterial injury produced by a balloon passed 4 times at 1.5 atm, while their surgical procedure for arterial injury was not shown in detail. Therefore, the discrepancy in the results might be due to these differences in experimental conditions. We cannot, however, deny the possibility that nifedipine, at higher than 20 mg/kg (b.i.d.), may be effective under our experimental conditions.

In conclusion, our present results demonstrate that benidipine inhibits arterial smooth muscle proliferation and it acts not only in the G1/G0 phase but also in the G1/S phase. Benidipine may be useful for the prophylactic treatment of vascular proliferative diseases, such as restenosis following percutaneous transluminal angioplasty and atherosclerosis.

REFERENCES