Mechanisms of the Hypolipidemic Effect of NIP-200 in Rats

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ABSTRACT—We studied the mechanisms of hypolipidemic effects of NIP-200 (3,5-dimethyl-4,6-diphenyl-tetrahydro-2H-1,3,5-thiadiazine-2-thione), a potent hypolipidemic compound, in cholesterol biosynthesis in the liver, cholesterol absorption in small intestine, and cholesterol catabolism and excretion in rats. NIP-200 did not reduce cholesterol biosynthesis and had no effects on cholesterol absorption in the small intestine. In the cholesterol catabolism and excretion, NIP-200 induced increases in cholesterol and bile acids levels in the bile and acidic steroids in the feces, and it enhanced cholesterol 7a-hydroxylase activity in the liver. These results suggest that NIP-200 increases the synthesis of bile acids as a result of the activation of cholesterol 7a-hydroxylase, the rate-limiting enzyme in the conversion of cholesterol to bile acids. Therefore, it is considered that one of the probable mechanisms of the serum total cholesterol lowering action of NIP-200 involves the enhancement of catabolism and excretion of cholesterol in the liver.

Keywords: NIP-200, Hypolipidemic effect, Cholesterol, 7a-Hydroxylase activity

It has been reported that NIP-200, 3,5-dimethyl-4,6-diphenyl-tetrahydro-2H-1,3,5-thiadiazine-2-thione, has a potent hypolipidemic effect on cholesterol-fed hyperlipidemic rats and rabbits. In cholesterol-fed hyperlipidemic rats and rabbits, NIP-200 induced a marked decrease in plasma total cholesterol (TC) and an increase in plasma high density lipoprotein-cholesterol (HDL-C). Furthermore, in the scanning electron microscopic study on the lumen surface of the aortic arch in cholesterol-fed rabbits, NIP-200 was found to reduce fatty plaques and prevented the loss of endothelial fissures and swelling of nuclei (1, 2).

In the previous report, we also demonstrated that NIP-200 prevents intimal thickening, proliferation of elastic fibers and fatty necrosis under the condition where the plasma lipid levels are not affected in cholesterol-fed rabbits, and we suggested that the improvement of lipoprotein metabolism in the arterial wall and prevention of migration and proliferation of arterial smooth muscle cells may also be important factors in the prevention of atherosclerosis (3).

In the present study, we examined the mechanisms of the hypolipidemic effects of NIP-200. In these investigations, NIP-200 was administered to the animals at a dose of 150 mg/kg or 50 mg/body (about 200–250 mg/kg), because NIP-200 clearly shows a potent hypolipidemic effect on cholesterol-fed hyperlipidemic rats at a dose of 150–300 mg/kg/day (1).

MATERIALS AND METHODS

Animals
Male Sprague Dawley rats obtained from Charles River Japan, Inc. (Atsugi), were used. The animals were selected for use in these studies after an acclimatization period of about one week.

Reagents
NIP-200 and clofibrate were synthesized at Nissan Chemical Industries, Ltd. [2-14C]Sodium acetate (56.2 mCi/mmol), [4-14C]cholesterol (58.4 mCi/mmol) and [1,2-3H]cholesterol (47.7 Ci/mmol) were obtained from Amersham International, Ltd. (Buckinghamshire, UK). All other chemicals were of the best quality commercially available.

Studies of hypolipidemic mechanisms
Cholesterol biosynthesis in rat liver: The rats, weighing 80–90 g, were fed commercial chow pellets ad libitum. NIP-200 was given to the rats via a stomach tube at a dose
of 150 mg/kg/day for 7 days. About 24 hr after the last dose, the rats were killed by decapitation in the middle of the dark period. The cholesterol biosynthesis activity was assayed by the method of Kuroda and Endo (5). Each incubation tube contained in a final volume of 0.2 ml: 1 mM ATP, 5 mM glucose 1-phosphate, 6 mM glutathione, 6 mM MgCl₂, 0.04 mM CoA, 0.25 mM NADP, 100 mM potassium phosphate buffer (pH 7.4), 0.25 mM [2-¹⁴C]-sodium acetate, and 0.1 ml of the cell-free homogenate. Protein was measured by the method of Lowry et al. (4).

Cholesterol absorption in rats: Experiment in thoracic-duct fistula rats: The rats, weighing 240-260 g and each with a cannula in the thoracic-duct (6), were fed commercial diet and 0.9% NaCl solution ad libitum overnight. NIP-200 (50 mg/body) dissolved in 0.5 ml of olive oil and 0.5 ml of lipid emulsion (1) were simultaneously given to the rats via a stomach tube. Lymph was collected for 24 hr after the drug administration. Total cholesterol in the lymph was determined by a modified Libermann Burchard reaction.

Experiment by the dual isotope method: The experiments were done according to the method of Zilversmit (7). Rats weighing 200-220 g were used. NIP-200 was given to the rats via a stomach tube at a dose of 50 mg/body. This was followed immediately by the administration of [¹⁴C]cholesterol (p.o.) and [³H]cholesterol (i.v.). Blood samples were collected from the jugular vein. The rats were fasted for 16 hr before and 4 hr after administration of the isotopes. The ¹⁴C or ³H radioactivity in the blood was measured individually by the combustion method.

Cholesterol catabolism and excretion in rats: Experiment in bile-duct fistula rats: The rats, weighing 150-160 g, were exposed to a reversed lighting pattern for 12 days. For the first 7 days, the rats were fed a commercial diet ad libitum. After that, the diet was replaced by a high cholesterol diet (1), and NIP-200 (150 mg/kg) was given to the rats via a stomach tube for 5 days. Between 16 and 17 hr after the last administration, the bile duct of each rat was cannulated, and then the bile was collected for 4 hr. Bile acids and cholesterol level in the bile were determined by the enzymatic method (8).

Experiment with labeled cholesterol: The rats, weighing 150-160 g, were fed a high cholesterol diet (1) and water ad libitum during the experimental period. NIP-200 was given to the rats via a stomach tube at a dose of 150 mg/kg for 9 days. On the seventh day, immediately after administration of the test compound, [¹⁴C]cholesterol was injected into the tail vein. A blood sample was collected from the jugular vein, and the radioactivity was determined by the combustion method. A feces sample was collected over a period of 72 hr. Neutral steroids were extracted from the feces sample by the method of Glundy et al. (9), and its radioactive steroids were determined by the combustion method.

Effect on cholesterol 7α-hydroxylase in rats: The rats, weighing 90-110 g, were exposed to a reversed lighting pattern, and fed a commercial diet and water ad libitum for 14 days. For the last 7 days, NIP-200 was given to the rats via a stomach tube at a dose of 150 mg/kg. Cholesterol 7α-hydroxylase activity was measured by the modified method of Goodwin et al. (10, 11). About 24 hr after the last dose, the rats were killed by decapitation, and the liver microsomes were prepared by centrifugation at 105,000 x g for 60 min. The microsomes were finally suspended in 40 mM potassium phosphate buffer (pH 7.2) containing 100 mM sucrose, 30 mM EDTA, 20 mM dithiothreitol and 50 mM KCl and used for the assay of cholesterol 7α-hydroxylase. All incubations included 0.4 mg of microsomal protein, [4-¹⁴C]cholesterol, an NADPH-generating system and 10 mM β-mercaptoethanolamine in a final volume of 0.5 ml assay buffer. A boiled enzyme control was run with each experiment.

Statistical analysis
Data were expressed as the mean±S.E. The statistical significance of the difference was evaluated by Student's t-test or Aspin-Welch's t-test and indicated by *: P<0.05.

RESULTS
Cholesterol biosynthesis in rat liver
The incorporation of [2-¹⁴C]acetate into sterols after daily oral doses of 150 mg/kg for 7 days is shown in Table 1. In the group treated with clofibrate, the incorporation of radioactive acetate into nonsaponifiable lipids and digitonin-precipitated sterols were inhibited by 39% and 82% (P<0.05), respectively.

NIP-200 did not reduce the incorporation of acetate into nonsaponifiable lipids and digitonin precipitated

Table 1. Effect of NIP-200 on the incorporation of [2-¹⁴C]acetate into sterols in rat liver homogenate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>No. of rats</th>
<th>Radioactivity (dpm/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>N.S.L.</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>6</td>
<td>15019±2376</td>
</tr>
<tr>
<td>Clofibrate</td>
<td>150</td>
<td>6</td>
<td>9136±550 (-39)</td>
</tr>
<tr>
<td>NIP-200</td>
<td>150</td>
<td>6</td>
<td>12707±1091 (-18)</td>
</tr>
</tbody>
</table>

N.S.L.: non-saponifiable lipids, D.P.S.: digitonin-precipitated sterols. Each value represents the mean±S.E. Figures in parentheses represent the % change from the control. *: Significantly different from the control (P<0.05).
Sterols.

Cholesterol absorption in rats

Table 2 shows the effects of β-sitosterol and NIP-200 on cholesterol absorption in thoracic-duct fistula rats. Amount of absorbed cholesterol in lymph collected during periods from 0–4, 4–8 and 8–24 hr after administration of 0.5 ml of lipid emulsion was not influenced by simultaneous administration of 50 mg/body of NIP-200 as compared with the oil vehicle-administered group (control). On the other hand, β-sitosterol lowered cholesterol levels in the lymph in the period of 4–8 hr.

Furthermore, cholesterol absorption was determined by using a technique involving the simultaneous administration of intravenous [1,2-3H]cholesterol and oral [4-14C]-cholesterol. As shown in Table 3, the percent absorption at 24 or 48 hr after administration of the labeled sterols was not significantly different between the group treated with 50 mg/body of NIP-200 and the control group.

Cholesterol catabolism and excretion in rats

The effect of NIP-200 on cholesterol excretion was studied in bile-duct fistula rats. As shown in Table 4, bile acids in the bile from each period were increased by administration of NIP-200. Particularly, the sample from 0–0.5 hr showed a significant (P<0.05) increase of as large as 58%. Total cholesterol levels in the bile from the period of 0–4 hr were increased to almost the same extent as compared with the oil vehicle-administered group (control).

Table 2. Effect of NIP-200 on cholesterol absorption in thoracic-duct fistula rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/body)</th>
<th>No. of rats</th>
<th>Recovered cholesterol in lymph (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0–4</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>4</td>
<td>9.65±0.89</td>
</tr>
<tr>
<td>β-Sitosterol</td>
<td>100</td>
<td>4</td>
<td>7.13±0.61</td>
</tr>
<tr>
<td>NIP-200</td>
<td>50</td>
<td>7</td>
<td>9.37±0.97</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E. *: Significantly different from the control (P<0.05).

Table 3. The determination of cholesterol absorption in rats by the dual isotope method

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/body)</th>
<th>No. of rats</th>
<th>Percent absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 hr</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>6</td>
<td>34.7±3.7</td>
</tr>
<tr>
<td>NIP-200</td>
<td>50</td>
<td>5</td>
<td>37.3±3.0</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E. Percent absorption: (% total ^14C in blood)/(% total ^3H in blood) × 100.

Table 4. Effect of NIP-200 on cholesterol excretion in bile-duct fistula rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>No. of rats</th>
<th>Bile acids (μmol/100 g B.W.)</th>
<th>Bile cholesterol (μg/100 g B.W.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0–0.5</td>
<td>0.5–1</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>4</td>
<td>13.8 ± 1.1</td>
<td>10.8 ± 2.2</td>
</tr>
<tr>
<td>NIP-200</td>
<td>150</td>
<td>3</td>
<td>21.8 ± 2.6</td>
<td>13.5 ± 1.0</td>
</tr>
</tbody>
</table>

B.W.: body weight. Each value represents the mean±S.E. *: Significantly different from the control (P<0.05).

Table 5. Changes of ^14C radioactivity in blood from 4 hr to 72 hr and half life from 24 hr to 72 hr after intravenously administered ^14C-cholesterol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>No. of rats</th>
<th>^14C Radioactivity (× 10^3 dpm/ml blood)</th>
<th>T1/2 (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>5</td>
<td>12.2±0.4</td>
<td>15.4±1.0</td>
</tr>
<tr>
<td>NIP-200</td>
<td>150</td>
<td>4</td>
<td>11.2±0.1</td>
<td>13.1±0.9</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E.
as the bile acid levels. In addition, an experiment using labeled cholesterol was done to follow-up the changes in the radioactivity in the blood and excretion into the feces. As shown in Table 5, NIP-200 had no significant effect on the changes in radioactivity from 4 hr to 72 hr and the half-life of that from 24-72 hr in the blood. As shown in Table 6, the total steroids were significantly (P < 0.05) increased by NIP-200. This effect of NIP-200 was attributed to a significant (P<0.05) increase in the excretion of acid ic steroids. On the other hand, neutral steroids were not influenced by NIP-200.

**Effect on cholesterol 7α-hydroxylase in rats**

The following studies examined the effect of NIP-200 on the 7α-hydroxycholesterol synthesis in rat liver microsomes. Table 7 shows the effect of NIP-200 treatment for 7 days on cholesterol 7α-hydroxylase. The rate of the hydroxylation was significantly (P < 0.05) enhanced by 28.4% in the liver microsomes treated with NIP-200.

**DISCUSSION**

The previous studies showed that NIP-200 had a potent hypolipidemic effect in hyperlipidemic rats and rabbits (1, 2). In the rats, NIP-200 significantly decreased serum TC in a dose dependent manner; and moreover, it elevated serum HDL-C at all doses. From the dose-response curves, NIP-200 was considered to be two to three times more potent than clofibrate.

In general, the mechanisms of the decrease in serum TC are considered to involve the following factors: 1) inhibition of cholesterol biosynthesis in the liver, 2) inhibition of intestinal absorption, 3) inhibition of secretion of lipoprotein from the liver, 4) enhancement of catabolism of cholesterol, 5) increased excretion of sterols in the feces, and 6) enhancement of incorporation of low density lipoprotein (LDL) in the liver and peripheral cells.

It has been reported that the hypolipidemic effects of clofibrate are based on the inhibition of cholesterol biosynthesis in the liver (12), secretion of lipoprotein into the blood from the liver (13), and enhancement of plasma lipoprotein lipase (LPL) activity (14), while clofibrate does not affect the cholesterol absorption in the small intestine (15). In cholesterol-fed rats, the ability of cholesterol biosynthesis in the liver is depressed by external cholesterol (16). Consequently, the main mechanism is considered to be the inhibition of secretion of lipoprotein into the blood from the liver and enhancement of LPL activity.

In our study, NIP-200 did not show inhibition of cholesterol biosynthesis (Table 1). On the other hand, clofibrate showed inhibition of cholesterol biosynthesis under the same conditions. Therefore, it is suggested that the inhibition of cholesterol biosynthesis does not involve the mechanisms of the hypolipidemic effect of NIP-200. As to the cholesterol absorption, β-sitosterol, which is known to inhibit cholesterol absorption (17), lowered the cholesterol level in the lymph, while NIP-200 showed no effects throughout the experimental period (Tables 2 and 3). The above observations suggest that NIP-200 does not affect the cholesterol absorption.

As to cholesterol catabolism and excretion, NIP-200 increased the excretion of bile acids and cholesterol into the bile in bile-duct fistula rats (Table 4). In this study, we started the experiment at 16–17 hr after the last administration of NIP-200. Under this condition, NIP-200 significantly increased the excretion of bile acid into the bile at an early time. If we start the experiment at an early time after the last administration, NIP-200 may more clearly show these effects.

Continuously, we did an experiment with labeled cholesterol to follow-up on the above results. We found that although NIP-200 had no significant effect on the changes
of radioactivity in blood (Table 5), the excreted radioactive acidic steroids in the feces were significantly increased by NIP-200 (Table 6). In this study, if the catabolism and excretion of cholesterol were enhanced by NIP-200, elimination of lipoprotein-cholesterol from the blood would be rapid in the NIP-200-treated group as compared with that in the control group. In this point, the difference between the theory and results was recognized.

However, it was anticipated that radioactivity in the blood includes the radioactivity of cholesterol transferred to the blood cell membrane and catabolites of cholesterol as well as plasma lipoprotein-cholesterol. Therefore, the change of radioactivity in the blood may not exactly reflect the change of plasma lipoprotein-cholesterol in the blood. Moreover, the level of radioactivity in the blood was low, and the difference of the excreted radioactive total steroids in the feces (between control and NIP-200 group) were small because of the small amount of administered radioactive cholesterol.

The above reasons may therefore explain why there was no observed difference in the change of radioactivity in the blood between the control animals and the NIP-200-administered group.

The above results suggested that NIP-200 may increase the synthesis of bile acids. Consequently, the activity of cholesterol 7α-hydroxylase, the rate-limiting enzyme in the conversion of cholesterol to bile acids, was estimated. NIP-200 significantly enhanced the rate of cholesterol hydroxylation (Table 7).

In view of the present results, it was considered that administration of NIP-200 increases the synthesis of bile acids through the activation of cholesterol 7α-hydroxylase. Thus, one of the probable mechanisms for the hypolipidemic effect of NIP-200 based on the present observations would be the enhancement of the catabolism and excretion of cholesterol in the liver.

REFERENCES


