Chlorpromazine Inhibits Arterial ACAT and Reduces Arterial Cholesterol and Cholesteryl Ester Accumulation in Cholesterol-Fed Rabbits

Frank P. Bell and Robert G. Schaub

Rabbits were fed an atherogenic diet concurrently with chlorpromazine for 2 weeks (10 mg/kg/day in the diet) or 12 weeks (20 mg/kg/day given orally in a single capsule). After 2 weeks, arterial acyl CoA: cholesterol acyltransferase (ACAT) activity tended to be reduced by chlorpromazine treatment with no affect on net arterial cholesterol. After 12 weeks of treatment, arterial ACAT activity was significantly reduced and was paralleled by a reduction in net arterial cholesterol, a reduction in the esterified cholesterol/unesterified cholesterol ratio, and a reduction in lipid staining intensity as determined histologically with oil red O staining of aortic cross sections. Paradoxically, there was no histological evidence for a reduction in the size of atheromatous lesions with chlorpromazine treatment as determined by morphometric analysis of tissue cross sections. The results support the hypothesis that the increased esterification of cholesterol that characteristically accompanies the atherogenic process serves as a biochemical trapping mechanism for cholesterol entering the vessel wall and suggest that regulation of the enzyme in vivo can reduce the net accumulation of arterial cholesterol. (Arteriosclerosis 6:42–49, January/February 1986)

Methods

Animals and Tissue Preparation

Male New Zealand rabbits (weighing approximately 2.4 kg) were fed an atherogenic diet consisting of Purina Chow supplemented with 3% peanut oil and 1% cholesterol (wt/wt/wt). Chlorpromazine-HCl (Sigma Chemical Corporation, St. Louis, Missouri) was administered daily to the rabbits as an admixture with the diet to provide 10 mg/kg body wt (2-week study) or orally in a No. 1 gelatin capsule at a level of 20 mg/kg body wt (12-week study). The capsules were administered between 8:30 and 9:30 A.M. with a balling gun that placed the capsule at the back of the throat for obligatory swallowing; powdered gelatin was substituted in the capsules given to control rabbits. All animals received measured amounts of feed daily so that consumption by control and treated animals could be kept equivalent; this was usually in the range of 90 to 100 g per day. Animal weights were unaffected by chlorpromazine. The rabbits were killed by exsanguination following intravenous (marginal ear vein) pentobarbital anesthesia (30 mg/kg). The aortas were quickly excised and rinsed in chilled 0.9% NaCl solution; a standardized section of the thoracic aorta was taken for histology as described below. The remaining tissue

Arteries undergoing atherogenic change characteristically show an increase in cholesterol esterifying activity by ACAT (acyl CoA: cholesterol acyltransferase, EC 2.3.1.26) and a progressive accumulation of cholesteryl esters. The significance of the ACAT reaction to the development of atherosclerotic vessel disease, however, is not well understood because of a lack of suitable inhibitors. We recently reported that chlorpromazine was an inhibitor of ACAT in vitro in arteries from rabbits fed an atherogenic diet; in the present studies, we determined that chlorpromazine will also inhibit arterial ACAT in vivo in cholesterol-fed rabbits. On the basis of biochemical and histological assessment of the experimental results, the data support the hypothesis that arterial ACAT serves as a biochemical trapping mechanism for cholesterol entering the artery. Additionally, the data suggest that arterial lipid accumulation and atheromatous lesion size may be independently determined.

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Received November 28, 1984; revision accepted July 3, 1985.
(i.e., the entire aorta minus the segment removed for histology) was opened longitudinally, stripped of adventitial tissue with fine forceps, and subjected to the procedures described below.

**Tissue Incubation and Analysis of Tissue and Plasma**

The freshly obtained tissue was either lipid-extracted directly by homogenization in CHCl₃-MeOH (2:1, vol/vol)³ (2-week study) or incubated for 3 hours at 37°C in 9.0 ml of a medium consisting of Medium 199 (Grand Island Biological Company, Grand Island, New York) and pooled normal rabbit serum in a ratio of 1:1 (vol/vol).² The medium contained 1.1 μCi/ml of 1-¹⁴C-oleic acid (S.A. 52.6 Ci/Mol, New England Nuclear Corporation, Boston, Massachusetts), which was added as the sodium salt.² After incubation, the tissues were rinsed in five 100 ml changes of 0.9% NaCl solution and were extracted with CHCl₃-MeOH as noted above. A portion of the arterial extracts was used to determine the total arterial cholesterol, unesterified cholesterol, and esterified cholesterol by difference.⁴ In tissues that were incubated with ¹⁴C-oleate, another portion of the extracts was fractionated by thin-layer chromatography on silica gel G-coated glass plates in a solvent system consisting of n-hexane/diethyl ether/glacial acetic acid (146:50:4, vol/vol/vol)⁵ for separation of the various lipid classes. Lipid bands were scraped from the chromatoplates into vials containing 15 ml of Liquifluor (New England Nuclear Corporation, Boston, Massachusetts) and were assayed for radioactivity by liquid scintillation counting in a Packard Model 3375 liquid scintillation spectrometer. Quench corrections were made by use of an external standard; the counting efficiency for ¹⁴C was approximately 90%.

**Plasma Analysis**

Plasma was obtained from blood drawn by cardiac puncture into heparinized syringes at the time of sacrifice and was analyzed for triglycerides⁶ and total cholesterol.⁶ In the 12-week study, plasma cholesterol was also measured in blood taken by venapuncture (marginal ear vein) at 4 weeks.

**Histology**

A portion of the thoracic aorta caudal to the aortic arch at the first intercostal arteries was removed and immersion-fixed in a 1% formaldehyde/1% glutaraldehyde fixative made in Tyrode’s solution (pH 7.4).⁷ Total frozen cross sections were made from each aorta at 8 μ increments with a Damon/IEC cryostat until the entire segment was sectioned. Each section was stained with oil red O and counter-stained with hematoxylin on the same day by using the same batches of stains. Lesion size (area) was determined as a percentage of the total cross-sectional area with a Zeiss Videoplan image analysis system. Intensity of oil red O stain was also assessed for each section containing atherosclerotic lesions. Each stained section was completely photographed with a Zeiss photomicroscope III, and colored slides were prepared. The slides were examined by two independent observers who had no knowledge of the experimental groups. Slides were graded as follows: 0 = no stain; 1 = <50% light intensity stain; 2 = >50% light intensity stain; 3 = medium intensity stain to <50% deep red stain; 4 = deep red stain to <50% medium intensity stain; 5 = complete deep red stain. Scores for each section were summed and averaged. A total of 190 sections of aortas from control rabbits and 200 sections from chlorpromazine-treated rabbits were analyzed.

**Results and Discussion**

We recently reported⁸ that cholesterol esterification by ACAT was inhibited in arterial tissue and in isolated arterial microsomes that were incubated in vitro in the presence of chlorpromazine. The purpose of the present studies was to determine in rabbits whether: 1) arterial ACAT activity can be modified in vivo by the administration of chlorpromazine and 2) inhibition of ACAT in vivo affects the development of atherosclerosis. The latter objective is of particular interest because cholesterol-esterifying activity is enhanced in arteries undergoing athrogenic change,⁹¹¹ and this is thought to contribute to the net accumulation of esterified cholesterol within the artery.¹²¹³ On this basis, the esterification reaction has been viewed as a possible biochemical trapping mechanism for cholesterol entering the artery from plasma.¹⁴¹⁵ In making this statement, we acknowledge the evidence that arterial cholesterol esters are not metabolically inert (i.e., cholesteryl esters can undergo hydrolysis¹⁶²⁰ and can egress from vessels.²¹²⁵ On the other hand, the progressive net accumulation of sterol esters in arteries undergoing athrogenic changes becomes the dominating aspect of sterol ester metabolism²⁶²⁷ and within that context, the ester represents a "trapped" form of the cholesterol molecule. If the "biochemical trap" hypothesis is valid, a reduction in ACAT activity under athrogenic conditions should result in a decreased rate of net sterol accretion by arterial tissue.

In these studies, rabbits were given chlorpromazine daily either as an admixture with the diet (calculated to provide 10 mg/kg body wt/day by monitoring feed consumption) or as an oral dose in a gelatin capsule (calculated to provide 20 mg/kg body wt). Table 1 shows that the addition of chlorpromazine to the athrogenic diet for 2 weeks did not significantly affect body weight gain, plasma lipid levels (cholesterol and triglyceride), aortic weight, or aortic total cholesterol levels. There was, however, a 27% decrease in the mean ratio of esterified cholesterol/unesterified cholesterol in the aortas of the group receiving chlorpromazine. Although the change did not reach a statistical level of significance (0.22 ± 0.03
Figure 1 A. Aorta from control New Zealand rabbit. Representative section of the least intensely stained tissue.

Figure 1 B. Aorta from chlorpromazine-treated New Zealand rabbit. Representative section of the least intensely stained tissue. The lesion size was similar in A and B, but the stain intensity was reduced in aortas from chlorpromazine-treated rabbits. Oil red O and hematoxylin stains. Original magnification, × 530. Bar = 53 μ.
Figure 1 C. Aorta from control New Zealand rabbit. Representative section of the most intensely stained tissue.

Figure 1 D. Aorta from chlorpromazine-treated New Zealand rabbit. Representative section of the most intensely stained tissue. The lesion size was similar in A, B, C, and D, but the stain intensity was reduced in aortas from chlorpromazine-treated rabbits. Oil red O and hematoxylin stains. Original magnification, × 530. Bar = 53 μ.
Table 1. Effect of a 2-Week Administration of Chlorpromazine on Body Weight, Arterial Cholesterol, and Plasma Lipid Levels in the Cholesterol-Fed Rabbit

<table>
<thead>
<tr>
<th>Rabbit group</th>
<th>Body wt (g)</th>
<th>Plasma lipids (mg/dl)</th>
<th>Aortic cholesterol (µg/mg wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2374 ±64</td>
<td>2763 ±33</td>
<td>390</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>±19</td>
</tr>
<tr>
<td>CPZ</td>
<td>2364 ±53</td>
<td>2760 ±77</td>
<td>416</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>±20</td>
</tr>
</tbody>
</table>

Male New Zealand rabbits were fed the atherogenic diet alone (control) or with added chlorpromazine (CPZ) at a level providing 10 mg/kg body wt/day based on daily feed consumption measurements. Values are means ± SEM of seven animals per group.

Table 2 shows the response of rabbits to the atherogenic diet and chlorpromazine (20 mg/kg body wt/day orally) for a longer time (12 weeks). As had been observed in the 2-week study (using 10 mg/kg body wt/day of chlorpromazine), the dose of 20 mg/kg body wt/day did not significantly affect body weight, plasma cholesterol, plasma triglyceride levels, or aortic wet weight when compared with control animals receiving the atherogenic diet alone. Interim plasma cholesterol levels determined at 4 weeks were also similar in the two groups (control, 1325 ± 160 mg/dl; chlorpromazine, 1300 ± 115 mg/dl). There was, however, a striking difference in the net cholesterol and esterified cholesterol content of the aortas of the two groups of animals at 12 weeks. Animals receiving chlorpromazine had a 40% reduction in total (net) arterial cholesterol (13.0 vs 21.5 µg/mg tissue wet wt, p < 0.05) and a 47% reduction in esterified cholesterol (6.4 vs 12.1 µg/mg tissue wet wt, p < 0.05). The reduction in esterified cholesterol was also reflected in a 25% reduction in the esterified/esterified cholesterol ratio.

Table 3 summarizes the pattern of lipids synthesized from 14C-oleate in the arteries of animals from the 12-week study. Chlorpromazine treatment did not significantly affect the total incorporation of 14C-oleate into esterified lipids, nor did it affect the percentage distribution of 14C-oleate into phospholipids or diglyceride. There was, however, an increase in the percentage distribution of 14C-oleate into triglycerides in the chlorpromazine group relative to the controls (27.4% vs 14.1%, p < 0.05) which remains unexplained, since oil red O staining intensity of arterial cross sections from the chlorpromazine-treated rabbits does not suggest an increased production of neutral lipid in vivo. Of particular interest was a significant decrease in the percentage distribution of 14C-oleate into the cholesteryl ester fraction of the chlorpromazine-treated group (26.2% vs 44.4%, p < 0.02). This decreased incorporation of 14C-oleate into radiolabeled cholesteryl ester (Table 3) together with the decreased level of chemically determined cholesteryl ester present (Table 2) and the reduced esterified/esterified cholesterol ratio (Table 2) are evidence that chlorpromazine administration does, in fact, inhibit ACAT in arterial tissue in vivo. Furthermore, the reduction of net sterol in arteries associated with chlorpromazine treatment is consistent with

Table 3. Effect of a 12-Week Administration of Chlorpromazine on Body Weight, Arterial Cholesterol, and Plasma Lipid Levels in the Cholesterol-Fed Rabbit

<table>
<thead>
<tr>
<th>Rabbit group</th>
<th>Body wt (g)</th>
<th>Aortic wet wt. (mg)</th>
<th>Plasma lipids (mg/dl)</th>
<th>Aortic cholesterol (µg/mg/wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Cholesterol</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>Control</td>
<td>2415 ±64</td>
<td>3273 ±80</td>
<td>448</td>
<td>2022 ±161</td>
</tr>
<tr>
<td></td>
<td>±62</td>
<td>±18</td>
<td>±18</td>
<td>±18</td>
</tr>
<tr>
<td>CPZ</td>
<td>2397 ±53</td>
<td>3244 ±109</td>
<td>483</td>
<td>1629 ±76</td>
</tr>
<tr>
<td></td>
<td>±53</td>
<td>±44</td>
<td>±44</td>
<td>±44</td>
</tr>
</tbody>
</table>

Male New Zealand rabbits weighing approximately 2.4 kg were fed the atherogenic diet along with the daily oral administration of a capsule filled with powdered gelatin (control) or with chlorpromazine (CPZ) at a dose level of 20 mg/kg body wt for 12 weeks. Values are means ± SEM of data from seven animals per group.

*Significantly different from control values (p < 0.05) by Student's t test for independent variates.
Table 3. Effect of a 12-Week Administration of Chlorpromazine on the Incorporation of \(^{14}\)C-Oleate Into Arterial Lipid in the Cholesterol-Fed Rabbit, In Vitro

<table>
<thead>
<tr>
<th>Rabbit group</th>
<th>(^{14})C Oleate incorporation into total esterified lipid (dpm/mg wet wt)</th>
<th>Distribution of (^{14})C-oleate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>620 ± 60</td>
<td>Phospholipid 31.8 ± 2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diglyceride 9.7 ± 1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Triglyceride 14.1 ± 2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Steryl ester 44.4 ± 4.2</td>
</tr>
<tr>
<td>CPZ</td>
<td>760 ± 110</td>
<td>Phospholipid 28.7 ± 5.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diglyceride 12.9 ± 1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Triglyceride 27.4* ± 1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Steryl ester 26.2† ± 4.7</td>
</tr>
</tbody>
</table>

Aortas from rabbits described in the footnote to Table 2 were incubated for 3 hours at 37°C in 9.0 ml of M199: normal rabbit serum (1:1, vol/vol) which contained 1 \(^{14}\)C-oleate (1.1 \(\mu\)Ci/ml). Arterial lipids were extracted with CHCl\(_3\)-MeOH (2:1 vol/vol) and fractionated by thin-layer chromatography as described under Methods. Values are means ± SEM of data from seven animals per group.

\(\*p < 0.05; \tp < 0.02; \) significantly different from control values by Student's \(t\) test for independent variates.

the hypothesis that a reduction in ACAT activity can result in a lowering of net cholesterol accumulation in arteries under atherogenic conditions.

The possibility that chlorpromazine reduces arterial ACAT activity indirectly by stimulating free cholesterol efflux\(^{28,29}\) from the arterial cells was also considered as an explanation for ACAT inhibition. This possibility seems unlikely, however, in view of the fact that the free cholesterol levels in arteries from the control and treated animals were similar at 7 and 9 \(\mu\)g/mg wet weight, respectively (derived from Table 2, total minus ester) and were greatly in excess of the free cholesterol levels in normal rabbit aortas (i.e., about 1 \(\mu\)g/mg wet weight\(^{30,31}\)). The results presented here are similar to our previous results\(^8,32\) concerning the effect of lidocaine, another ACAT-inhibitor, on cholesterol and cholesteryl ester accumulation in Fu5AH cells exposed to hyperlipemic plasma lipoproteins.\(^{33}\) Under culture conditions that lead to net cholesterol uptake and cholesteryl ester deposition in the cells, lidocaine inhibited ACAT activity, thereby reducing cholesteryl ester deposition by 40% and reducing net cellular sterol accumulation by 25%.

The reductions in net cholesterol and esterified cholesterol in aortas from the chlorpromazine-treated rabbits were also paralleled by reductions in oil red O staining intensity, as well. Examples of sections showing the least extent of oil red O staining in arteries are given in Figure 1A for control animals and in Figure 1B for treated animals. Examples of sections showing the most extensive staining in arteries are presented in Figure 1C for control animals and in Figure 1D for treated animals. The mean

Figure 2. The relationship between total aortic cholesterol and lesion area in aortas from rabbits fed the atherogenic diet alone (control) or atherogenic diet and chlorpromazine (20 mg/kg body wt) for 12 weeks (see text and footnote to Table 2).
staining intensity score of all the control tissues was 2.14 ± 0.80 (190 tissue sections) vs 1.75 ± 0.69 (200 tissue sections) (p < 0.001) in the drug-exposed tissues. Paradoxically, however, there was no histological evidence that lesion size was affected by chlorpromazine treatment (Figures 1A vs 1B and 1C vs 1D). Mean lesion area calculated as a percentage of total cross-sectional area of all sections from control aortas was 26.9% ± 7.0% (190 tissue sections) vs 33.7% ± 12.2% (200 tissue sections) in aortas from the chlorpromazine-treated group. This dichotomy between lipid accumulation and lesion size seen in the chlorpromazine group is an unexpected feature since lesion severity is usually paralleled by increased lipid accumulation, particularly the accumulation of cholesterol and its esters, in humans and in virtually all species used to study experimental atherosclerosis. This correlation was confirmed by our control group (Figure 2) where linear regression analysis indicated that lesion area and arterial cholesterol were highly positively correlated (r = 0.95, p < 0.001); the correlation was weak and not significant in the chlorpromazine group (r = 0.50, p > 0.05).

As a final comment, the studies presented here confirm through biochemistry and histology the interesting observations of LaFrance and LeBlanc that chlorpromazine treatment in rabbits during progression of atherosclerosis reduced aortic sudanophilia as determined visually.

Acknowledgments

The authors are grateful to Edna V. Hubert and Carol A. Simmons for laboratory assistance and to Jeanne L. Obreiter for secretarial assistance.

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Index Terms: cholesterol-fed rabbit • arterial cholesterol esterification • chlorpromazine • arterial cholesterol level • inhibition of arterial acyCoA:cholesterol acyltransferase (ACAT) • oil red O
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doi: 10.1161/01.ATV.6.1.42

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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