Effect of Metoprolol on Diet-Induced Atherosclerosis in Rabbits

Ann-Margret Östlund-Lindqvist, Peter Lindqvist, John Bräutigam, Gun Olsson, Göran Bondjers, and Claes Nordborg

The effect of metoprolol, a β₁-blocker, on atherogenesis was evaluated in rabbits fed a diet supplemented with 0.25% cholesterol and 3% coconut oil for 21 weeks. After 7 weeks on the diet, the rabbits were randomly divided into treated (n = 22) and untreated (n = 22) groups. Treated animals received metoprolol subcutaneously by an osmotic pump for 14 weeks, resulting in a plasma level of 774 ± 69 nM during the investigation. Plasma concentrations of cholesterol, triglycerides, and phospholipids did not differ between the two groups. Nor were there any significant differences between the two groups in plasma concentrations of apolipoprotein A-I, apolipoprotein B, apolipoprotein C-III, and apolipoprotein E measured by electrophoresis. At the end of the study, the aortas were cut into three portions and the extent of atherosclerosis was determined by morphometry. The group that had received metoprolol had significantly (p < 0.015) less atherosclerosis in the aorta (ascending plus arch 37.8 ± 6.8%, thoracic 32.9 ± 6.1%, abdominal 19.8 ± 6.1% of total intimal area; mean ± SEM) than the controls (ascending plus arch 54.9 ± 7.1%, thoracic 48.0 ± 6.2%, abdominal 25.9 ± 5.5%). (Arteriosclerosis 8:40–45, January/February 1988)

Experimental Design

A total of 44 male, colored rabbits (British Halfpipp, Foxfield SPF Rabbits, Petersfield, Hampshire, United Kingdom) weighing about 3.0 kg and aged approximately 12 weeks at the start of the study were used. They were housed individually in plastic cages (width 45 cm × 55 cm, height 42 cm). The room temperature was kept at between 18°C and 22°C with a 12/12-hour light/dark cycle. The rabbits were fed a standard rabbit diet (EWOS Brood Stock Feed, EWOS AB, Södertälje, Sweden) during a 4-week conditioning period. At the end of this period, blood samples for initial plasma analysis of lipids and apolipoproteins were drawn for initial plasma analysis of lipids and apolipoproteins. For 21 weeks the rabbits were then fed a standard diet supplemented with 2.5 g cholesterol and 30 g coconut oil per 1,000 g fed. Feeding was restricted to 80 g per day.

After 7 weeks on the atherogenic diet, the animals were randomly divided into treated (n = 22) and untreated (n = 22) groups. Treated animals received metoprolol (metoprolol tartrate) at a dose of 0.35 mg/kg/hr administered subcutaneously by an osmotic pump 2ML4 (Alza Corporation, Palo Alto, California). The control rabbits received isotonic saline by osmotic pumps during 8 weeks. During the last 6 weeks, they did not have any pumps, but went twice through the procedure to change the pumps (anesthesia and surgery) at the same time as the pumps were changed in the treated rabbits. Every fourth week the pumps were replaced, and blood samples for plasma anal-

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ysis were drawn from the central ear artery into tubes containing heparin. During surgery and exsanguination, the animals were anesthetized with a mixture of xylazine (20 mg/ml), ketamine (50 mg/ml), and saline (1:2:7, vol/vol/vol) intravenously (2 to 3 ml/kg body weight). The animals were weighed at 4-week intervals throughout the investigation (Figure 1). The experimental procedures were approved by the Ethics Committee of Göteborg, Sweden.

**Tissue Preparation**

At the end of the study, after exsanguination of the rabbit, the heart and the aorta were dissected and removed from the animal.

Adventitial tissue of the isolated aortas were stripped off in chilled 0.9% NaCl solution. The aortas were opened longitudinally and cut transversely between the orifices of the eighth and the ninth intercostal arteries and approximately 10 mm caudally to the orifice of the left renal artery and thereby divided into three standardized portions: ascending aorta plus arch of aorta, thoracic aorta, and abdominal aorta. The hearts were fixed in 10% buffered formalin solution, pH 7.0.

The main branches of the left and right coronary arteries were dissected under a microscope as far distally as technically feasible. The preparations of the left artery were 20 mm or longer, and the right arterial segments were at least 8 mm long. The arteries were opened longitudinally and were then stained with Sudan red for 2.5 hours. Perivascular soft tissues were carefully trimmed. Longitudinal cuts, at the most four per circumference, were made through thick atheromatous plaques to allow complete unfolding of the vessels. The specimens were then mounted in glycerine gelatine under glass. A constant pressure was applied to keep the vessels flat during hardening of the medium.

**Macroscopic and Microscopic Examination of the Aorta**

The three portions of the aorta were pinned flat to a paraffin wax layer in a Petri dish, were fixed in 10% neutral formalin solution, and were then lipid-stained with oil red O. By blind reading, the degree of atherosclerosis was determined by measuring the percentage of lipid-stained area in each of the three portions of the aorta. For this purpose a grid was used, in which each point corresponded to 0.30 mm².

Eight lesions (four each from the treated and untreated groups) from the abdominal and from the thoracic aorta were cut out, rinsed in physiological saline, and freeze-sectioned in an ordinary cryostat. The 8 μm sections were stained with oil red O and Mayer's haemalum, and studied under the light microscope. By blind reading, the sections were described with regard to the relationship between intracellular and extracellular lipids, lipid-filled and lipid-free cells, and the presence of leucocytes and endothelium.

**Microscopic Examination of the Coronary Arteries**

The total area and the percentage area of atheromatous plaques were determined by blind reading in the 20 mm and 8 mm most proximal part of the left and right arteries, respectively. An eyepiece with a square lattice grid in a dissection microscope was used for point counting. The calibrated 121-point grid was moved stepwise along the

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**Figure 1.** Experimental design.
vessels. Each point corresponded to 0.30 mm² at a magnification of × 20.

**Plasma Analyses**

For all the plasma analyses, plasma from nonfasting rabbits was used. The plasma concentration of metoprolol was determined by high-resolution gas chromatography and electron capture detection.¹⁷  

The concentration of lipids in plasma and high density lipoprotein fractions were determined in a Cobas Bio Centrifugal Analyzer by using a kit for cholesterol (Monotest, Boehringer Mannheim GmbH, FRG), kits for phospholipids and fatty acids (Wako Chemicals GmbH, FRG), and a kit for triglycerides (Rapid Test, Roche Diagnostica, Switzerland). The variations within and between assays were: cholesterol, 2.8% and 3.2%; phospholipids, 2.4% and 3.6%; free fatty acids, 2.1% and 4.2%; and triglycerides, 2.1% and 2.8%, respectively.

The high density lipoprotein (HDL) fraction was prepared by NaPT-MgCl₂ precipitation of plasma at room temperature.¹⁸ The supernatant containing HDL was used for lipid analysis.

Plasma concentrations of apolipoproteins were determined by an electroimmunoassay (unpublished observations), using purified apolipoproteins as standards for the apolipoprotein A-I (apo A-I) and apolipoprotein C-III (apo C-III) assays. The apolipoprotein B (apo B) assay was standardized with a narrow density cut of LDL (d = 1.030 to 1.055), containing only apo B as protein. The apolipoprotein E (apo E) assay was standardized by a reference serum. The variations within and between assays were: apo A-I, 3.5% and 4%; apo B, 3.5% and 6.9%; apo C-III, 2.3% and 5.8%; and apo E, 2.1% and 4.6%, respectively.

**Statistical Methods**

In the calculation of differences in atherosclerosis between the treated group and the control group, contrasts in a fixed two-way crossed analysis of variance model with factors for group and part of aorta was used. For other statistical evaluation Student's t test was used. All results are expressed as means ± SEM.

**Results**

**Body Weights**

The food consumption of all animals was 80 g/day/rabbit. There was no significant difference in the food intake of the two groups at any time during the investigation. A slight increase in body weight of the control rabbits was noticed during the 21 weeks of the experiment. At the end of the study there was a difference between the body weights of the two groups, p < 0.05 (Table 1).

**Plasma Analyses**

Administration of metoprolol subcutaneously by osmotic pumps resulted in a plasma level of 774 ± 69 nM (mean ± SEM) during the investigation.

**Table 1. Body Weights of Two Experimental Groups**

<table>
<thead>
<tr>
<th>Weeks</th>
<th>0</th>
<th>7</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control animals (n = 22)</td>
<td>3.2 ± 0.06</td>
<td>3.4 ± 0.08</td>
<td>3.5 ± 0.10</td>
</tr>
<tr>
<td>Metoprolol-treated animals (n = 22)</td>
<td>3.1 ± 0.06</td>
<td>3.3 ± 0.07</td>
<td>3.2 ± 0.09</td>
</tr>
</tbody>
</table>

The results represent means ± SEM and are given in kilograms. n.s. = not significant.

Feeding an atherogenic diet containing 0.25% cholesterol and 3% coconut oil to normolipidemic rabbits for 21 weeks resulted in a noticeable increase in the plasma concentration of cholesterol and phospholipids and also an increase in the concentration of triglycerides and free fatty acids in the plasma (Figure 2). Comparing the control group with the metoprolol-treated group, there were no significant differences in the concentrations of plasma cholesterol, phospholipids, or triglycerides, but the plasma level of free fatty acids showed a tendency to increase in the metoprolol-treated group. This increase was, however, not statistically significant.

Further, there were no differences between the treated and untreated group regarding the lipid constituents of the HDL fraction after precipitation of very low density (VLDL) and low density lipoproteins (LDL) in the plasma at the end of the investigation (Table 2).

The plasma concentration of both apo B and apo E was increased several times by the atherogenic diet. No differences could be shown between the metoprolol-treated and the untreated group (Table 3).

The plasma concentration of apo A-I did not change during the study, but a slight increase in plasma concentration of apo C-III was found. There were, however, no differences in the plasma concentrations of apo A-I and apo C-III between the metoprolol-treated group and the control group.

**Atherosclerosis**

Atherosclerosis was most extensive in the ascending aorta plus the arch of aorta. In the thoracic aorta, the atherosclerosis was less extensive, while it was least developed in the abdominal aorta. One animal in the control group did not respond to the diet with increased levels of lipids, nor did it develop any atherosclerosis. This nonresponding animal had a plasma cholesterol concentration of 0.71 mM after 21 weeks, but was still included in the data analyses of the investigation.

Sections of aortic lesions were examined by light microscopy. Both extracellular and intracellular lipids were seen in the lesions. Intercellular connective tissue was found especially in the subendothelial area. Round cells adhering to the surface of the plaques were seen, especially in the periphery of the lesions. Judging by nuclear morphology, these cells were primarily monocytes and lymphocytes. There were no differences between the examined sections with regard to the structure of the lesions. However, as only a small number of sections were examined, the observations do not permit us to extrapolate to groups.
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Figure 2. Plasma lipid concentrations during the investigation. Metoprolol-treated group (○—○), untreated group (●—●). The arrow at 7 weeks indicates the start of metoprolol treatment. Values are means ± SEM.

Table 2. Lipid Constituents of High Density Lipoprotein Fraction at End of Investigation (Week 21)

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 22)</th>
<th>Metoprolol (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>0.74 ± 0.07</td>
<td>0.71 ± 0.06</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>0.32 ± 0.04</td>
<td>0.33 ± 0.04</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.71 ± 0.04</td>
<td>0.69 ± 0.05</td>
</tr>
</tbody>
</table>

The results represent means ± SEM and are given in millimolar concentration.

Table 3. Apolipoprotein Concentration in Plasma

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th>Week 7</th>
<th>Week 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo A-1 (mg/ml) C</td>
<td>1.24 ± 0.08</td>
<td>1.43 ± 0.09</td>
<td>1.39 ± 0.07</td>
</tr>
<tr>
<td>Me</td>
<td>1.33 ± 0.06</td>
<td>1.46 ± 0.09</td>
<td>1.24 ± 0.08</td>
</tr>
<tr>
<td>Apo B (mg/ml)    C</td>
<td>0.18 ± 0.02</td>
<td>1.29 ± 0.12</td>
<td>1.67 ± 0.16</td>
</tr>
<tr>
<td>Me</td>
<td>0.15 ± 0.02</td>
<td>1.20 ± 0.14</td>
<td>1.68 ± 0.14</td>
</tr>
<tr>
<td>Apo C-III (mg/ml) C</td>
<td>0.17 ± 0.07</td>
<td>0.25 ± 0.08</td>
<td>0.25 ± 0.07</td>
</tr>
<tr>
<td>Me</td>
<td>0.17 ± 0.01</td>
<td>0.25 ± 0.03</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>Apo E (% of ref. serum)</td>
<td>87 ± 6</td>
<td>612 ± 68</td>
<td>1033 ± 131</td>
</tr>
<tr>
<td>Me</td>
<td>81 ± 4</td>
<td>515 ± 63</td>
<td>944 ± 102</td>
</tr>
</tbody>
</table>

Results represent means ± SEM of 22 animals.

C = control animals; Me = metoprolol-treated animals.

A considerable variation in the percentage of aortic atherosclerosis was noted in the rabbits of both the metoprolol-treated and the untreated group. As seen in Figure 3, there was a marked shift toward less atherosclerosis in the metoprolol-treated group (Panel B) compared with the control group (Panel A). The aortas of the metoprolol-treated group had significantly less surface affected by atheromatous lesions (ascending aorta plus arch of aorta 37.8 ± 6.8%, thoracic aorta 32.9 ± 6.1%, abdominal aorta 19.8 ± 6.1%) than the control group (ascending aorta plus arch of aorta 54.9 ± 7.1%, thoracic aorta 48.0 ± 6.2%, and abdominal aorta 25.9 ± 5.5%). For the total aorta, this was significant at p < 0.015.

Diet-induced atherosclerosis of the coronary arteries was most pronounced in the proximal part of the coronary arteries. A correlation between the atherosclerosis of the coronary arteries and the aortas was found (r = 0.67, 95% confidence interval, 0.46 to 0.81). In the 8 mm segment of the right coronary artery, the degree of atherosclerosis in the control group was 41.1 ± 5.9% (n = 20), and in the metoprolol-treated group, 32.6 ± 5.0% (n = 21). In the 20 mm segment of the left coronary artery, the degree of atherosclerosis in the control group was 22.1 ± 4.3% (n = 22), and in the metoprolol-treated group, 18.3 ± 3.0%
Figure 3. Distribution of animals according to degree of aortic area afflicted with atherosclerosis. A. Control group (n = 22). B. Metoprolol-treated group (n = 22).

Discussion

Response to Diet

In animal models of diet-induced atherosclerosis, a 1% to 2% cholesterol-supplemented diet is usually given for 8 to 12 weeks of investigation, which leads to very high (50 to 75 mM) plasma levels of cholesterol as well as to macroscopic liver damage at the end of the study. In the present investigation, we intended to avoid these effects and to overload the experimental animals with exogenous cholesterol. Hence, a considerably lower degree of cholesterol supplement (0.25%) was used. This was combined with a supplement of saturated fat in the form of 3% coconut oil to contribute to an increase of endogenous cholesterol. Nevertheless, in this investigation the livers of the animals also showed macroscopic steatosis, although the mean value of plasma cholesterol was only 32 mM at the end of the investigation.

As found in other studies, the lesions of the aorta and of the coronary arteries were more extensive proximally than distally. Furthermore, the lesions showed extra- as well as intracellular lipid accumulation. Compared with lesions induced by cholesterol feeding only, those seen in the present study were more fibrous. It has been pointed out that cholesterol-induced lesions in rabbits differ from spontaneous lesions in humans by being primarily foam cell lesions. However, if a regimen of intermittent or protracted cholesterol feeding is used, a more fibrous type of lesion is found. Variation in the type of dietary fat can induce variations in the fibrotic content of the lesion. In the present study, the fibrotic nature of the intimal lesions could have been induced by the protracted feeding of cholesterol (21 weeks), as well as by the supplement of saturated fat in the diet.

Effects of $\beta_1$-Adrenoceptor-Selective Antagonists

Nonselective, as well as $\beta_1$-adrenoceptor-selective, antagonists have been associated with effects on fasting serum lipids in both human and animal studies. The main findings are a moderate increase in total triglyceride levels and a slight reduction of HDL cholesterol levels. In studies in humans, metoprolol has decreased the HDL cholesterol level, but there are also reports where no effect on the HDL cholesterol was observed. In our animal study using a fat-rich diet no effect of metoprolol could be shown on HDL cholesterol levels or plasma apo A-I concentrations. Nor was there any effect of metoprolol on the levels of total cholesterol and triglycerides. The slight increase in plasma levels of free fatty acids seen in the metoprolol-treated group is consistent with the findings of other investigators. The mechanism behind this is unknown.

The development of experimental atherosclerosis was significantly reduced by metoprolol. The cause behind this effect was not investigated in this study, but there are several theories about the mechanisms behind $\beta$-blockers in this respect, including actions other than the $\beta$-receptor inhibitory action. For example, $\beta$-adrenoceptor antagonists have been shown to influence cellular metabolism by inhibiting ACAT activity in aorta, but that was at very high concentrations of the drugs. The plasma level of metoprolol in this study was within the high range of that found during antihypertensive therapy in humans.

We conclude from this investigation that the $\beta_1$-adrenoceptor antagonist metoprolol significantly reduced the development of atherosclerosis in aortas of rabbits fed an atherogenic diet, whereas no influence on lipid or apolipoprotein levels was shown.

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