Prostacyclin Agonists Reduce Early Atherosclerosis in Hyperlipidemic Hamsters

Octimibate and BMY 42393 Suppress Monocyte Chemotaxis, Macrophage Cholesterol Ester Accumulation, Scavenger Receptor Activity, and Tumor Necrosis Factor Production


We determined the effects of two prostacyclin agonists (octimibate and BMY 42393) on the progression of the fatty streak in vivo and on macrophage function in vitro. Hamsters were fed chow plus 0.05% cholesterol and 10% coconut oil. Control hamsters were compared with animals receiving either octimibate (10 or 30 mg/kg per day) or BMY 42393 (30 mg/kg per day). After 10 weeks of treatment, octimibate decreased plasma total cholesterol and triglycerides by 43% and 32%, respectively. Neither agonist affected blood pressure or heart rate. Lesion-prone aortic arches were stained with hematoxylin and oil red O and examined en face. Compared with controls, octimibate and BMY 42393 on average decreased mononuclear cells attached to the luminal surface by 44% and reduced subendothelial macrophage–foam cell number by 56%, foam cell size by 38%, and fatty streak area by 63%. Since octimibate is a putative inhibitor of acyl coenzyme A cholesterol acyltransferase, we studied the effect of both agents on cholesteryl ester metabolism in murine macrophages. At 10 μM, octimibate and BMY 42393 decreased cholesteryl ester accumulation in macrophages by 90% and 41%, respectively. Octimibate inhibited cholesteryl ester synthesis by 96% and increased the rate of cholesteryl ester degradation by 52%. Both prostacyclin agonists reduced macrophage scavenger receptor–mediated uptake of acetylated low density lipoprotein by 24–66% and increased cyclic adenosine monophosphate levels. Octimibate and BMY 42393 inhibited the secretion of tumor necrosis factor by 80–88% when macrophages were activated with lipopolysaccharide. At 10 μM, both agents decreased human monocyte chemotaxis to N-formyl-methionyl-leucyl-phenylalanine by 64–79%. The in vitro results with octimibate and BMY 42393 are consistent with the low number of small foam cells quantified in vivo. We suggest that octimibate and BMY 42393 suppress monocyte–macrophage atherogenic activity and cytokine production and thus inhibit the development of early atherosclerosis.

Key Words • prostacyclin • atherosclerosis • macrophages • cholesterol • blood pressure • chemotaxis • scavenger receptor • tumor necrosis factor • acyl coenzyme A cholesterol acyltransferase • hamsters

Early atherosclerosis is characterized by the accumulation of low density lipoproteins (LDLs) and liposomes in arteries, followed by monocyte adhesion to the endothelium and migration into the subendothelial space. The monocyte-derived macrophages collect modified arterial LDL via the scavenger receptor and are transformed into foam cells. Large numbers of arterial foam cells lead to the formation of the fatty streak, which is the first recognizable lesion of atherosclerosis. The link between the cellular events of atherosclerosis and inflammation was recently reemphasized and reviewed. Inflammation involves a myriad of factors that orchestrate the tissue response to injury; one class of mediators, the prostaglandins, appears to be intimately involved in the inflammatory process. Several investigators demonstrated that patients with ischemic heart disease have an imbalance of two functionally opposing prostaglandins in which plasma prostacyclin levels decrease and thromboxane A₂ (TXA₂) concentrations increase. However, there is little consensus regarding the production of prostacyclin by atherosclerotic arteries. Several investigators suggest prostacyclin production decreases, remains unchanged, or increases. On the other hand, TXA₂ biosynthesis appears to increase. Anti-inflammatory agents such as cortisone blocked prostaglandin and leukotriene synthesis and reduced atherosclerosis in monkeys, whereas cyclooxygenase inhibitors such as aspirin had an inconclusive effect.
Treating hypercholesterolemic rabbits with a selective TxA2 synthetase inhibitor decreased the extent of macrophage–foam cell lesions in the aorta.22 In vitro, the same agent promoted metabolism of arachidonic acid from TxA2 to prostaglandin E2 (PGE2) in monocytes.22 Therefore, switching monocyte–macrophage prostaglandin metabolism from TxA2 to PGE2 was associated with the reduction of the fatty streak, and the study highlighted the important role that prostaglandins play in atherogenesis.

Octimibate was originally identified as an inhibitor of acyl coenzyme A cholesterol acyltransferase (ACAT), but recently it was found to act as a prostacyclin partial agonist.23,24 BMY 42393 was developed as a partial prostacyclin receptor agonist that inhibited platelet aggregation25 and increased platelet cyclic adenosine monophosphate (cAMP) levels (S.M. Seiler, personal communication). Macrophages respond to prostacyclin receptor agonists or PGE2 by increasing their cAMP levels.26,27 Based on this information, it was feasible that the partial prostacyclin agonists octimibate and BMY 42393 could modulate macrophage activity. Therefore, we first determined whether these agents altered the progression of the fatty streak in hyperlipidemic hamsters, a lesion in which macrophages predominate.28 Second, the effect of the same agonists on several macrophage functions related to atherosclerosis was examined in vitro.

**Methods**

Blood Pressure and Atherosclerosis Studies

Fifty-six 12-week-old male F3B hamsters were used in this study (Bio-Breeder Inc., Fitchburg, Mass.). The animals were maintained according to the recommendations in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council (Department of Health, Education, and Welfare publication No. NIH 85-23, 1985). Two separate in vivo experiments were undertaken. The first study assessed the effects of octimibate and BMY 42393 on blood pressure and heart rate. The second experiment determined the effects of the same agents on plasma lipids and the development of early atherosclerosis. Octimibate is known as NAT 04-152 or as 8-[1,4,5-triphenyl-1H-imidazol-2-yl]oxy] octanoic acid, while the chemical name for BMY 42393 (which is an analogue of octimibate) is 2-[3-[2-(4,5-diphenyl-2-oxazoly)ethyl]phenoxy]acetic acid.

In the blood pressure study, 19 hamsters received a mild atherogenic diet of chow (No. 5001, Purina, St. Louis, Mo.) containing 0.05% cholesterol and 10% coconut oil for 3 weeks. The animals were divided into three sets: controls, octimibate (30 mg/kg per day), and BMY 42393 (30 mg/kg per day). The drugs were delivered in the same atherogenic diet. After 10 weeks of treatment, the hamsters had their mean arterial pressure and heart rate measured as described previously.28

In the atherosclerosis study, 37 hamsters received the mild atherogenic diet for 3 weeks. Their plasma lipids were measured, and the animals were placed into four groups so that the average plasma total cholesterol for each group was similar. The first set of hamsters were controls, the second and third groups received 10 and 30 mg/kg body wt per day octimibate, respectively, and the fourth set was treated with BMY 42393 30 mg/kg per day. Drugs were mixed in the same atherogenic diet, and all groups were run for another 10 weeks. During the 10-week period, hamsters were fasted overnight and bled for plasma lipid analysis at weeks 3, 4, and 8. The three measurements of total, LDL plus very low density lipoprotein (VLDL), and high density lipoprotein (HDL) cholesterols and total triglycerides for each animal were averaged. At 10 weeks the aortic arches were fixed by perfusing formalin, and the extent of early atherosclerosis was determined as described previously.28

Measurement of Blood Pressure, Plasma Lipids, and Atherosclerosis

In the blood pressure experiment, nonfasted hamsters were anesthetized with methoxyflurane (Pittman-Moore Inc., Washington Crossing, N.J.), and a catheter (PE-10, Clay Adams, Parsippany, N.J.) was inserted into the right carotid artery. Mean arterial pressure and heart rate were recorded on a polygraph (Grass Instruments, Quincy, Mass.) when the animal was in a semi-conscious state. These measurements correspond to values obtained from conscious unrestrained hamsters.28 The hamsters were then perfused with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The aortic arches were stained with hematoxylin and oil red O and then photographed.

In the atherosclerosis study, plasma total cholesterol and triglycerides were measured with commercial enzyme kits on a Beckman System 700 automated analyzer. HDL cholesterol was determined after lipoproteins containing apolipoprotein B were precipitated from the plasma with phosphotungstate.29

After perfusing the hamsters with formalin, the aortic arch was removed, cleaned, and stained with hematoxylin and oil red O (0-0625, Sigma Chemical Co., St. Louis, Mo.) as outlined previously.6 The arch was cut open with the luminal surface facing up and examined en face by light microscopy. Mononuclear cells on the endothelial surface (adherent cells) and subendothelial macrophage–foam cells (intimal cells) were counted and divided by the area of the specimen to give cells per square millimeter. The average size of foam cells in square micrometers was determined by image analysis (Sigma Scan, Corte Madera, Calif.). The area of the fatty streak in square micrometers (i.e., the total area of foam cells) was determined by multiplying the number of foam cells by the average foam cell size in each specimen. The amount of neutral lipid in the aortic arch was determined indirectly by measuring intimal oil red O staining (micrograms oil red O per square millimeter). Using the method of Nunnari et al.30

In Vitro Macrophage Studies

The culture media and certified fetal calf serum (FCS) were purchased from Gibco Laboratories (Grand Island, N.Y.). Radioisotopes were from New England Nuclear (Boston). Brewer's thiglycollate medium came from BBL Microbiology System (Cockeysville, Md.). All other reagents were from Sigma.

Outbred female Swiss CD-1 mice (20–25 g) from Charles River Breeding Laboratories (Rockville, Md.) provided the source of the peritoneal macrophages. Sterile 4% Brewer's modified thiglycollate medium (2 mL) was injected into the peritoneal cavity, and 5 days
later macrophages were collected by peritoneal lavage with 8 mL phosphate-buffered saline (PBS) containing 3 mM EDTA (pH 7.4).

The macrophages were pelleted by centrifugation (400g for 5 minutes). Red blood cells were removed by hypotonic lysis (water for 30 seconds followed by 10-fold excess PBS). The cells were collected by centrifugation and resuspended in Dulbecco's modified Eagle's medium (high glucose; DMEM) with 10% FCS. Purity was 95% as determined by differential staining. The macrophages were plated into 12-well dishes (Costar, Cambridge, Mass.) at 1 x 10^6/mL per well; they were allowed to adhere for 2 hours in a 37°C, humidified, 5% CO_2 incubator. Nonadherent cells were removed with two washes of DMEM.

Scavenger receptor activity was determined by measuring the uptake of fluorescent acetylated LDL (Dil ac-LDL; Biotechnology Inc., Boston, Mass.) by macrophages. Briefly, macrophage monolayers were incubated for 2 hours with either octimibate, BMY 42393, or CL 277082 (ACAT inhibitor, Lederle) in the presence of 2 µg/mL Dil ac-LDL and DMEM. The extracellular Dil ac-LDL was removed by three washes of cold medium containing 10% fetal bovine serum (FBS), and the macrophages were fixed with 4% formalin for 15 minutes. The amount of intracellular fluorescent material was determined by quantifying the average fluorescence intensity in 25–50 cells. Six separate fields from duplicate and triplicate monolayers were measured and averaged. The fluorescence signal was detected by a video camera on a microscope, and the image was transferred to a computer. Fluorescence intensity was measured by image analysis software (JAVA, Jandel Scientific, Corte Madera, Calif.).

The effect of octimibate, BMY 42393, and the ACAT inhibitor CL 277082 on the accumulation of cholesteryl esters in macrophages was determined by using the method of Schmitz et al. Briefly, macrophages were incubated with acetylated LDL and drug for 20 hours, then [3H]oleate (1 µCi/mL; New England Nuclear) was added for the last 2 hours. After three washes with a solution of DMEM and 0.2% bovine serum albumin, the macrophage lipids were solubilized using the Bligh-Dyer extraction procedure. The lipids were separated on thin-layer chromatography plates (G-60, Merck). Thin-layer plates were measured with radiochromatography scanners (Vanguard Auto Scanner or Ambis Imager), and these lipids were compared with the migration of radioactive lipid standards (New England Nuclear). To quantify the effect of octimibate on cholesteryl ester synthesis, macrophages were incubated with octimibate, acetylated LDL, and [3H]oleate for 2–4 hours and then washed; the amount of radiolabel incorporated into cholesteryl esters was measured as outlined previously. The effect of octimibate on the removal (or degradation) of cholesteryl esters was determined by incubating macrophages with acetylated LDL and [3H]oleate, after which the cells were washed and incubated with octimibate for 2–4 hours. The macrophages were washed again, and the amount of radiolabeled cholesteryl ester remaining in the cells was measured as described previously.

Intracellular cAMP levels in cultured macrophages were measured with radioimmunoassay kits from Amersham (Arlington Heights, Ill.).

Monocyte Chemotaxis

Fresh human blood was collected in 3.8% sodium citrate and centrifuged at room temperature, and the platelet-rich plasma was removed. The remaining blood was centrifuged at 650g for 10 minutes, and the buffy coat was mixed in Hank's balanced salt solution containing 5% FBS and layered over 4.5 mL Lymphoprep (Accurate Chemical Co., Westbury, N.Y.). The solution was centrifuged for 20 minutes at 830g, and the interface layer containing a mixture of lymphocytes, monocytes, and platelets was removed and pooled. The cells were centrifuged again for 10 minutes at 470g, and the cell pellets were resuspended and pooled in a PBS/citrate/albumin buffer. The cells were then washed twice, resuspended in RPMI 1640–tris(hydroxymethyl) aminomethane (Tris) buffer (GIBCO) containing 10% FBS, and incubated at 37°C for 30 minutes. The cells were centrifuged at 470g for 5 minutes, then resuspended in RPMI 1640 (Tris-citrate, 10% FCS), and layered over Nycodenz 1.068 (Accurate). After centrifugation, the monocytes were collected and washed twice in RPMI 1640 (Tris-citrate, 10% FCS). Trypan blue and Turk's stain (nuclear stain) were used to determine the viability and purity of the cell preparation. The viability of all preparations used was >80%.

Boyden chambers were used to measure monocyte chemotaxis. A 50-µL aliquot of monocytes (5 x 10^4 cells) was added to one side of a chemotactic chamber, and 25 µL of N-formyl-methionyl-leucyl-phenylalanine (FMLP) (10^-7 M) was added to the other side (Neuroprobe Inc., Cabin John, Md.). The chambers were separated by a Nucleopore filter with pore diameters of 5 µm. Equal concentrations of octimibate or BMY 42393 were applied to both chambers, and the cells were allowed to migrate for 30–90 minutes at 37°C with 5% CO_2. The filter was then gently removed and washed three times in PBS. The cells that passed through the pores and attached to the filter were fixed in methanol, air dried, and stained with Diff-Quik rapid blood stain (Baxter Health Care Corp., McGraw Park, Ill.). In each experiment there were four replicates (or wells) per group, and the total number of monocytes in five oil-immersion fields per well was counted.

Tumor Necrosis Factor Assay

Biologically active tumor necrosis factor (TNF) secreted by macrophages into the medium was measured with the L929 fibroblast cytotoxicity assay as previously outlined. Murine peritoneal macrophages were obtained from outbred female Swiss CD-1 mice as described previously. L929 cells were obtained from American Type Culture Collection (Bethesda, Md.). Macrophages were incubated for 20 hours at 37°C with either octimibate or BMY 42393 and 200 ng/mL lipopolysaccharide. L929 fibroblasts, 2 x 10^3 /100 µL, were seeded in Costar plates for 24 hours at 37°C with medium containing 5 µg/mL actinomycin D. Samples of macrophage culture medium were added to the fibroblasts for 18 hours at 37°C. The percentage of surviving cells in each well was determined by washing the cells with PBS, fixing them with 3.7% formaldehyde, staining
them with 0.0075% crystal violet, and then quantifying the optical density of the residue dissolved in 33% acetic acid at 590 nm on a Dynatech M600 plate reader.

Statistics

For the in vivo studies, a one-way analysis of variance (ANOVA) followed by a Tukey's test was used to compare blood pressure, heart rate, plasma lipid, and atherosclerosis parameters. Data were transformed where necessary to pass Bartlett's test for homogeneity of group variances. The BMY 42393 group had one hyperresponder, and since the data set failed the Bartlett's test, some of the atherosclerosis variables were analyzed by a nonparametric Mann-Whitney U test. With the in vitro experiments, each data point (i.e., the mean of at least four experiments) was divided by its standard error to calculate a t statistic, which determines whether the data point was significantly greater than zero.

Results

In Vivo Studies

After 10 weeks of treatment neither octimibate nor BMY 42393 affected arterial blood pressure or heart rate (Table 1). In the atherosclerosis study, octimibate at 10 mg/kg per day decreased total cholesterol and LDL plus VLDL cholesterol by 25% and 41%, respectively, compared with controls (Table 2). Octimibate 30 mg/kg per day reduced total cholesterol, LDL plus VLDL cholesterol, and total triglycerides by 43%, 65%, and 32%, respectively (Table 2). Octimibate failed to alter HDL cholesterol levels. The partial prostacyclin agonist BMY 42393 had no significant effect on plasma lipids (Table 2).

In the aortic arch of control hamsters, mononuclear cells were randomly attached to the intact endothelial surface and adherent platelets were rare. On the other hand, many subendothelial macrophage–foam cells accumulated along the inner curvature of the aortic arch (Figure 1A), a region previously shown to be susceptible to atherosclerosis. The foam cells were engorged with neutral lipid stained with oil red O (Figure 1B) and were often located near clusters of small extracellular lipid droplets.

Hamsters treated with octimibate and BMY 42393 had fewer and smaller macrophage–foam cells, whereas the extent of the oil red O–stained lipid droplets in the subendothelial space was variable. Quantitative analyses indicated that compared with controls, 10 mg/kg per day octimibate decreased the number of adherent mononuclear cells per square millimeter, the number of intimal macrophage–foam cells per square millimeter, foam cell size, fatty streak area, and intimal oil red O staining by 48%, 46%, 34%, 59%, and 53%, respectively. The 30 mg/kg per day octimibate dose reduced these parameters by 50%, 77%, 54%, 89%, and 71% (Figures 2A, 2B, and 4; Table 3). BMY 42393 inhibited mononuclear cell adhesion by 35%, decreased foam cells per square millimeter by 44%, reduced foam cell size by 26%, reduced fatty streak area by 40%, and decreased intimal oil red O staining by 39% compared with the control group (Figures 3A, 3B, and 4; Table 3). Included in the BMY 42393 group was one hyperresponder that had far more foam cells and a larger fatty streak than any of the control animals (Figure 4).

In Vitro Studies

Since octimibate and BMY 42393 decreased mononuclear cells attached to the luminal surface of the aortic arch, the effect of these prostacyclin agonists on monocyte chemotaxis to fMLP was determined. Human monocyte chemotaxis was inhibited by 40–64% with octimibate concentrations of 0.01–10 μM, respectively. The same range of BMY 42393 concentrations decreased migration by 40–79% (Figure 5).

Octimibate has been reported to be an inhibitor of ACAT. Therefore, we compared the effects of octimibate, BMY 42393, and the ACAT inhibitor CL 277082 on cholesteryl ester accumulation in murine macrophages. Cholesteryl ester synthesis was stimulated by incubating macrophages with acetylated LDL. After 20 hours of incubation with 10 μM octimibate, BMY 42393, or CL 277082, there was a decrease in the accumulation of cholesteryl esters of 90%, 41%, and 78%, respectively (Figure 6). To determine whether octimibate inhibited cholesteryl ester synthesis, we added octimibate, radiolabeled oleate, and acetylated LDL to the macrophages. After 2 and 4 hours, 10 μM octimibate reduced the amount of radiolabeled cholesteryl esters by 96% and 92%, respectively (Figure 7). We also investigated the effect of octimibate on degrading cholesteryl esters in macrophages. These cells were preloaded by incubation with acetylated LDL and

### Table 1. Mean Arterial Pressure and Heart Rate of Control and Treated Hamsters

<table>
<thead>
<tr>
<th></th>
<th>Mean arterial pressure (mm Hg)</th>
<th>Heart rate (beats/min)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>134±3</td>
<td>386±10</td>
<td>7</td>
</tr>
<tr>
<td>Octimibate 30 mg/kg per day</td>
<td>136±5</td>
<td>378±12</td>
<td>6</td>
</tr>
<tr>
<td>BMY 42393 30 mg/kg per day</td>
<td>136±4</td>
<td>386±12</td>
<td>6</td>
</tr>
</tbody>
</table>

Data are mean±SEM.

### Table 2. Plasma Lipids of Control and Treated Hamsters in the Atherosclerosis Study

<table>
<thead>
<tr>
<th></th>
<th>Total cholesterol (mg/dL)</th>
<th>LDL+VLDL cholesterol (mg/dL)</th>
<th>HDL cholesterol (mg/dL)</th>
<th>Total triglycerides (mg/dL)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>239±8</td>
<td>162±10</td>
<td>77±3</td>
<td>200±18</td>
<td>10</td>
</tr>
<tr>
<td>Octimibate 10 mg/kg per day</td>
<td>179±7*</td>
<td>95±6*</td>
<td>84±1</td>
<td>157±8</td>
<td>8</td>
</tr>
<tr>
<td>Octimibate 30 mg/kg per day</td>
<td>137±3*</td>
<td>57±2*</td>
<td>80±2</td>
<td>137±4*</td>
<td>10</td>
</tr>
<tr>
<td>BMY 42393 30 mg/kg per day</td>
<td>234±26</td>
<td>157±25</td>
<td>77±1</td>
<td>254±42</td>
<td>9</td>
</tr>
</tbody>
</table>

LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein. *p<0.002 compared with control group (Tukey's test). Data are mean±SEM.
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FIGURE 1. Photomicrographs of the aortic arch viewed en face from a hyperlipidemic control hamster. Blood flow is from left to right. Panel A: At low magnification there are many lipid inclusions located along the inner curvature, i.e., the lesion-prone area. Bar=100 μm. The numerous purple dots are endothelial cell nuclei. Panel B: The same area as in panel A but at higher power. Arrowheads indicate individual macrophage-foam cells loaded with lipid. Bar=20 μm; hematoxylin and oil red O stains.

FIGURE 2. Photomicrographs of the aortic arch from a hamster treated with octimibate (30 mg/kg per day) for 10 weeks. Panel A: There is less lipid in the lesion-prone area compared with the control in Figure 1A. Bar=100 μm. Panel B: The macrophage-foam cells (arrowheads) are fewer and smaller than those of controls. Note the high density of endothelial cell nuclei. Bar=20 μm; hematoxylin and oil red O stains.

FIGURE 3. Photomicrographs of the lesion-prone area from a hamster receiving the partial prostacyclin agonist BMY 42393 (30 mg/kg per day) for 10 weeks. Panel A: There is a reduction of intimal lipid compared with Figure 1A. Bar=100 μm. Panel B: The number and size of macrophage-foam cells (arrowheads) has decreased compared with those of controls. Bar=20 μm; hematoxylin and oil red O stains.

[14C]oleate, washed, and treated with octimibate. Cholesteryl ester synthesis ceases when acetylated LDL is washed away, and the intracellular lipid levels gradually decrease, presumably through the action of hydrolytic enzymes. Octimibate (10 μM) increased the removal (or degradation) of cholesteryl esters within 2–4 hours by 33–52%, respectively (Figure 7).

Cholesteryl ester storage could also be modulated by changes in the activity of the macrophage scavenger receptor. In experiments in which scavenger receptor
TABLE 3. Fatty Streak Parameters of Control and Treated Hamsters in the Atherosclerosis Study

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Adherent mononuclear cells (per mm²)</th>
<th>Intimal foam cells (per mm²)</th>
<th>Average foam cell size (µm²)</th>
<th>Fatty streak area (µm² x 1,000)</th>
<th>Intimal oil red O (µg/mm²)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.0±0.7</td>
<td>126±9</td>
<td>141±8</td>
<td>268±27</td>
<td>0.072±0.005</td>
<td>10</td>
</tr>
<tr>
<td>Octimibate 10 mg/kg per day</td>
<td>3.1±0.5*</td>
<td>68±9*</td>
<td>93±7*</td>
<td>110±22*</td>
<td>0.034±0.005*</td>
<td>8</td>
</tr>
<tr>
<td>Octimibate 30 mg/kg per day</td>
<td>3.0±0.3*</td>
<td>29±13*</td>
<td>65±3*</td>
<td>30±4*</td>
<td>0.021±0.003*</td>
<td>10</td>
</tr>
<tr>
<td>BMY 42393 30 mg/kg per day</td>
<td>3.9±0.3*</td>
<td>71±30†</td>
<td>105±11*</td>
<td>160±93†</td>
<td>0.044±0.021†</td>
<td>9</td>
</tr>
</tbody>
</table>

*p<0.001 compared with control group (Tukey's test).
fp<0.008 compared with control group (Mann-Whitney U test).

Data are mean±SEM.

activity was determined, 10 µM of either octimibate or BMY 42393 inhibited the uptake of Dil ac-LDL by 66% and 24%, respectively, whereas CL 277082 (10 µM) had no effect (Figure 8). Octimibate required at least 5 µM concentration to depress scavenger receptor activity and decrease cholesteryl ester accumulation (Figure 9).

Measurement of intracellular cAMP in macrophages indicated that 10 µM of either octimibate or BMY 42393 produced increases of 80% and 140%, respectively, compared with untreated cells (Table 4). Since elevation of macrophage cAMP has been reported to affect macrophage activation, we investigated the effect of the prostacyclin agonist on macrophage production of TNF in response to a challenge with endotoxin. A minimum concentration of 0.5 µM of either octimibate or BMY 42393 inhibited TNF production by 25%, whereas 10 µM of either agent reduced the production of this cytokine by 80–88% (Figure 10).

Discussion

The Hamster Model

Male F,B hamsters fed moderate amounts of cholesterol and saturated fat become hyperlipidemic, and most of the cholesterol and triglycerides are carried in the LDL and the VLDL fractions, respectively. With hyperlipidemia, macrophage–foam cells and extracellular lipid droplets consistently accumulate in a well-defined region along the inner curvature of the aortic arch; hence the term "lesion-prone" area. Severe hypercholesterolemia ultimately leads to the development of fibrofatty plaques in the same region. Oil red O stains neutral lipids such as cholesteryl ester and triglycerides; since cholesteryl ester is the predominant lipid in LDL, it suggests that the extracellular lipid particles were aggregates of LDL and VLDL, and that the macrophages contained droplets of cholesteryl ester and triglyceride. Thus, the hamster model has a similar lipoprotein profile to humans, and with mild hyperlipidemia, atherosclerosis rapidly develops in the aortic arch where the lesions are reproducible and easily quantified by morphometry. This model allows for meaningful studies on the progression or regression of early atherosclerosis.

The decrease in arterial macrophage–foam cell number, size, and fatty streak area in hamsters receiving octimibate and BMY 42393 confirms the reduction of atherosclerosis in cholesterol-fed rabbits that were treated with either octimibate or the prostacyclin agonist cicaprost. Our in vivo and in vitro data suggest...
several possible mechanisms through which prostacyclin agonists affect the development of the fatty streak.

Plasma LDL Cholesterol

Chow-fed hamsters with low levels of plasma cholesterol and triglyceride had few arterial lipid droplets and low numbers of small macrophage-foam cells. Hypocholesterolemic hamsters treated with cholestyramine had a fall in plasma LDL cholesterol and VLDL triglycerides and a subsequent reduction in foam cell number and size. These findings suggest that low concentrations of circulating lipoproteins reduce the number of lipid particles accumulating in the subendothelial space and decrease the source of cholesterol and triglycerides for macrophage-foam cells to scavenge. Octimibate appeared to produce the same effect as cholestyramine, i.e., a reduction in plasma cholesterol and the fatty streak. The mechanism behind the hypolipidemic action of octimibate could be due to its putative inhibition of ACAT, an enzyme implicated in the absorption of cholesterol. Surprisingly, foam cell number and size

\[ 	ext{Cholesteryl Ester Accumulation} \]

\[ \text{Ac-LDL Uptake} \]

\[ 	ext{Octimibate} \]

\[ 	ext{BMY 42393} \]

\[ \text{CL 277082} \]
and the area of the fatty streak also decreased in the BMY 42393 group, despite persistent elevation of plasma lipids and normal blood pressure, suggesting that the prostacyclin agonists may modulate atherosclerosis through additional mechanisms.

**Mononuclear Cell Adhesion to Endothelium and Monocyte Chemotaxis**

Cell counts of mononuclear cells on the luminal surface of the aortic arch indicated that octimibate and BMY 42393 reduced the number of leukocytes attached to the endothelium in vivo. Both prostacyclin agonists inhibited the chemotactic response of human monocytes to FMLP in vitro. Prostacyclin had a similar effect on monocyte chemotaxis, although it had no effect on monocyte adhesion to endothelial monolayers in vitro.

Taken together these findings suggest that octimibate and BMY 42393 inhibited mononuclear cell adhesion and/or chemotaxis to the artery wall and, in turn, diminished monocyte diapedesis into the aorta. This notion partially explains the reduced number of intimal macrophage–foam cells accumulated in vivo.

**Cholesteryl Ester Accumulation in Macrophages**

Modification of LDL in the artery wall and subsequent uptake by the macrophage scavenger receptor lead to the formation of foam cells and, ultimately, fatty streaks. In murine peritoneal macrophages, octimibate and BMY 42393 decreased the uptake of acetylated LDL by the scavenger receptor. Since the macrophage scavenger receptor recognizes acetylated and oxidized LDL, our results suggest that in vivo the prostacyclin agonists may have reduced the size of foam cells by inhibiting the uptake of oxidized LDL by macrophages.

In macrophages challenged with acetylated LDL, octimibate and BMY 42393 decreased the accumulation of cholesteryl esters, and this was associated with an elevation of cAMP. Other prostacyclin analogues such as RS 93427 decreased the cholesteryl ester content of human macrophages. Carbacyclin and 6β-prostaglandin I₂ reduced cholesteryl ester and increased cAMP levels in cells derived from human atherosclerotic plaques.

Thus, octimibate and BMY 42393 decreased cholesteryl ester accumulation in macrophages by reducing scavenger receptor activity. In addition, octimibate inhibited cholesteryl ester synthesis and accelerated cholesteryl ester hydrolysis. It is possible that BMY 42393 also modulated cholesteryl ester synthesis and degradation, since the agent mimics prostacyclin and elevates cAMP. The reduced amount of cholesteryl ester stored in macrophages treated with octimibate or BMY 42393 is consistent with the presence of small macrophage–foam cells in hamsters receiving the same agents.

**Macrophage Production of Tumor Necrosis Factor**

The cytokine TNF has been localized in human atherosclerotic lesions and is released by activated macrophages. TNF is chemotactic for monocytes and induces the expression of vascular cell adhesion molecules and monocyte chemotactic protein by the endothelium. These actions indicate that TNF is probably involved in the recruitment of monocytes during atherogenesis. When macrophages were treated with either octimibate or BMY 42393 and activated with lipopolysaccharide to stimulate TNF production, the amount of TNF released was reduced as determined by the L929 fibroblast cytotoxicity assay. It is conceivable that in vivo the prostacyclin agonists decreased the production of TNF by macrophages in the aorta and, in turn, reduced the TNF chemotactic gradient, the expression of adhesion molecules, and the synthesis of monocyte chemotactic protein by the endothelium. This concept could in part explain the fall in the number of monocyte–macrophages being recruited into the aorta.

Therefore, octimibate and BMY 42393 directly impeded the functions of activated monocyte–macrophages, such as adhesion, chemotaxis, scavenger receptor activity, and cholesteryl ester accumulation. In addition, prostacyclin agonists suppressed the production of mediators by macrophages that may promote the atherogenic process, i.e., the cytokine TNF and macrophage–derived growth factor (Table 5). Prostacyclin
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and PGE₂ also depressed the activation of macrophages, and increased monocyte production of PGE₂ was associated with a reduction of the fatty streak in rabbits treated with a TXA₂ synthetase inhibitor.

Octimibate and BMY 42393 inhibited human monocyte chemotaxis at 0.01 μM concentrations, whereas in murine macrophages, up to 1,000 times higher concentrations of these agents were required to decrease cholesterol ester accumulation, scavenger receptor activity, and TNF production as well as increase cAMP levels. The only possible explanations are that human cells are more responsive to these agonists than murine cells or that monocytes are more easily stimulated by prostacyclin agonists than are macrophages.

To summarize, the partial prostacyclin agonists octimibate and BMY 42393 decreased macrophage-foam cell number, foam cell size, and fatty streak area in the aortic arch of hamsters. Octimibate reduced plasma LDL cholesterol and VLDL triglyceride, which partially accounted for this effect. Both prostacyclin agonists diminished mononuclear cell adhesion to endothelium and TNF production as well as increase cAMP levels. The only possible explanations are that human cells are more responsive to these agonists than murine cells or that monocytes are more easily stimulated by prostacyclin agonists than are macrophages.

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