Effect of Dietary Omega-3 Fatty Acid on Serum Lipids, Platelet Function, and Atherosclerosis in Watanabe Heritable Hyperlipidemic Rabbits

Fred J. Clubb, Jr., James M. Schmitz, Michelle M. Butler, L. Maximilian Buja, James T. Willerson, and William B. Campbell

Omega-3 fatty acids have been shown to lower plasma cholesterol and triglyceride concentrations in humans. However, the effects of these fatty acids on the interactions among lipid concentrations, platelet activity, and atherogenesis have not been characterized in humans or in animals with low density lipoprotein (LDL) receptor deficiencies. To test the hypothesis that omega-3 fatty acids exert a protective effect in LDL receptor-deficient animals by lowering hyperlipidemia, reducing platelet aggregation, and reducing the severity of atherosclerosis, we evaluated young homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits that were fed omega-3 fatty acids. One-month-old male and female WHHL rabbits were placed on either a control diet (standard laboratory rabbit chow) or a diet supplemented with Menhaden fish oil (MFO), which contained eicosapentaenoic acid (EPA). Consumption measurements during the 5 months of the study indicated that the MFO-fed group received 150 to 200 mg/kg/day of EPA. Six-month-old, MFO-fed, female WHHL rabbits had significantly lower plasma concentrations of total cholesterol (582±20 mg/dl vs. 856±44 mg/dl, control, p<0.05) and triglycerides (266±21 mg/dl vs. 459±15 mg/dl, control, p<0.05), with lower serum/plasma lipoprotein concentrations [very low density lipoprotein (VLDL), LDL, high density lipoprotein (HDL)] compared to control female WHHL rabbits. Male MFO-fed rabbits had only significantly lower VLDLs (46±9 mg/dl) compared to control male WHHL rabbits (156±9 mg/dl, p<0.05). The platelet fatty acid composition in MFO-fed rabbits had significantly greater amounts of omega-3 fatty acids and lesser amounts of omega-6 fatty acids. Additionally, the threshold concentration of collagen and arachidionate required to aggregate platelets in MFO-fed rabbits was nearly double that of controls. In contrast, the platelet aggregation response to the thromboxane analogue, U-46619, was similar in both groups of animals. Morphometric evaluation of intimal surface of the aortas failed to demonstrate significant differences in lipid accumulation or intimal or medial thickening between MFO-fed and control WHHL rabbits, even when the potential effects of gender were considered. In conclusion: 1) omega-3 fatty acids exert hypolipidemic and antithrombotic effects in LDL receptor-deficient animals, and 2) despite these actions, omega-3 fatty acids do not reduce the severity of atherosclerosis in WHHL rabbits. (Arteriosclerosis 9:529-537, July/August 1989)
Additionally, the effects of omega-3 fatty acids on arteriosclerosis have been studied in cholesterol-fed pigs, some of which had endothelial denudation of the left anterior descending coronary artery. The results of the latter study demonstrated that omega-3 fatty acids not only lowered platelet arachidonic acid and serum thromboxane concentrations, but also had inhibitory effects on both coronary atherosclerosis and intimal hyperplasia after endothelial disruption. It was concluded that the reduction in coronary artery disease was a possible result of altered prostaglandin metabolism. Thus, experimental evidence from certain animal models suggests that omega-3 fatty acids may have a beneficial effect in reducing the severity of atherosclerosis through lipid-lowering effects and reduction in arterial intimal lesions.

To further elucidate the effects of omega-3 fatty acids on the development of atherosclerosis, we evaluated the effects of dietary supplementation with omega-3 fatty acids in an animal model of familial hypercholesterolemia, the Watanabe heritable hyperlipidemic (WHHL) rabbit. The WHHL rabbit produced by serial inbreeding has elevated plasma cholesterol concentrations secondary to a deficiency of functional low density lipoprotein (LDL) receptors. These rabbits develop accelerated atherogenesis similar to their human counterparts with familial hypercholesterolemia and show established lesions in the aortas by 5 months of age.

The purpose of this study was to evaluate the effects of omega-3 fatty acid administration (dietary supplementation with fish oil) on the evolution of atherosclerosis in growing WHHL rabbits. To evaluate the effects of omega-3 fatty acids, we assessed the following: 1) plasma lipids and lipoproteins, 2) platelet fatty acid composition, 3) platelet aggregation, and 4) quantitative and qualitative atherosclerotic changes in the aorta. The results showed decreased plasma cholesterol, increased omega-3 fatty acids in platelet membranes, and increased concentrations of collagen and arachidonic acid needed to cause platelet aggregation. However, when we quantitated the extent of atherosclerosis in the aortas from fish oil-fed and control rabbits, there was no overall difference in the extent of atherosclerotic development between Menhaden fish oil (MFO)-fed and control WHHL rabbits.

**Methods**

**Animal Model**

Weanling WHHL rabbits were obtained from breeders housed at the University of Texas Southwestern Medical Center at Dallas animal facility. This colony originated from a mated pair of homozygous WHHL rabbits acquired from Yoshio Watanabe and given to us by Joseph Goldstein and Michael Brown. At 5 weeks of age, the rabbits were randomly divided into two major groups: one group which was given omega-3 fatty acid supplementation and a control group which was not. To determine if differences in atherogenesis existed between genders, male and female rabbits were studied separately within each group. The control group received a standard rabbit diet (Laboratory Rabbit Diet, Teklad, Madison, WI). Rabbits in the omega-3 fatty acid-supplemented group were fed the control diet supplemented with MFO (Zapata-Haynie, Reedville, VA). This diet provided 5 mg eicosapentaenoic acid (EPA) per gram of food. The rabbit diet was prepared bi-weekly, was divided into daily aliquots, and was stored frozen (-80°C) in an argon atmosphere. Rabbits from both groups were fed ad libitum and were housed in secured, environmentally controlled rooms, with lighting maintained at a constant 12 hours of light and 12 hours of dark. In six animals receiving MFO, daily food consumption was measured for the 5-month duration of the study; the amount of EPA consumed averaged 150 to 200 mg/kg/day. The diet also contained small amounts of cholesterol and vitamin E, so that the amount of cholesterol and vitamin E in the MFO supplement consumed by the rabbits was 3.3 to 4.4 mg cholesterol/kg/day and 47 to 62 mg vitamin E/kg/day. The plasma EPA was not measured. The feeding regimen was continued for 5 months or until the animals were 6 months old.

**Measurement of Plasma Lipids and Lipoproteins**

Plasma lipid and lipoprotein concentrations were measured at monthly intervals during the study. Animals were fasted overnight, and blood samples (20 ml) were collected into tubes containing ethylenediaminetetraacetic acid (EDTA). Lipoprotein quantification was performed by standard techniques. Plasma cholesterol and triglyceride assays were performed with commercially available enzymatic methods.

**Platelet Fatty Acid Composition**

At 6 months of age, a blood sample was collected. The animal was then killed, and the aorta was fixed by perfusion as outlined below. Platelet-rich plasma (PRP) was obtained by centrifuging blood for 10 minutes at 200 g at room temperature. The PRP was removed, and the platelets were isolated by centrifuging for 10 minutes at 1500 g at room temperature. After the plasma was decanted, the platelets were resuspended in distilled water and were stored under an argon atmosphere at -80°C. A total lipid extract of an aliquot of the platelet suspension was prepared by using the technique of Bligh and Dyer. Hydrolysis and methylation of the component fatty acids were performed by using benzene and boron trifluoride in methanol. The fatty acid-methyl esters were extracted with hexane. During the methylation and extraction procedures, all solvents contained the antioxidant butylated hydroxytoluene (5 mg/100 ml solvent). Separation and identification of the fatty acid-methyl esters were performed by using a gas chromatograph (Hewlett-Packard model 5830), equipped with a flame ionization detector and digital integrator. A 6-foot glass column packed with 10% SP2230 on 100/200 Chromosorb W (Supelco, Bellefonte, PA) was used. The injector and detector temperatures were 240°C and 250°C, respectively. The column temperature was 180°C for 2 minutes, rising at 1.8°C per minute to 200°C. The carrier gas was nitrogen at a flow rate of 20 ml per minute. The retention times of fatty acid-methyl esters were determined using authentic standards (Supelco).


**Platelet Aggregation Studies**

Blood was obtained from an 18-gauge plastic catheter inserted in the central auricular artery. Samples were collected from 6-month-old animals (five controls and four MFO-treated WHHL rabbits). Blood was mixed with 0.1 volume of 3.5% sodium citrate (pH 7.4) and was centrifuged for 10 minutes at 200 g at room temperature. The supernatant or the PRP was removed. The pellet was centrifuged again for 10 minutes at 1500 g to obtain platelet-poor plasma (PPP). Platelet aggregation was performed using the technique of Born on a dual-channel aggregometer (Sienco, Morrison, CO). Zero and 100% light transmittance was determined by using PRP and PPP, respectively. Concentration-response curves were performed to arachidonic acid (Sigma, St. Louis, MO), collagen (Hormon-Chemie Munich, West Germany), and the thromboxane A₂-mimetic, U-46619 (The Upjohn Company, Kalamazoo, MI). The threshold concentrations to stimulate aggregation were determined. The threshold concentration was the lowest concentration of the aggregating agent that produced a detectable change in light transmission.

**Tissue Collection and Preparation**

At 6 months of age, 12 animals (6 males and 6 females) from each of the two groups were treated intravenously with heparin (1000 U heparin, heparin sodium, LyphoMed, Melrose Park, IL) and then anesthetized (Nembutal, Abbott Laboratories, North Chicago, IL). Via a left thoracotomy, the heart was isolated, the left ventricle was catheterized, and an overdose of anesthetic was injected. The animal was perfused first with a modified Joklik solution (Eagle's Minimum Essential Medium, Joklik-Modified, Gibco Laboratories, Lawrence, MA) and was gassed with 95% O₂ and 5% CO₂ for 5 minutes, and this was followed by a 2% paraformaldehyde, 2% glutaraldehyde fixative for 25 minutes. Perfusion pressure was maintained at a constant 60 mm Hg. After perfusion fixation, the heart and aorta (1 cm distal to the bifurcation of the iliac arteries) were removed. The aorta was cleaned of excess adventitial tissue, the lumen was exposed via a ventral incision along the entire longitudinal length, and the vessel was pinned open (endothelial side up) to a wax base. After an additional 24 hours postfixation in formalin (Millonig's modified phosphate-buffered formalin, Surgipath, Northbrook, IL), the aorta was stained with Sudan IV (Sudan IV, S-8756, Sigma) in order to identify grossly the lipid distribution. All animals were coded so that the gender or treatment group was not known to the person measuring the aortas. Aortas were then photographed using 35-mm slide film (Kodachrome 64, Kodak Canada Incorporated, Toronto, Ontario, Canada).

Estimates of the percent lipid-positive areas present were made with a computer-assisted planimetry system. Photographs of the aorta were projected onto a digitizer pad (Jandel Scientific, Sausalito, CA), and the lipid-positive areas were measured. The aorta was divided into four regions: 1) proximal thoracic (area from the aortic valve to proximal to the first intercostal ostia), 2) proximal abdominal (from the crura of the diaphragm to the distal lip of the caudal mesenteric artery ostia), and 4) distal abdominal (from the ostia of the caudal mesenteric artery to the bifurcation of the iliac arteries, Figure 1). After calibration, the areas of both the vessel and the lipid-positive zones were traced, with the final results expressed as the percentage of lipid-positive area.

After photography, two 2- to 3-mm wide longitudinal sections encompassing the second intercostal artery ostia and one 2- to 3-mm wide longitudinal section of aorta 0.5 cm distal to the aortic valves were acquired. One section from the intercostal artery region was first placed in osmium tetroxide (Electron Microscopy Sciences, Fort Washington, PA) for 4 hours; then it was dehydrated in acetone and embedded in glycol methacrylate, and 1 μm-thick sections were obtained with a Reichert 2050 microtome. The second section was processed as outlined above except without exposure to osmium tetroxide. Sections were stained with either nuclear fast red, hematoxylin and eosin (H&E), or Masson's trichrome. The section from the proximal aorta was processed for electron microscopy. Sections were dehydrated through acetones and embedded in an Epon/Araldite resin mixture (E.F. Fullam Incorporated, Schenectady, NY). Sections in the
silver-grey interference color area were placed on a copper grid, were stained with uranyl acetate and lead citrate, and were examined with a JOEL 100S transmission electron microscope. The morphologic criteria for identifying the cell types were based on our experience and descriptions by other investigators.25,32

Morphometric evaluation of the histologic sections was obtained by using the same digitizer system as for the gross morphometrics. Histologic sections were projected by a Zeiss microprojector onto the digitizer pad. From the osmium-nuclear fast red sections, the area of subendothelial lipid was traced in relation to the overall area of aorta on the slide, and the final results were expressed as percentages of total area occupied by subendothelial lipid. The intimae and medial areas were measured by using the H&E sections, and these data were expressed as the percent of total area occupied by intima.

Data reduction and analyses were accomplished with an IBM/PC computer and the Statistical Analysis System/PC statistics program (SAS Institute, Cary, NC). The results were summarized as the means±SEM, and the differences among means were determined by using analysis of variance with ranking according to Duncan’s Multiple Mean Comparison test.33

### Results

Data supplementation with MFO resulted in significant reductions of 35% and 50%, respectively, in total cholesterol and triglyceride concentrations in female rabbits when compared to female control WHHL rabbits (Table 1). Although cholesterol and triglyceride concentrations were consistently lower in MFO-fed male rabbits compared to male controls, these concentrations were not significantly different. The plasma lipoproteins—very low density (VLDL), LDL, and high density (HDL) lipoproteins—were significantly reduced in MFO-fed females when compared to female controls, whereas males had a significant reduction only in VLDL compared to male controls.

The platelet fatty acid composition was significantly altered in WHHL rabbits supplemented with omega-3 fatty acids (Table 2). The MFO-fed group had a significantly greater percentage of EPA (14.3%) in lipid composition of their platelets than did control rabbits (0%). MFO-fed rabbits showed decreased levels of omega-9 fatty acids as well as omega-6 fatty acids. Arachidonic acid was significantly reduced by 64% when compared to control animals. To a lesser extent, linoleic acid (36%) and oleic acid (31%) were also lower in MFO-fed rabbits. There was no significant alteration in the platelet saturated fatty acid composition between MFO-fed and control WHHL rabbits. Platelet aggregation in MFO-fed rabbits required a significantly greater concentration of collagen (4.6±0.5 vs. 1.5±0.3 µg/ml for controls) and arachidonic acid (28±1.7 vs. 15±0 µg/ml for controls) to produce aggregation (Table 3). Thus, platelets from the MFO-fed animals were less responsive to these aggregating agents. However, no difference occurred between MFO-fed and control rabbits in concentrations of the thromboxane A2 mimetic (U-46619) needed to stimulate platelet aggregation.

Gross and microscopic evaluation of the aortas from both groups revealed marked accumulations of atherosclerotic lesions by 6 months of age. Except for the ascending limb of the aorta, most of these positive areas
Table 3. Concentrations of Aggregating Agents Required to Induce Threshold Aggregation in Platelets from Control and Menhaden Fish Oil-treated WHHL Rabbits

<table>
<thead>
<tr>
<th>Animals</th>
<th>N</th>
<th>Collagen</th>
<th>Arachidonate</th>
<th>U-46619</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>4</td>
<td>1.5±0.3</td>
<td>15±0</td>
<td>0.40±0.2</td>
</tr>
<tr>
<td>MFO diet</td>
<td>5</td>
<td>4.6±0.5*</td>
<td>28±1.7*</td>
<td>0.36±0.1</td>
</tr>
</tbody>
</table>

Values are expressed as means±SEM and are in μg/ml.

*p<0.01, control diet versus Menhaden fish oil diet.

WHHL=Watanabe heritable hyperlipidemic rabbit, MFO=Menhaden fish oil.

Discussion

The mechanism for antiatherogenic action of omega-3 fatty acids, although not fully understood, has been variably ascribed to: 1) reductions in plasma lipid or lipoprotein concentrations, 2) reductions in platelet thromboxane or platelet-activating factor formation, 3) formation of the leukotrienes with five double bonds, 4) increases in membrane fluidity, and 5) reductions in the production or action of either procoagulant or fibrinolytic factors.1-18 The purpose of this study was to evaluate the effects of omega-3 fatty acids on the progression of atherosclerosis in an animal model with a genetic LDL receptor deficiency. The dietary supplementation with MFO started at a point when WHHL rabbits have hypercholesterolemia but negligible atherosclerosis (i.e., at 1 month of age).22,23 The results show that supplementation of the diet of immature WHHL rabbits with omega-3 fatty acids for 5 months will: 1) lower plasma cholesterol and triglyceride concentrations, 2) incorporate omega-3 fatty acids into platelets, and 3) increase the threshold concentration of collagen or arachidonic acid required to aggregate platelets. However, these potentially

Figure 2. Photomicrograph of the aorta at the distal lip of the second intercostal artery ostium from a Menhaden fish oil-fed Watanabe heritable hyperlipidemic rabbit showing lipid-laden foam cells (arrows) in the intima (I) and lipid-positive smooth muscle cells (arrowheads) in the media (M). O₄O₂ and nuclear fast red stains. ×200
beneficial effects of omega-3 fatty acids do not reduce the extent of atherosclerosis in these animals.

Although the mechanism by which omega-3 fatty acids lower plasma cholesterol and triglyceride is not known, evidence suggests that the lowering is due to inhibition of hepatic triglyceride synthesis, alteration of enzyme activity in cell membranes that contain omega-3 fatty acids, or inhibition of saturable binding of LDL by hepatocytes. With regard to the lipid-lowering effect of omega-3 fatty acids, studies in normal volunteers and in patients with hyperlipidemia indicate that the effects of omega-3 fatty acids on plasma triglyceride and VLDL cholesterol concentrations are more pronounced than the effects on total cholesterol or LDL cholesterol concentrations. This finding suggests that omega-3 fatty acids primarily affect the synthesis or clearance of triglycerides and VLDL. Moreover, HDL cholesterol concentrations are maintained or even increased in humans, and increased in rats on diets supplemented with omega-3 fatty acids. In contrast to their effects in subjects with intact LDL receptor-dependent metabolism, omega-3 fatty acids appear to have limited effects on plasma lipids in male WHHL rabbits. In these animals, omega-3 fatty acids exert a significant lowering effect only on VLDL. In contrast, female MFO-fed rabbits demonstrated lower cholesterol and triglyceride concentrations. Although in humans, females with intact LDL receptors have lower cholesterol and triglyceride levels compared to age-matched males, this trend has not been elucidated in studies with MFO-supplementation in which both men and women have been studied. Neither has this been compared in animal studies. This is the first time that such a difference between males and females supplemented with omega-3 fatty acids has been noted. This suggests that female WHHL rabbits may possess an independent pathway for metabolizing LDL that is enhanced by omega-3 fatty acids.

Another antiatherogenic mechanism implicated in omega-3 fatty acid supplementation is altered platelet aggregation. Our data on platelet fatty acid content and aggregation are similar to the findings of other investigators in humans and swine that were given diets supplemented with marine lipids. The results of these studies show a significant increase in platelet omega-3 fatty acids and a decrease in platelet omega-6 fatty acids. Additionally, an increase in threshold concentrations of collagen and arachidonic acid, but not the thromboxane A2 mimetic, U-46619, were required to stimulate platelet aggregation. These findings suggest that omega-3 fatty acids may exert their antiplatelet action on cyclooxygenase activity. The incorporation of omega-3 fatty acids into the platelet may compete with arachidonic acid, replacing the active thromboxane A2 with the more inactive thromboxane A3. The result is reduced platelet aggregability at sites of endothelial damage, thus removing a potentially important mechanism for atherosclerosis. The omega-3 fatty acids do not appear to generally depress platelet function, since the response to U-46619 was similar in control and MFO-fed animals. Similarly, it may be concluded that omega-3 fatty acids do not inhibit the thromboxane A2/PGH2 receptor interaction or the events distal to activation of the thromboxane A2/PGH2 receptor.

Although omega-3 fatty acids are linked with lowering the incidence of myocardial infarction in humans, the effects in animal studies have been inconsistent. Pigs with diet-induced hypercholesterolemia supplemented with omega-3 fatty acids have less severe atherosclerosis than do hypercholesterolemic controls. The difference occurs without a concomitant reduction in plasma triglyceride, total cholesterol, or lipoprotein concentrations. This suggests that omega-3 fatty acids can exert their antiatherogenic properties even under hypercholesterolemic conditions. In rhesus monkeys fed either fish-oil cholesterol or cholesterol diets, the fish-oil group has reductions in serum cholesterol, aortic intimal atherosclerosis, and atherosclerotic plaque size. Similarly, the cynomolgus monkey on a hypercholesterolemic diet supplemented with fish-oil shows reduction in severity of atherosclerosis in the aorta, coronary, and common carotid artery; however, the carotid bifurcation has more severe atherosclerosis. Also, Japanese quail fed a hypercholesterolemic diet and supplemented with fish-oil have reduced serum cholesterol and no early genesis of coronary artery atherosclerosis. However, rats that have dietary-induced hypercholesterolemia...
actually have enhanced monocyte adhesion and fatty streak formation with fish oil supplementation. Likewise, omega-3 fatty acid supplementation of New Zealand White rabbits fed a hypercholesterolemic diet causes increased lipid-positive areas on the aorta. Although omega-3 fatty acid supplementation in WHHL rabbits reduces serum triglycerides, the degree of aortic atherosclerosis is not affected. Of interest in this study is the trend for omega-3 supplemented WHHL rabbits to show slight increases in lipid-positive areas in all four regions compared to WHHL controls, with significant increases in the distal abdominal aorta. This is also seen at the microscopic level with MFO-fed WHHL rabbits, as indicated by a greater accumulation of subendothelial lipid. However, there is not a significant increase in intimal area, thus indicating no effect either beneficial or detrimental on atherosclerotic plaque development. The results from these animal studies indicate that the rabbit may respond differently to omega-3 fatty acid supplementation than other animal models or humans. Two different mechanisms may be involved. First, the rabbit may be more susceptible to lipid accumulation with omega-3 fatty acids. However, unlike the rat, this may not be reflected by a significant increase in fatty streak formation. Second, the severe hypercholesterolemia in WHHL rabbits may be an overriding mechanism, and this hypercholesterolemia may not be reduced sufficiently by omega-3 fatty acids to produce a protective effect. Thus, even with reduction in plasma cholesterol concentrations by 30% to 50%, there is still severe hypercholesterolemia, and progression of atherosclerosis is not altered.

This resistance of WHHL rabbits to potential antiatherogenic interventions has also been observed in other studies. Slow channel calcium antagonists delay atherogenesis in rabbits ingesting a high cholesterol diet. Nevertheless, verapamil and nifedipine have no influence on atherogenesis in growing WHHL rabbits. These rabbits demonstrated the same extent of aortic lipid accumulation as did control WHHL rabbits. Thus, the protective effect of either omega-3 fatty acids or calcium antagonists observed in hypercholesterolemic animals does not occur in the WHHL rabbit. The reason for the differing effects of some interventions in WHHL rabbits versus cholesterol-fed rabbits is unclear. Since hypercholesterolemia down-regulates LDL receptors, both groups of animals may be deficient in LDL receptors. The results may be related to the severity of hypercholesterolemia, the type of associated lipoprotein derangements, or genetic differences. In contrast to the effects of other interventions, treatment with probucol does reduce atherosclerosis in WHHL rabbits, possibly by preventing the local oxidation of LDL and uptake of altered LDL in the vessel wall. Thus, probucol may attenuate the formation of early foam cell lesions in hypercholesterolemic WHHL rabbits by preventing or limiting the oxidation of LDL. From the results of this study, we conclude that dietary supplementation with MFO in WHHL rabbits is associated with: 1) the incorporation of omega-3 fatty acids into platelet lipids with demonstrable reductions in platelet function, and 2) a lowering of plasma lipids and lipoproteins. However, these alterations have no effect on reducing the extent of atherosclerosis in these animals deficient in LDL receptors. Thus, treatment outside the LDL receptor pathway (i.e., probucol) appears to have beneficial effects in reducing atherosclerosis in the WHHL rabbit. Conversely, treatment directed toward modifying conventional risk factors (i.e., reducing serum cholesterol and platelet adhesion) does not prevent or reduce atherosclerosis in the LDL receptor-deficient WHHL rabbit.

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