Dietary Cod-liver Oil Improves Endothelium-Dependent Responses in Hypercholesterolemic and Atherosclerotic Porcine Coronary Arteries

Hiroaki Shimokawa, MD, and Paul M. Vanhoutte, MD

This study examined the effects of dietary supplementation with cod-liver oil on impaired endothelium-dependent relaxations in hypercholesterolemia and in atherosclerosis in porcine coronary arteries. Sixteen male Yorkshire pigs underwent balloon endothelium removal of the left coronary arteries and were fed a 2% high-cholesterol diet for 10 weeks, with or without dietary supplementation of cod-liver oil (30 ml/day) (oil-fed and cholesterol-fed groups, respectively). This model allowed the simultaneous examination of the effects of dietary cod-liver oil on vascular reactivity in hypercholesterolemia alone (right coronary artery) and in atherosclerosis (left coronary artery). After 10 weeks of feeding, the dietary treatment with cod-liver oil caused an increase in plasma levels of eicosapentaenoic acid and a decrease in the plasma levels of arachidonic acid, whereas the treatment had no significant effect on the increases in plasma lipid levels induced by the high-cholesterol feeding. Morphometric analysis showed significant inhibition of coronary atherosclerosis by the treatment. Endothelium-dependent responses were examined in vitro in ring preparations and in bioassay experiments. Endothelium-dependent relaxations to bradykinin, serotonin, and adenosine 5’-diphosphate were larger in both right and left coronary arteries from oil-fed than from cholesterol-fed animals. Aggregating platelets from cholesterol-fed and oil-fed pigs induced comparable, larger endothelium-dependent relaxations in rings from oil-fed than from cholesterol-fed pigs. The contractions induced by serotonin or aggregating platelets were significantly inhibited in rings with endothelium from oil-fed pigs, whereas they were comparable in rings without endothelium in both groups. Relaxations to sodium nitroprusside and contractions to potassium chloride or serotonin were comparable in rings without endothelium in both groups. The bioassay experiments revealed that the release of endothelium-derived relaxing factor in response to bradykinin and the relaxations of vascular smooth muscle to the endothelial factor were greater after the fish-oil diet. These results indicate that dietary supplementation of cod-liver oil delays the impairment of endothelium-dependent relaxations in hypercholesterolemia and in atherosclerosis, partly because of an improved release of endothelium-derived relaxing factor and partly because of an improved relaxation of coronary smooth muscle to the factor. (Circulation 1988;78:1421–1430)

Injury or dysfunction of endothelial cells plays a major role in the etiology of atherosclerosis.1,2 Impaired endothelium-dependent relaxations in atherosclerotic arteries have been reported in both experimental animals3–5 and humans.5,6 In porcine coronary arteries, endothelium-dependent relaxations to aggregating platelets and related vasoactive substances (serotonin and adenosine 5’-diphosphate [ADP]) are impaired by hypercholesterolemia and are impaired further by atherosclerosis.7 Dietary fish oils are one of the possible antithrombotic agents in ischemic heart disease.8–12 Previous studies showed that dietary supplementation with cod-liver oil augments endothelium-dependent relaxations in porcine coronary arteries13 and that eicosapentaenoic acid (EPA) may be the major component of the fish oil responsible for the augmentation, by
increasing the synthesis or release of endothelium-derived relaxing factor or both. Endothelium-derived relaxing factor is not only a vasodilator but also a potent inhibitor of platelet aggregation. An augmented release of endothelium-derived relaxing factor by dietary fish oils could partly explain their antiatherogenic effects. Therefore, the present study was designed to examine whether dietary supplementation with cod-liver oil would improve endothelium-dependent responses in hypercholesterolemic and atherosclerotic porcine coronary arteries.

Materials and Methods

Animal Preparations

Sixteen normal Yorkshire pigs, 7–10 weeks of age (22.5 ± 1.0 kg), were used. The pigs were randomly divided into two groups: eight pigs were fed a 2% high-cholesterol diet (TD 86019 with 19% lard and 2% cholesterol, Teklad, Madison, Wisconsin) (cholesterol-fed group), and another eight pigs were fed the atherogenic diet and 30 ml cod liver oil each day (E.R. Squibb & Sons, Princeton, New Jersey) (oil-fed group). The cod-liver oil contained 9% EPA and 0.4% arachidonic acid, whereas the atherogenic diet contained 0.1% EPA and 6.5% arachidonic acid. The daily food intake was limited to an amount equal to 3% of the body weight per day to prevent excessive weight gain. After 2 weeks of feeding, the animals of both groups underwent balloon endothelial denudation of the left anterior descending (LAD) and circumflex coronary arteries (LCx) to allow the rapid development of coronary atherosclerosis. The procedure was performed without knowledge of whether or not the animal was treated with cod-liver oil. After this, they were fed for 8 more weeks. In this model, the effects of dietary cod-liver oil on vascular reactivity in hypercholesterolemia (right coronary artery, RCA) and in atherosclerosis (left coronary arteries) could be evaluated in the same animal in the oil-fed group by comparing the data with those from the cholesterol-fed group. Before and after the 10 weeks of feeding, the following variables were measured: plasma concentrations of lipid (enzymatic method), platelet count (Model S-plus IV, Coulter Electronics, Hialeah, Florida), and fatty acid profiles of plasma lipids (gas chromatographic analysis).

Organ Chamber Experiments

Experiments were performed in vitro in one cholesterol-fed pig and one oil-fed pig in parallel. The hearts were removed after anesthesia with ketamine hydrochloride (300 mg i.m.) and sodium pentobarbital (12.5 mg/kg i.v.). The three coronary arteries were removed from the heart and immersed in cold modified Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl 118.3, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, CaCl2 2.5, NaHCO3 25.0, Ca-EDTA 0.016, and glucose 11.1 (control solution). The experiments were performed on rings (3–4 mm long) of proximal LAD and RCA. At maximum, 10 rings were obtained from each coronary artery; they were numbered from proximal to distal portions, and rings with the same number taken from the two pigs were studied in parallel. In some rings, the endothelium was removed by gently rubbing the luminal surface with a cotton swab wetted with control solution. The rings were suspended horizontally between two stirrups in organ chambers filled with 25 ml control solution (37° C, pH 7.4) gassed with 95% O2-5% CO2. The preparations were attached to a strain gauge (UC2, Gould Statham, Oxnard, California), and isometric tension was recorded. The rings were then progressively stretched until the contractile response evoked by 20 mM KCl was maximal (optimal tension). They were allowed to equilibrate for 30 minutes.

After the equilibration, all rings were exposed to bradykinin (concentration-response curve [10-10 to 10-7 M] or one dose [10-7 M]) during a contraction caused by prostaglandin F2α (2 × 10-6 M) to confirm the presence or absence of functional endothelial cells. No relaxation was present in rings without endothelium. Relaxations were then examined during a contraction to prostaglandin F2α (2 × 10-6 M), and contractions were examined in quiescent rings in the following order: set A: (relaxation), 1) serotonin and 2) platelets taken from a cholesterol-fed pig; set B: (relaxation), 1) ADP and 2) platelets from an oil-fed pig; set C: (contraction), 1) platelets taken from a cholesterol-fed pig (followed by 60 mM KCl) and 2) serotonin; set D: 1) platelets taken from an oil-fed pig (contraction), 2) potassium chloride (contraction), and 3) sodium nitroprusside (relaxation). All four sets of protocol were tested in LAD (atherosclerosis), whereas sets A, B, and D (potassium chloride and sodium nitroprusside only) were tested in RCA (hypercholesterolemia). While relaxations were being determined, the rings were incubated with indomethacin (10-5 M) for 40 minutes to inhibit the synthesis of endogenous prostaglandins. Similarly, when determining relaxations to serotonin and aggregating platelets, the rings were incubated with the 5-hydroxytryptamine (5-HT3)-serotonergic antagonist ketanserin (10-6 M) for 40 minutes to inhibit the direct activating effects of the monoamine on vascular smooth muscle. Samples of fluid (0.5 ml) were withdrawn from the chambers 7 minutes after addition of platelets (75,000 platelets/μl) and were added to 120 μl cysteine (1% by weight in distilled water). Proteins were then precipitated by adding ZnSO4 and NaOH and centrifuging at 1,400g for 30 minutes. The resulting supernatant was frozen for later analysis. The concentration of serotonin was determined by reverse-phase high-pressure liquid chromatography.
Bioassay

The biological activity of endothelium-derived relaxing factor released from segments (4 cm long) of LCx was bioassayed using a ring of the proximal LAD from which the endothelium had been removed mechanically (bioassay ring). Coronary segments taken from cholesterol- and oil-fed pigs were studied in parallel. The segments were perfused at constant flow (2 ml/min) by means of a roller pump (Minipuls 2, Gilson Medical Electronics, Middleton, Wisconsin) with control solution maintained at 37°C. The bioassay rings were suspended by means of two stainless steel stirrups, one of which was connected to an isometric force transducer (FTO3C; Grass Instruments, Quincy, Massachusetts). The assembly of bioassay ring, stirrups, and force transducer could be moved freely below the organ chamber, allowing the preparation to be superfused with the perfusate either from the coronary segment with endothelium (endothelial superfusion, endothelial line) or from the stainless steel tube (direct superfusion, direct line).

The bioassay rings were first superfused through the stainless steel cannula with control solution for 60 minutes. During this interval, they were stretched in a stepwise manner to approximately 8 g, the optimal tension of isolated porcine coronary arteries. Indomethacin (10^{-6} M) was present in the perfusate throughout the experiment to prevent the synthesis of endogenous prostaglandins. Relaxations were examined during a contraction evoked by prostaglandin F_{2a} (2 \times 10^{-6} M); the contractions of bioassay rings were not statistically different between the two groups (6.3±0.6 g in the cholesterol-fed group and 7.3±0.8 g in the oil-fed group). Bradykinin was used to cause release of endothelium-derived relaxing factor because its direct actions on the vascular smooth muscle of the bioassay rings were minimal compared with those of serotonin or ADP, respectively.

Drugs and Platelets

The following drugs were used: ADP, bradykinin, 5-hydroxytryptamine creatinine sulfate (serotonin), indomethacin, potassium chloride, prostaglandin F_{2a}, sodium nitroprusside (all from Sigma Chemical, St. Louis, Missouri), and ketanserin tartrate (Janssen Pharmaceutica, Beerse, Belgium). All drugs were prepared daily with distilled water except for indomethacin, which was dissolved in Na_{2}CO_{3} (10^{-7} M). The concentrations are expressed as final molar concentration in the bath or superfusion solution. A platelet-rich solution was prepared by centrifugation.

Morphology

The hearts were divided into five horizontal blocks and were examined macroscopically for the presence or absence of myocardial infarction.

The rings used in the organ chamber study were examined histologically by hematoxylin and eosin staining for determination of endothelial lining and general observation, by van Gieson’s elastic staining for determination of the thickness of the intima, and by Sudan IV staining for confirmation of lipid deposition in the blood vessel wall. Morphometric determination was performed with a computer-assisted image analyzer (IBAS 2000, Zeiss, Oberkochen, FRG) to evaluate cross-sectional area of the intima and percent medial involvement by the atherosclerotic plaques.

Data Analysis

Results are expressed as mean±SEM. Unless otherwise specified, n refers to the number of animals. In rings contracted with prostaglandin F_{2a}, responses are expressed as percent changes from the contracted levels, and in quiescent rings, responses are expressed as percentage of the maximal response to KCl (60 mM). For relaxations, the negative logarithm of the effective molar concentration of agonist causing 25% or 50% inhibition (IC_{25} or IC_{50}) of the contractions to prostaglandin F_{2a} was calculated for each concentration-response curve, and the mean of these values is presented. For contractions evoked by potassium chloride or serotonin, the effective concentration producing 50% or 40% of the maximal response (ED_{50} or ED_{40}) was calculated, respectively. The relation between IC_{50} values (bradykinin and ADP) or maximal percent relaxation (serotonin) and degrees of coronary atherosclerosis in LAD (intimal cross-sectional area and percent medial involvement by atherosclerotic plaque) was examined by linear regression analysis. Maximal percent relaxation was chosen for serotonin because the relaxations to the monoamine were so impaired in the cholesterol-fed group that even IC_{25} values could not be calculated. Statistical evaluation of the data was performed by Student’s t test for either paired or unpaired observations. When more than two means were compared, a one-way analysis of variance was used. If a significant value was found, Scheffe’s test for multiple comparisons was used to identify differences among groups. Values were considered statistically different when p was less than 0.05.

Results

Baseline Data

After 10 weeks of feeding, body weight increased significantly but in a similar manner in both groups (68.6±2.0 kg in the cholesterol-fed group and 70.1±1.8 kg in the oil-fed group). There was no difference in platelet count (×10^{9}/mm^{3}) between the two groups (366±25 for cholesterol-fed and 374±48 for oil-fed). The plasma concentrations of cholesterol significantly increased in all fractions in both groups, and there was no difference in each fraction between the two groups (Table 1). The
plasma concentration of triglyceride was unaltered in both groups (Table 1).

Fatty Acid Profiles of Plasma Lipids

Among the major fatty acids, the plasma levels of oleic acid significantly increased, and those of docosahexaenoic acid significantly decreased after 10 weeks of feeding; there was no significant difference between the two groups (Table 2). In contrast, the plasma levels of EPA significantly increased and those of arachidonic acid significantly decreased in the oil-fed group, whereas in the cholesterol-fed group, EPA significantly decreased with no change in arachidonic acid (Table 2). The ratio of EPA to arachidonic acid significantly increased in the oil-fed group and significantly decreased in the cholesterol-fed group.

Morphology

In 16 pig hearts, no macroscopic lesion of myocardial infarction was noted. In LAD that had been affected by endothelial denudation and hypercholesterolemia, intimal thickening with lipid deposition (atheroma) was noted in all rings of both groups. However, the extent of coronary atherosclerosis, as expressed in intimal cross-sectional area or percent medial involvement, was signifi-
cantly less in the oil-fed compared with the cholesterol-fed group (Figure 1). In contrast, in RCA that was exposed to hypercholesterolemia only, mild intimal lesions were noted, the extent of which was also less in the oil-fed group (Figure 1).

Organ Chamber Experiments

Smooth muscle characteristics. There was no statistically significant difference in optimal tension or contractions evoked by prostaglandin F2a (2 × 10⁻⁶ M) between the two groups. KCl (5–60 mM) caused comparable, concentration-dependent contractions in rings without endothelium in both groups. Sodium nitroprusside (10⁻⁴ to 10⁻⁶ M) caused comparable relaxations in rings without endothelium in both groups (Table 3).

Relaxations. Bradykinin (10⁻¹⁰ to 10⁻⁷ M), serotonin (10⁻⁹ to 3 × 10⁻⁶ M), and ADP (10⁻⁸ to 10⁻⁴ M) caused endothelium-dependent, concentration-dependent relaxations in both groups; the relaxations to all three vasoactive substances were significantly larger in the oil-fed group in either RCA or LAD compared with those in the cholesterol group (Figures 2–4 and Table 4). When comparing the relaxations between RCA and LAD in each group, those to the three vasoactive substances were significantly impaired in the LAD in the

<table>
<thead>
<tr>
<th>TABLE 1. Plasma Lipid Levels</th>
<th>Cholesterol (n=8)</th>
<th>Cholesterol and fish oil (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>10 Weeks</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>93±18</td>
<td>571±68*</td>
</tr>
<tr>
<td>Low density lipoprotein</td>
<td>2±1</td>
<td>95±47*</td>
</tr>
<tr>
<td>Low density lipoprotein</td>
<td>68±11</td>
<td>412±90*</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>23±6</td>
<td>65±7*</td>
</tr>
<tr>
<td></td>
<td>29±6</td>
<td>45±7</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. Cholesterol, animals fed the atherogenic diet only; cholesterol and fish oil, animals fed the atherogenic diet and cod-liver oil.

*tp<0.05 compared with levels before initiation of diet.

<table>
<thead>
<tr>
<th>TABLE 2. Fatty Acid Profiles of Plasma Lipids</th>
<th>Cholesterol (n=8)</th>
<th>Cholesterol and fish oil (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid</td>
<td>Before</td>
<td>10 Weeks</td>
</tr>
<tr>
<td>16:0 (palmitate)</td>
<td>15.6±0.7</td>
<td>15.1±0.5</td>
</tr>
<tr>
<td>18:0 (stearate)</td>
<td>12.4±1.3</td>
<td>10.0±1.3</td>
</tr>
<tr>
<td>18:1 (oleate, ω 6)</td>
<td>14.7±2.6</td>
<td>26.9±1.4*</td>
</tr>
<tr>
<td>18:2 (linoleate, ω 6)</td>
<td>23.9±1.1</td>
<td>23.3±1.5</td>
</tr>
<tr>
<td>20:4 (arachidonate, ω 6)</td>
<td>7.8±0.7</td>
<td>6.2±0.5</td>
</tr>
<tr>
<td>20:5 (eicosapentaenoic acid, ω 3)</td>
<td>1.4±0.2</td>
<td>0.2±0.1*</td>
</tr>
<tr>
<td>22:6 (docosahexaenoic acid, ω 3)</td>
<td>5.0±1.0</td>
<td>2.3±1.2*</td>
</tr>
<tr>
<td>Others</td>
<td>19.3±3.3</td>
<td>16.1±2.2*</td>
</tr>
<tr>
<td>20:5/20:4</td>
<td>0.19±0.04</td>
<td>0.02±0.01*</td>
</tr>
</tbody>
</table>

Data (percentage of total) are expressed as mean±SEM. Fatty acids are expressed by chain length: number of double bonds. Number in parentheses represents the carbon atoms between the terminal bond and the methyl group.

Cholesterol, animals fed the atherogenic diet only; cholesterol and fish oil, animals fed the atherogenic diet plus cod-liver oil.

*p<0.05 compared before initiation of diet; †p<0.05 compared with cholesterol-fed group.
cholesterol-fed group, whereas in the oil-fed group, a significant difference was noted in response to serotonin and ADP but not to bradykinin (Table 4). In contrast, ADP caused comparable relaxations in rings without endothelium in both groups in either RCA or LAD (Figure 4).

Aggregating platelets (25,000–75,000 platelets/μl) caused endothelium-dependent relaxations of either RCA or LAD, which were significantly larger in the oil-fed group compared with the cholesterol-fed group, regardless of whether the platelets were taken from the cholesterol- or the oil-fed group (Figure 5).

Contraction. In quiescent rings without endothelium from LAD, serotonin (10^{-9} to 10^{-5} M) caused comparable, concentration-dependent contractions in both groups (Figure 6); the ED_{50} value (-log M) and maximal contraction (percent maximal contraction to potassium chloride) were 6.87±0.13 M and 68±4% in the cholesterol-fed group and 6.73±0.26 M and 66±7% in the oil-fed group, respectively. In rings with endothelium of the cholesterol-fed group, contractions to serotonin were pronounced, and the contractions were greater in rings with than in those without endothelium at higher concentrations of the monoamine (Figure 6). In contrast, the contractions were significantly inhibited in rings with endothelium of the oil-fed group (Figure 6); ED_{50} value (-log M) and maximal percent contraction were 6.69±0.14 M and 78±5% in the cholesterol-fed group and 6.20±0.30 M and 57±6% in the oil-fed group, respectively (p<0.05).

In quiescent rings without endothelium from LAD, aggregating platelets (75,000 platelets/μl) caused comparable contractions in both groups, regardless of whether the platelets were taken from a cholesterol- or an oil-fed pig (Figure 7). In quiescent rings with endothelium, platelets caused comparable contractions in the cholesterol-fed group as they did in rings without endothelium, whereas the platelet-induced contractions were significantly inhibited in the oil-fed group, regardless of the source of platelets (Figure 7). Aggregating platelets (75,000 platelets/μl) taken from the cholesterol- and oil-fed pigs released comparable amounts of serotonin into the bath solution (30.1±39 ng/ml [1.8×10^{-6} M] and 321±49 ng/ml [1.9×10^{-6} M], respectively, n=6).

Bioassay
Bradykinin caused no relaxations of bioassay rings when infused during direct superfusion (n=6).

### Table 3. Characteristics of Vascular Smooth Muscle

<table>
<thead>
<tr>
<th></th>
<th>Right coronary artery</th>
<th>Left anterior descending artery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol</td>
<td>Cholesterol and fish oil</td>
</tr>
<tr>
<td>Optimal tension (g)</td>
<td>8.2±0.3 (26)</td>
<td>8.2±0.3 (26)</td>
</tr>
<tr>
<td>Developed tension to</td>
<td>2×10^{-6} M PGF_{2α} (g)</td>
<td>9.6±1.1 (26) 10.6±0.7 (26)</td>
</tr>
<tr>
<td>Contraction to potassium chloride</td>
<td>ED_{50} (mM)</td>
<td>Max contraction (g)</td>
</tr>
<tr>
<td>ED_{50} (mM)</td>
<td>10.2±0.4</td>
<td>10.2±0.4</td>
</tr>
<tr>
<td>Max contraction (g)</td>
<td>19.3±2.6</td>
<td>17.9±2.4</td>
</tr>
<tr>
<td>Relaxation to sodium nitroprusside</td>
<td>IC_{50} (-log M)</td>
<td>Max (%) relaxation</td>
</tr>
<tr>
<td>IC_{50} (-log M)</td>
<td>8.06±0.12</td>
<td>8.03±0.17</td>
</tr>
<tr>
<td>Max (%) relaxation</td>
<td>117±6</td>
<td>129±4</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. Numbers in parentheses are the numbers of rings tested in each experimental period. Potassium chloride and sodium nitroprusside were tested in four animals for right coronary artery and in six animals for left anterior descending coronary artery in each group.

Cholesterol or cholesterol and fish oil, cholesterol-fed pigs without or with dietary treatment with cod-liver oil; PGF_{2α}, prostaglandin F_{2α}; ED_{50}, effective concentration producing 50% of the maximal response to KCl; Max contraction, maximal contraction to KCl; IC_{50}, effective concentration causing 50% inhibition of the contractions to prostaglandin F_{2α} (2×10^{-6} M); Max % relaxation, maximal relaxation in percentage of the contraction induced by prostaglandin F_{2α} (2×10^{-6} M).
When the rings were moved to be superfused from the endothelial line (with $2 \times 10^{-6}$ M prostaglandin F$_{2\alpha}$), small but significant relaxations were noted (basal release of endothelium-derived relaxing factor); these relaxations were significantly larger when the bioassay rings were superfused through the donor segment from an oil-fed pig than through the segment from a cholesterol-fed pig (Figure 8). Bradykinin ($10^{-9}$ to $10^{-7}$ M) was then infused through donor segments and caused concentration-dependent relaxations of the bioassay rings, which were significantly larger when infused through segments from oil-fed than from cholesterol-fed pigs, regardless of whether the bioassay ring was taken from the cholesterol- or the oil-fed pig (Figure 8). In addition, the relaxations caused by higher concentrations of bradykinin ($10^{-8}$ and $10^{-7}$ M) were significantly larger in rings from an oil-fed pig than in rings from a cholesterol-fed pig when perfused through the donor segment from an oil-fed pig (Figure 8).

**Correlation Between Endothelium-Dependent Relaxation and Coronary Atherosclerosis**

There was a significant correlation in the cholesterol-fed group between the IC$_{50}$ values to bradykinin ($n=16$) and to ADP ($n=8$) or the maximal percent relaxations to serotonin ($n=8$) and variables of coronary atherosclerosis ($r=0.62, 0.65$, and $0.66$ for intimal cross-sectional area and $r=0.67, 0.77$, and $0.76$ for percent medial involvement, respectively; $n$ is the number of rings with endothelium). In contrast, in the oil-fed group there was no significant correlation between those values ($r<\pm 0.2$ for all correlations).

**Discussion**

This study demonstrates that dietary supplementation with cod-liver oil improves endothelium-dependent responses in hypercholesterolemic and atherosclerotic porcine coronary arteries. In the present pig model, hypercholesterolemia (for 10 weeks) impairs endothelium-dependent relaxations to serotonin and ADP but not to bradykinin, whereas atherosclerosis further impairs relaxations to serotonin and ADP and impairs those to bradykinin. Compared with the responses with normal coronary arteries from animals fed a normal diet, the present results indicate that the dietary cod-liver oil delays the process of impairment of endothelium-dependent relaxations by normalizing the responses in hypercholesterolemia and by improving the impaired responses in atherosclerosis to the levels obtained with hypercholesterolemia alone.
Changes in Fatty Acid Levels

These findings regarding plasma lipids and fatty acids are in agreement with the previous study by Weiner et al.,29 in which dietary supplementation with cod-liver oil (30 ml/day) in cholesterol-fed pigs had no significant effect on the increases in plasma lipids but increased the EPA levels and decreased the arachidonic acid levels of platelets. Plasma free fatty acids may be the main source of the fatty acids that are esterified and incorporated into membrane phospholipids.30 This would suggest that a change in the plasma free fatty acids would be reflected in the fatty acid composition of the membrane phospholipids.30 Therefore, the present results may indicate that EPA is the major component of cod-liver oil responsible for the augmented endothelium-dependent relaxations, together with the significant decrease in plasma levels of arachidonic acid. The increase in plasma EPA and the decrease in plasma arachidonic acid may also be responsible for the augmented endothelium-dependent relaxations in normal pigs.14 Because the changes in the plasma levels of oleic acid and docosahexaenoic acid were comparable between the cholesterol- and oil-fed groups, these changes may reflect the effects of a high cholesterol diet but not those of dietary cod-liver oil.

Morphology

As reported previously,21,24 a combination of balloon endothelium removal and high cholesterol feeding (for 10 weeks) resulted in rapid development of

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**Table 4. Endothelium-Dependent Relaxations of Porcine Coronary Arteries**

<table>
<thead>
<tr>
<th></th>
<th>Right coronary artery</th>
<th>Left anterior descending artery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol</td>
<td>Cholesterol and fish oil</td>
</tr>
<tr>
<td>Bradykinin (n=8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC50 (~log M)</td>
<td>8.33±0.11</td>
<td>8.71±0.14*</td>
</tr>
<tr>
<td>Max relaxation (%)</td>
<td>115±4</td>
<td>108±4</td>
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<tr>
<td>Serotonin (n=8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC25 (~log M)</td>
<td>7.03±0.50</td>
<td>8.49±0.14*</td>
</tr>
<tr>
<td>Max relaxation (%)</td>
<td>58±12</td>
<td>89±13*</td>
</tr>
<tr>
<td>Adenosine 5'-diphosphate (n=8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC50 (~log M)</td>
<td>5.71±0.16</td>
<td>6.57±0.26*</td>
</tr>
<tr>
<td>Max relaxation (%)</td>
<td>95±7</td>
<td>113±8</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. All rings were treated with indomethacin (10^-5 M). In the experiments of serotonin, rings were treated with ketanserin (10^-6 M) to inhibit the direct effect on vascular smooth muscle. Cholesterol or cholesterol and fish oil, cholesterol-fed pigs without or with dietary treatment with cod-liver oil; IC50 or IC25, effective concentration causing 50% or 25% inhibition of the contractions to prostaglandin F2a (2×10^-6 M); max relaxation, maximal relaxation in percent of the response to prostaglandin F2a (2×10^-6 M); ——, responses did not attain the IC25 level in five out of eight cases. 

*p<0.05 compared with cholesterol-fed group; †p<0.05 compared with right coronary artery in the same dietary group.

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**Figure 5.** Plots of cumulative concentration-response curves to aggregating platelets during a contraction evoked by prostaglandin F2a (2×10^-6 M) in the right coronary artery (RCA) and the left anterior descending coronary artery (LAD). All rings were treated with indomethacin (10^-5 M) and ketanserin (10^-6 M). Responses are expressed as percent changes in tension from the contraction evoked by prostaglandin F2a. Data are mean±SEM. Asterisk denotes a statistically significant difference (p<0.05) between rings from cholesterol- and oil-fed groups.

**Figure 6.** Plots of cumulative concentration-response curves to serotonin in quiescent rings from cholesterol-(left) and oil-fed (right) groups. Contractions are expressed as percent increase in tension compared with maximal contractions caused by KCl (60 mM). Data are mean±SEM. Asterisk denotes a statistically significant difference (p<0.05) between rings with and without endothelium.
selective lesions of coronary atherosclerosis, whereas hypercholesterolemia alone caused minimal intimal lesions. The dietary treatment with cod-liver oil significantly inhibited the intimal lesions both in atherosclerosis and in hypercholesterolemia. The present model provides a relatively early stage of coronary atherosclerosis in contrast with the pig model of Weiner et al.,29 in which advanced lesions of coronary atherosclerosis (severe intimal thickening with areas of calcification or necrosis or both) were induced by 8 months of high-cholesterol feeding. With this model, dietary supplementation with cod-liver oil also significantly inhibits coronary atherosclerosis.29

Mechanisms of Improved Relaxations

Previous studies in the same pig model showed that the decreased release of endothelium-derived relaxing factor is responsible for the impaired endothelium-dependent relaxations in hypercholesterolemia.7 In more advanced atherosclerosis, the concomitant release of vasoconstrictor prostaglandins or their intermediate(s) from the endothelium and intimal thickening, causing a functional barrier, may also play additional pathogenetic roles.7

The bioassay experiments revealed that the major mechanism for the improved relaxations by the dietary treatment is the improved release of endothelium-derived relaxing factor. This finding is in agreement with the previous finding that the augmented release of endothelium-derived relaxing factor accounts for the augmented endothelium-dependent relaxations by the dietary unsaturated fatty acids in normal animals.14 In addition, the significant inhibition of the serotonin-induced endothelium-dependent contractions (which are sensitive to cyclooxygenase blockers7) suggests that the altered balance between vasoconstrctor and vasodilator prostaglandins or their intermediate(s)10,31 may also be involved in the improved relaxations. A decreased intimal thickening or the anti-inflammatory action of fish oil or both32 may also favor the diffusion of endothelium-derived relaxing factor. Finally, the bioassay experiments also suggest that the sensitivity of the vascular smooth muscle to endothelium-derived relaxing factor is improved. A similar tendency was noted also in a previous study on normolipemic pigs treated with dietary unsaturated fatty acids.14 Because, in both studies, there was no difference between control and oil-fed animals in the relaxations of the vascular smooth muscle to sodium nitroprusside (which induces relaxations through activation of guanylate cyclase as endothelium-derived relaxing factor does33), these findings indicate that there may be some differences in the mechanisms of relaxations between endothelium-derived relaxing factor and sodium nitroprusside.

Further mechanisms of the impaired release of endothelium-derived relaxing factor in hypercholesterolemia and in atherosclerosis remain to be examined. Regenerated endothelial cells (4 weeks
after endothelium removal) have a reduced ability to release endothelium-derived relaxing factor in response to serotonin. In the chronic stage (8 weeks after endothelium removal), the endothelium-dependent relaxations to ADP also are impaired (H. Shimokawa and P. M. Vanhoutte, unpublished observation). Hypercholesterolemia per se injures endothelial cells, resulting in the increased turnover of the cells. Therefore, the reduced ability of regenerated endothelial cells to release endothelium-derived relaxing factor could partly explain the endothelial dysfunction in hypercholesterolemia and atherosclerosis, and dietary fish oils may have beneficial effects on endothelial function under these conditions. In the oil-fed group, there was no significant correlation between endothelium-dependent relaxations and variables of intimal thickening, indicating that dietary cod-liver oil improves endothelial function, irrespective of the degree of intimal thickening. Dietary treatment of atherosclerosis in monkeys also restores endothelium-dependent relaxations even if the intimal thickening remains. Low-density lipoproteins administered on a short-term basis nonspecifically inhibit endothelium-dependent relaxations in the rabbit aorta. Although such an action may explain endothelial dysfunction in hypercholesterolemia or atherosclerosis, the improvement of endothelial function by the diet with fish oil cannot be related to it because the plasma levels of low-density lipoproteins were not decreased by the dietary treatment.

### Aggregating Platelets

Endothelium-dependent responses to aggregating platelets are the global expression of the responses to several platelet-derived products and their interactions. In porcine coronary arteries, endothelium-dependent relaxations to aggregating platelets are due to activation of P<sub>2</sub>-purinergic receptors (by adenosine 5'-triphosphate and ADP) and of 5-HT<sub>1</sub>-serotonergic receptors, and platelet-induced contractions of the smooth muscle are due to activation of 5-HT<sub>1</sub>-serotonergic receptors with little contribution of thromboxanes. In the present study, aggregating platelets taken from both groups released comparable amounts of serotonin. Because in the absence of the endothelium the platelet-induced contractions were comparable in the cholesterol- and oil-fed groups, the augmented endothelium-dependent responses to ADP and serotonin by cod-liver oil explain the augmented relaxations to aggregating platelets.

### Clinical Implications

Endothelium-derived relaxing factor not only relaxes vascular smooth muscle but also is a potent antiaggregatory substance. Impaired endothelium-dependent responses in atherosclerosis are observed not only in experimental animals but also in humans. Impaired interactions between platelets and the atherosclerotic blood vessels would favor the occurrence of platelet aggregation and platelet-induced contractions of coronary smooth muscle, leading to ischemic events such as coronary vasospasm and coronary thrombosis. The antiatherogenic actions of fish oils may depend on several mechanisms. The present results indicate that dietary treatment with fish oil may protect endothelial function and thus delay the process of atherosclerosis, demonstrating a new aspect of the antiatherogenic actions of fish oils.

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### References

33. Rapoport RM, Murad F: Agonist induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cyclic GMP. Circ Res 1983;52:352–357

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