Dietary \( \omega-3 \) Fatty Acids and Endothelium-Dependent Responses in Porcine Cerebral Arteries

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Purpose: We sought to determine the effect of dietary \( \omega-3 \) polyunsaturated fatty acids on cerebrovascular endothelium-dependent responses in studies performed on isolated porcine basilar arteries.

Methods: Male Yorkshire pigs (6–8 weeks old) were kept for 4 weeks on a standard diet (control group, \( n = 12 \)) or on chow supplemented with polyunsaturated fatty acids (eicosapentaenoic acid, 3.5 g/day, or docosahexaenoic acid, 1.5 g/day; treated group, \( n = 12 \)). Isometric tension recording of the basilar artery was carried out and responses were compared between the two groups.

Results: The regimen resulted in a decrease in the plasma arachidonic acid level and an increase in eicosapentaenoic acid. Endothelium-dependent relaxations induced by bradykinin and adenosine diphosphate were augmented in the basilar arteries of the treated group. Incubation with indomethacin (10\(^{-5}\) M) prevented the augmentation of the relaxations induced by bradykinin, but not those caused by adenosine diphosphate. The indomethacin-sensitive, endothelium-dependent contractions to arachidonic acid remained comparable in the two groups, indicating that the activity of cyclooxygenase was not affected by the diet.

Conclusions: Dietary supplementation with \( \omega-3 \) polyunsaturated fatty acids enhances endothelium-dependent relaxations in the basilar artery by two mechanisms: 1) replacement of endogenous arachidonic acid and suppression of the concomitant release of vasoconstrictor prostaglandins from the endothelium, and 2) enhancement of the release of endothelium-derived relaxing factor. (Stroke 1992;23:407–413)

Key Words • basilar artery • endothelium-derived relaxing factor • fatty acids, unsaturated • pigs

The dietary intake of large amounts of marine oils rich in \( \omega-3 \) polyunsaturated fatty acids probably accounts for the low incidence of coronary artery disease among Greenland Eskimos. High contents of \( \omega-3 \) polyunsaturated fatty acids, especially eicosapentaenoic acid, are found in the plasma and platelets of Eskimos. Eicosapentaenoic acid is transformed into trienoic prostanoids, such as thromboxane \( A_3 \) and prostaglandin \( I_3 \). Thromboxane \( A_3 \) does not possess proaggregatory activity, unlike the dienoic prostanoid thromboxane \( A_2 \) and prostaglandin \( I_2 \) has effects comparable to those of prostacyclin. Hence, supplementation with \( \omega-3 \) fatty acids inhibits platelet aggregation and prolongs bleeding time, presumably due to the decreased production of thromboxane \( A_2 \).

Previous studies demonstrate that dietary supplementation with cod-liver oil or purified \( \omega-3 \) fatty acids augments endothelium-dependent relaxations in isolated porcine coronary arteries. Endothelium-dependent relaxations probably play an important role in maintaining a patent circulation in the event of thromboembolism. Substances released from aggregating platelets, such as adenosine diphosphate and serotonin, evoke contractions of damaged arterial wall when the medial layer is exposed. In the presence of endothelium, these substances cause the release of endothelium-derived relaxing factor (EDRF) and dilate coronary arteries. Aggregating platelets evoke endothelium-dependent relaxations in the cerebral artery as well. During intravascular thrombosis, the kallikrein pathway is activated, causing the release of bradykinin, a potent inducer of EDRF release. In the cerebral circulation, eicosapentaenoic acid may be effective in preventing hypoperfusion and edema during reperfusion after carotid occlusion. During cerebral arterial spasm after subarachnoid hemorrhage, endothelium-dependent relaxations are severely impaired and the resulting failure to maintain normal dilator responses may contribute to the pathological constriction. In a comparative epidemiological study of stroke incidence in fishing and farming communities in Japan, higher plasma eicosapentaenoic acid levels were found in the former, where prevalence of cerebral infarct was significantly lower (2.4/1,000 versus 7.2/1,000) and that of cerebral hemorrhage slightly higher. Interestingly, the incidence of subarachnoid hemorrhage was found to be

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four times higher in Greenland Eskimos than in the Danish controls. The present study was designed to examine whether a dietary supplementation with ω-3 polyunsaturated fatty acids alters responses mediated by the endothelium in cerebral arteries.

**Materials and Methods**

Twenty-four male Yorkshire pigs, 6–8 weeks old (27.5±0.8 kg), were used. The pigs were randomly divided into two groups. In the control group (n=12), the animals were kept on a regular diet (30 g/kg/day; Hog Finisher, Bedtke Brothers Feed and Seed Co., Dover, Minn.). In the treated group (n=12), eicosapentaenoic acid (3.5 g/day) and docosahexaenoic acid (1.5 g/day) (Promega, Parke-Davis, Morris Plains, N.J.) were added to the regular diet for 4 weeks. At the beginning and the end of the 4-week period, the plasma concentration of lipids (enzymatic method) and the fatty acid profiles (gas chromatographic method) were determined. After 4 weeks, the animals were euthanized with ketamine (300 mg i.m.), pentobarbital (12.5 mg/kg i.m.), and exsanguination. The brain and the basilar artery were dissected free and immediately placed in modified Krebs-Ringer bicarbonate solution of millimolar composition NaCl 118.3, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0, CaEDTA 0.026, and glucose 11.1 (control solution) at 4°C. Under a dissecting microscope (Zeiss), the basilar artery was separated from the brain stem and cut into rings (approximately 4 mm long). The procedures and the care of animals complied with the “Principles of Laboratory Animal Care” and the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health publication No. 80-23, revised 1985) and were approved by the Institutional Animal Care and Use Committee.

Rings were prepared in a given order from the proximal to the distal portion of the basilar artery. In every other ring, the endothelium was removed mechanically by gentle rubbing of the intimal surface with a stainless wire. Pharmacological experiments were carried out on pairs of adjacent rings (with and without endothelium) to minimize the possible effect of varying diameter along the artery. Each ring was connected to an isometric force transducer (Gould UC-2) with two triangular stainless wire stirrups (0.035 gauge) inserted in the lumen and suspended in an organ chamber (37°C) filled with control solution bubbled with a 95% O₂–5% CO₂ gas mixture. The rings were equilibrated for 30 minutes at a length where the tension approximated 100 mg. This length is referred to as the initial length. The rings were then placed at the optimal point of their length–tension relationship measured in response to KCl (20 mM). The optimal point was reached when the rings were stretched 0.5±0.02 mm (n=48, four rings from each animal) from the initial length and was not different between the control and the treated groups. Removal of the endothelium did not affect this length (0.5±0.02 mm in both groups). A full concentration–response curve to prostaglandin F₂α was obtained in the first six animals in each group, and no significant difference in the responses was observed between the two groups. Therefore, in rings in which the relaxations were measured, plateau contractions were obtained by using the same concentration (2×10⁻⁶ M) of prostaglandin F₂α.

In rings in which responses to mechanical stretching were measured, the initial length was resumed after determining the optimal length, and the preparations were allowed to equilibrate for 30 minutes. The rings were then stretched to the optimal length in 3 seconds, and the subsequent generation of contractile force was recorded. The tension caused by the passive stretching to the optimal length (t₁) and the maximum active tension generated after stretch (t₂) were measured. In rings in which no active contraction was observed (rings without endothelium), t₁ was measured 4.7 minutes after the stretch, which corresponds to the mean peak latency of active tension (4.7±0.3 minutes, n=48, rings with endothelium).

The following drugs were used: adenosine diphosphate (ADP, Sigma), arachidonic acid (Nu-chek), bradykinin (Sigma), indomethacin (Sigma), nordihydroguaiaretic acid (NDGA; Sigma), papaverine hydrochloride (Merck), prostaglandin F₂α (Sigma), and sodium nitroprusside (Sigma). The concentrations of drugs are expressed as final molar (M) in the bath solution. Stock solution of indomethacin was prepared immediately before use with equimolar amounts of Na₂CO₃. Previous data demonstrated that addition of these solutions (100 ml) to the organ chamber did not change the pH measurably. NDGA was dissolved in polyethylene glycol; 50 ml of the stock solution was added to the organ chamber.

Pairs of rings with and without endothelium were assigned at random to one of three protocols to measure responses to several drugs. Between exposure to each drug, 30 minutes of incubation was allowed. The three protocols were as follows.

Protocol A: 1) KCl (20 mM) to determine length–tension curve; 2) ADP (10⁻⁴–10⁻⁶ M) during contraction to prostaglandin F₂α (2×10⁻⁶ M); and 3) bradykinin (10⁻⁶–10⁻⁵ M) during contraction to prostaglandin F₂α (2×10⁻⁴ M), followed by sodium nitroprusside (10⁻⁴ M).

Protocol B: 1) length–tension curve; and 2) sodium nitroprusside (10⁻⁹–10⁻⁴ M) during contraction to prostaglandin F₂α (2×10⁻⁶ M), followed by papaverine (3×10⁻⁶ M).

Protocol C: 1) length–tension curve; 2) bradykinin given as a single dose (10⁻⁸ M) during contraction to prostaglandin F₂α (2×10⁻⁶ M) to check the presence or absence of the endothelium; 3) mechanical stretching; and 4) arachidonic acid (10⁻⁸–10⁻⁵ M).

In rings in which protocol C was performed, dose–response curves to prostaglandin F₂α (10⁻⁸–10⁻⁵ M) and KCl (5–60 mM) were examined in the initial six animals from each group. All protocols were performed in the absence or presence of indomethacin (10⁻⁵ M, given 30 minutes before starting the concentration–response curves). In some experiments, responses to mechanical stretching were measured after exposure to an inhibitor of lipoxgenase, nordihydroguaiaretic acid (NDGA) (10⁻⁵ M), in addition to indomethacin. These rings were incubated for 20 minutes with NDGA initially; the agent was then washed out and rings were incubated with indomethacin for 30 minutes.

Relaxations to agonists were expressed as percentage changes of the maximal relaxation to sodium nitroprusside (10⁻⁴ M) unless otherwise mentioned. Contractions
Relaxations were augmented in the control group (Figure 2, left panel; at 10⁻⁹, 3 x 10⁻⁹ and 10⁻⁸ M, p < 0.05). After incubation with indomethacin, the relaxations were augmented in the control group (Figure 2, right panel; at 10⁻⁹, 10⁻⁸, and 10⁻⁷ M, p < 0.05), and the curves in the two groups became identical. In the treated group, relaxations were not augmented by incubation with indomethacin.

Adenosine diphosphate caused small relaxations both in rings with and without endothelium in the absence of indomethacin. No difference was observed between the control and the treated groups (Figure 3, left panel). After incubation with indomethacin, concentration-dependent relaxations were observed in rings with endothelium but not in rings without endothelium (Figure 3, right panel). The endothelium-dependent relaxations to adenosine diphosphate in the presence of indomethacin were significantly larger in the treated group.

In the absence of indomethacin, arachidonic acid caused concentration-dependent contractions in rings with, but not in those without, endothelium when indomethacin was absent (Figure 4, upper panel); the endothelium-dependent contractions were comparable between the control and the treated groups. The contractions were abolished after incubation with indomethacin (Figure 4).

Rapid stretching from the initial to the optimal length caused subsequent contractions in rings with, but not in those without, endothelium (Figure 5, upper panel). Indomethacin (10⁻⁷ M) did not affect the endothelium-dependent contractions in either group; the endothelium-dependent contractions were not altered by the dietary supplementation with ω-3 fatty acids (Figure 5, lower panel). The contractions were abolished after incubation with NDGA (10⁻³ M) plus indomethacin (10⁻³ M) (Figure 5).

Discussion
Dietary supplementation of ω-3 polyunsaturated fatty acids resulted in a decrease in plasma arachidonic acid in quiescent rings were expressed as percentage changes of the response to prostaglandin E₂ (2 x 10⁻⁶ M). Constrictions after mechanical stretch were expressed as the ratio of the maximal tension generated (t₁) and the tension required to stretch the ring to its optimal point (t₂). Comparisons between groups were made using Student's t test for paired or unpaired comparisons; values <0.05 were considered to be statistically significant.

Results
The increase in body weight over the 4-week period was comparable between the control and treatment groups. The plasma concentration of cholesterol decreased in the treatment group (from 122.4 ± 4.9 to 104.7 ± 4.4 mg/dl, n = 9) but not in the control group (from 117.6 ± 5.7 to 113.4 ± 4.7 mg/dl, n = 9). The plasma triglyceride level was unchanged in both groups. As regards the fatty acid profile of the plasma, arachidonic acid decreased relatively (from 11.8 ± 0.9 to 4.0 ± 0.3%, n = 9) and eicosapentaenoic acid increased (from 1.0 ± 0.2 to 8.5 ± 0.9%, n = 9) in the plasma of the treated pigs; no significant changes were observed in the control group.

Progressive stretching during the determination of length-tension relationship resulted in development of myogenic tone (Table 1). The resting tension was not altered by removal of endothelium and was not different between the control and the treated groups.

The maximal contractions to KCl in rings without endothelium (control group 2.2 ± 0.31 g, n = 9; treatment group 2.4 ± 0.25 g, n = 9) were smaller than those in rings with endothelium (control group 3.6 ± 0.45 g, n = 9; treatment group 3.4 ± 0.38 g, n = 9), but no difference was observed between the control and the treatment groups. The maximal contractions to prostaglandin E₂ (10⁻⁵ M) were comparable between the rings with endothelium (control group 2.2 ± 0.41 g, n = 9; treatment group 2.6 ± 0.53 g, n = 9) and those without endothelium (control group 2.5 ± 0.4 g, n = 9; treatment group 2.6 ± 0.42 g, n = 9).

Sodium nitroprusside caused concentration-dependent relaxations in rings without endothelium, which were comparable between the control and the treatment group (Figure 1). Bradykinin (10⁻¹⁰–10⁻⁹ M) caused concentration-dependent relaxations in rings with, but not in those without, endothelium (Figure 2 left, right). In the absence of indomethacin, the endothelium-dependent relaxations were significantly larger in the treated group.
acid and an increase in eicosapentaenoic acid. Eicosapentaenoic acid is a relatively poor substrate for cyclooxygenase, with a high affinity for binding to the enzyme; hence, it inhibits the conversion of arachidonic acid to prostaglandins. Cyclooxygenase converts the ω-3 polyunsaturated fatty acid into trienoic prostanoids, such as prostaglandin I₂ (PGI₂) and thromboxane A₃ (TXA₃). PGI₂ possesses antiaggregatory and vasodilator effects comparable to those of prostacyclin, but thromboxane A₃ does not possess the proaggregatory activity of thromboxane A₂. Endothelium-dependent contractions are mediated by products of the metabolism of arachidonic acid in some blood vessels. In the cerebral arteries, acetylcholine, ADP, arachidonic acid, and the calcium ionophore A23187 cause endothelium-dependent contractions, which are prevented by the inhibitor of cyclooxygenase, indomethacin. Agonists that elicit endothelium-dependent relaxations induce concomitant release of vasoconstrictor prostanoids from the endothelium. As a consequence, endothelium-dependent contractions are mediated by products of the metabolism of arachidonic acid in some blood vessels.

**FIGURE 2.** Left panel: Concentration–response curve to bradykinin in rings of porcine basilar artery during contractions to prostaglandin F₂α (2×10⁻⁶). Responses were obtained in the absence of indomethacin. Relaxations are expressed as percentage changes of maximal relaxations to sodium nitroprusside (10⁻⁴ M). Data are mean±SEM (n=8). *Significant difference (p<0.05) between control and treatment groups. Right panel: Similar responses measured after 30 minutes of incubation with indomethacin (10⁻⁵ M).

**FIGURE 3.** Left panel: Responses to adenosine diphosphate in rings of porcine basilar artery (number of preparations in parenthesis) in absence of indomethacin. Rings were contracted with prostaglandin F₂α (2×10⁻⁶ M) and responses expressed as percentage of maximal relaxation to sodium nitroprusside (10⁻⁴ M). Right panel: Responses in presence of indomethacin (10⁻⁵ M). *Significant difference (p<0.05) between treated and control groups.
In the present study, endothelium-dependent relaxations to bradykinin were larger in the treated group. It is likely that the difference was due to the replacement of arachidonic acid with eicosapentaenoic acid and a decrease in the concomitant release of vasoconstrictor prostanoids. This conclusion is prompted by the finding that indomethacin augments relaxations in the control arteries to the extent that, in the presence of the inhibitor cyclooxygenase, the concentration-relaxation curve to kinin was comparable in the control and treatment groups.

In the absence of indomethacin, endothelium-dependent relaxations to ADP were not observed, presumably because they are masked by the constrictor effect of simultaneously produced vasoconstrictor prostanoids. They became apparent only after incubation with indomethacin. In the presence of the cyclooxygenase blocker, the endothelium-dependent relaxations to ADP were larger in the treated group, suggesting that the dietary supplementation enhances the release of EDRF. Because our chosen concentration of indomethacin (10^{-3} M) abolished the endothelium-dependent contractions evoked by arachidonic acid in both groups, it is unlikely that the enhancement in ADP-induced relaxation can be attributed to differential effects of indomethacin on cyclooxygenase in the two groups.

A recent study demonstrated that topical application of ADP causes only small relaxations in the rat basilar artery, whereas it elicited marked dilatation in the pial arterioles. In this study, intravenous indomethacin did not enhance the relaxations, unlike the result of the present study. This discrepancy could be due to variation in the receptor distribution between the porcine
and the rat cerebral arteries or to the different methods of application of ADP (topical application in situ versus addition to the media in the organ chambers) and of indomethacin (intravenous versus incubation in the organ chambers). The relaxing effect of ADP observed in the present study was largely endothelium-dependent, and application to the outer surface of the artery would allow less access of the agonist to the intima.

Endothelium-independent relaxations to sodium nitroprusside were not affected by the intake of ω-3 polyunsaturated fatty acids. Sodium nitroprusside increases cyclic GMP and relaxes vascular smooth muscle, a mechanism shared with EDRF. If one assumes that the nitrovasodilator acts by the same mechanism in the porcine basilar artery, the present study suggests that the capacity of cerebrovascular smooth muscle to produce cyclic GMP (and to relax in consequence) is not affected by treatment with the ω-3 polyunsaturated fatty acids.

Arachidonic acid caused endothelium-dependent contractions that were abolished by indomethacin. The responses were comparable between the two groups, indicating that the activity of cyclooxygenase to convert arachidonic acid into vasoconstrictor prostaglandins was not altered by the dietary supplementation. As could be expected from previous work in the canine basilar artery, stretching resulted in subsequent increase in myogenic tone in rings with endothelium but not in those without. In the canine basilar artery, the stretch-induced contractions were suppressed by indomethacin, indicating that the factor responsible for the endothelium-dependent contractions was the cyclooxygenase product of arachidonic acid metabolism. However, in the porcine basilar artery, the contractions were not inhibited by indomethacin; they were abolished after exposure to NDGA, an irreversible nonspecific inhibitor of lipoygenase. The discrepancy suggests that the factors responsible for contraction due to different metabolites of arachidonic acid in the two species because the dose of indomethacin used in the present study was sufficient to abolish endothelium-dependent contractions to arachidonic acid. The observation that dietary supplementation with the ω-3 polyunsaturated fatty acids did not alter the indomethacin-resistant contractions evoked by stretch is compatible with the hypothesis that these fatty acids replace vasoconstrictor prostaglandins in the vessel wall.

The present results indicate that supplementation of ω-3 polyunsaturated fatty acids augments endothelium-dependent relaxations in the porcine basilar arteries by two mechanisms: first, the replacement of arachidonic acid and inhibition of the concomitant release of vasoconstrictor prostanooids from the endothelium and, second, the enhancement of the release of EDRF. The enhancement of the relaxations induced by ADP and bradykinin may be beneficial for the prevention of infarction in the event of thromboembolism; the former is released by aggregating platelets and responsible for the endothelium-dependent relaxations caused by aggregating platelets, and the latter is released by activation of kallikrein pathway during thrombosis. The replacement of dienoic with trienoic prostanooids by ω-3 polyunsaturated fatty acids results in inhibition of platelet aggregation, which may contribute to prevent thromboembolic events as well. These effects of ω-3 polyunsaturated fatty acids may help explain the reduced incidence of ischemic strokes observed in a Japanese fishing cohort whose plasma eicosapentaenoic acid was elevated. The antiaggregatory and provasodilator effect may contribute to the higher incidence of subarachnoid hemorrhage observed in Japanese fishermen and in Eskimos.

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References
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