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Dietary ω3 polyunsaturated fatty acids augment endothelium-dependent relaxation to bradykinin in coronary microvessels of the pig

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1 The effects of chronic dietary supplementation with ω3 polyunsaturated fatty acids on endothelium-dependent relaxations were examined in isolated coronary microvessels of the pig.

2 Animals were maintained for four weeks with or without dietary supplementation of purified eicosapentaenoic acid (3.5 g daily) and docosahexaenoic acid (1.5 g daily). Fatty acid profiles of plasma lipids showed that only the fraction of eicosapentaenoic acid increased by the treatment, together with a decrease of that of arachidonic acid.

3 In the treated group, endothelium-dependent relaxations to bradykinin were significantly augmented, while contractions to acetylcholine or relaxations to nitroprusside were unaltered.

4 These results indicate that dietary ω3 polyunsaturated fatty acids (mainly eicosapentaenoic acid) augment endothelium-dependent relaxations in coronary microvessels of the pig, without changing the ability of vascular smooth muscle to contract or relax.

Introduction

Studies on isolated arteries have demonstrated the important role of the endothelium in modulating the responsiveness of the underlying vascular smooth muscle (Furchgott, 1983; Vanhoutte *et al.*, 1986). The endothelium appears to modulate the vascular tone in response to changes in flow or shear stress (Busse *et al.*, 1985; Rubanyi *et al.*, 1986). This is particularly important at the microcirculatory level (Griffith *et al.*, 1987), because small blood vessels regulate peripheral vascular resistance and therefore determine the distribution of blood flow in the vascular beds, including the coronary circulation (Shepherd & Vanhoutte, 1979; Guyton, 1981). Dietary supplementation with cod-liver oil augments endothelium-dependent relaxations in large coronary arteries of the pig (Shimokawa *et al.*, 1987); the ω3 polyunsaturated fatty acids (mainly eicosapentaenoic acid; EPA) contained in cod-liver oil probably are responsible for the augmentation (Shimokawa & Vanhoutte, 1987). If this dietary treatment were to alter also endothelium-dependent responses in the coronary microcirculation, it may provide a means to modulate endothelial function at the level of microvessels. The present study was designed to investigate the effects of dietary ω3 polyunsaturated fatty acids on endothelium-dependent relaxations in porcine coronary microvessels.

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Methods

Fourteen normal male Yorkshire pigs (27.0 ± 1.2 kg) were randomly divided into two groups and maintained for four weeks: 7 pigs were fed a regular chow (30 g kg^{-1} daily, Hog Finisher, Bedtke Brothers Feed and Seed Co., Dover, Minnesota, U.S.A.) (control group) and 7 pigs were fed a regular chow plus purified eicosapentaenoic acid (EPA, 3.5 g daily) and docosahexaenoic acid (DHA, 1.5 g daily) (Promega,

Parke-Davis, Morris Plains, New Jersey, U.S.A.). The amount of EPA approximates that used in a previous study, where animals were given daily 1 ml kg^{-1} of cod-liver oil (which contains 9% EPA, Shimokawa *et al.*, 1987). All animals were housed individually under the same environmental conditions. Before and after four weeks of diet, the following variables were measured: plasma concentration of lipids (enzymatic method), blood cell counts (Model S plus IV, Coulter Electronics, Inc., Hialeah, Florida, U.S.A.) and fatty acid profile of plasma lipids (gas chromatographic analysis, Ellefson & Mason, 1964). Three of the control pigs and three treated pigs were used for another study (Shimokawa & Vanhoutte, 1987).

Organ chamber experiments

The hearts were removed following anaesthesia with ketamine hydrochloride (300 mg, i.m.) and sodium pentobarbitone (12.5 mg kg^{-1}, i.v.). The peripheral portion of the second diagonal branch of the left anterior descending coronary artery and the myocardium beneath it were removed 'en bloc' and immersed in cold modified Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, Ca-EDTA 0.016 and glucose 11.1 (control solution). The intramyocardial branch of the artery (internal diameter; 0.306 ± 0.022 mm in the control and 0.332 ± 0.022 mm in the treated group, $n = 7$, each) were dissected free under a microscope (Carl Zeiss Instruments, Oberkochen, F.R.G) and cleaned of loose connective tissue. Two rings (1.5–2.0 mm long) were obtained from the small coronary vessel. Histologically no intimal thickening was noted in the small vessels in either groups. In one of the two rings the endothelium was removed by perfusing the ring with 200 $\mu g\,ml^{-1}$ saponin for 30 s (Gospodarowicz *et al.*, 1980; DeMey & Gray, 1985). Preliminary experiments confirmed that this method is useful for removing the endothelium in microvessels of the pig heart without damaging the characteristics of their vascular smooth muscle (data not shown). The rings were suspended in specially designed organ chambers for microvessels (Myograph, Living Systems Instrumentation, Burlington, Vermont, U.S.A.) filled with control solution kept at 37°C and aerated with a 95% O_2 and 5% CO_2 gas mixture (Mulvany & Halpern, 1977). The preparations were attached to a force transducer and isometric tension was recorded. The rings were then progressively stretched until the contractile response evoked by 10^{-6} M acetylcholine was maximal (optimal point). This optimal point is expressed in terms of tension and effective transmural pressure; the latter was calculated from the equation $p = 2\pi T/L$ (where L is the internal circumference corresponding to the wall tension T), on the basis that the vessel wall was sufficiently thin for Laplace's equation to apply (Mulvany & Halpern, 1977). That is, p is an estimate of the transmural pressure which would have been required to maintain the vessel *in situ* at the same internal circumference and wall tension (Mulvany & Halpern, 1977). In preliminary studies, contractions to acetylcholine, KCl and prostaglandin $F_{2\alpha}$ were compared; acetylcholine caused greater and more stable contractions than the other two agonists ($n = 4$; data not shown). These studies showed that acetylcholine causes no endothelium-dependent relaxation in coronary microvessels of the pig, as is also the case in the large coronary arteries of the same species (Graser *et al.*, 1986; unpublished observations).

After an equilibration period of 30 min, the rings were first exposed to acetylcholine (10^{-9} to 10^{-5} M). Then relaxations to bradykinin and to sodium nitroprusside were examined in a cumulative fashion during contractions caused by the individual ED_{40} value for acetylcholine. Other preliminary studies had compared relaxations to bradykinin, 5-hydroxytryptamine and ADP (dietary cod-liver oil augments endothelium-dependent relaxations to all three agonists, Shimokawa *et al.*, 1987) and revealed that bradykinin causes more pronounced and more stable endothelium-dependent relaxations, with less direct actions on the vascular smooth muscle, than the other two agonists ($n = 3$, data not shown). Indomethacin (10^{-5} M) was present throughout the experiments in order to prevent synthesis of endogenous prostaglandins.

Drugs

The following drugs were used: acetylcholine, bradykinin, indomethacin, and sodium nitroprusside (all from Sigma). All drugs were prepared daily with distilled water except for indomethacin which was dissolved in Na_2CO_3 (10^{-5} M). The concentrations are expressed as final molar (M) concentration in the bath solution.

Data analysis

Results are expressed as mean ± s.e.mean. For contractions evoked by acetylcholine, the negative logarithm of the effective concentration producing 50% of the maximal response (EC_{50}) was calculated. For relaxations, responses are expressed as percentage changes from the contraction to acetylcholine; the negative logarithm of the effective molar concentration of agonist causing 50% inhibition (IC_{50}) of

Table 1 Fatty acid profiles of plasma lipids

Fatty acid	*Control* (n = 7) *Before*	*4 weeks*	*Treated* (n = 7) *Before*	*4 weeks*
16:0 (Palmitate)	14.7 ± 0.7	15.5 ± 0.9	12.3 ± 2.0	14.2 ± 0.8
18:0 (Stearate)	5.9 ± 0.8	6.8 ± 1.1	7.2 ± 0.7	4.8 ± 1.2
18:1 (Oleate,ω9)	17.4 ± 1.8	16.3 ± 2.4	17.1 ± 1.7	13.2 ± 1.8
18:2 (Linoleate,ω6)	29.8 ± 2.6	27.4 ± 2.7	26.3 ± 2.6	29.8 ± 3.1
20:4 (Arachidonate,ω6)	12.3 ± 1.2	9.3 ± 1.1	13.0 ± 1.2	4.1 ± 0.4*†
20:5 (EPA,ω3)	1.2 ± 0.2	1.3 ± 0.2	1.2 ± 0.2	8.8 ± 1.0*†
22:6 (DHA,ω3)	2.3 ± 0.6	1.9 ± 0.5	3.2 ± 0.6	2.4 ± 0.5
Others	16.7 ± 1.8	21.4 ± 3.3	17.8 ± 2.8	22.7 ± 3.9
20:5/20:4	0.10 ± 0.02	0.14 ± 0.02	0.09 ± 0.02	2.21 ± 0.25*†

Data (% of total) are expressed as mean ± s.e.mean.
Fatty acids are expressed by chain length:number of double bonds; the number in parentheses representing the carbon atoms between the terminal bond and the methyl group.
* Significant difference compared with before initiation of diet ($P < 0.05$).
† Significant difference compared with control group ($P < 0.05$).

the contraction to acetylcholine was calculated. Unless otherwise specified, *n* refers to the number of animals. Statistical evaluation of the data was performed by Student's *t* test for either paired or unpaired observations. When more than two values 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 to be statistically significantly different when *P* was smaller than 0.05.

Results

Baseline data

After four weeks of feeding, body weight increased significantly but in a similar manner in both groups; the final body weight was 37.7 ± 2.3 kg in the control and 36.1 ± 1.9 kg in the treated group. There was no difference in platelet count ($\times 10^3/mm^3$) between the two groups (505 ± 47, and 500 ± 43,

Table 2 Effects of dietary ω3 polyunsaturated fatty acids on vascular responses

	Control (+) E	(−) E	*Treated* (+) E	(−) E
Contractions to acetylcholine				
Max. contraction (mg)	632 ± 127	662 ± 92	656 ± 92	757 ± 204
EC_{50} (−log M)	6.56 ± 0.09	6.56 ± 0.03	6.51 ± 0.15	6.59 ± 0.09
Relaxations to bradykinin				
Max. relaxation (%)	73 ± 10*	11 ± 7	100 ± 0*†	8 ± 5
IC_{50} (−log M)	8.04 ± 0.38	—	9.08 ± 0.36†	—
Relaxations to sodium nitroprusside				
Max. relaxation (%)	90 ± 10	98 ± 2	89 ± 6	97 ± 2
IC_{50} (−log M)	6.29 ± 0.36*	7.00 ± 0.22	6.10 ± 0.26*	6.97 ± 0.20

Data are expressed as mean ± s.e.mean for seven experiments in each group, except for rings with endothelium in response to sodium nitroprusside in both groups ($n = 5$).
(+) E = rings with endothelium; (−) E = rings without endothelium.
Max. contraction = maximal contraction; ED_{50} = effective concentration producing 50% of the maximal response to acetylcholine; Max. relaxation (%) = maximal relaxation as a percentage of the contraction induced by acetylcholine; IC_{50} = effective concentration causing 50% inhibition of the contractions to acetylcholine.
* Significant difference compared with rings without endothelium ($P < 0.05$).
† Significant difference compared with control group ($P < 0.05$).

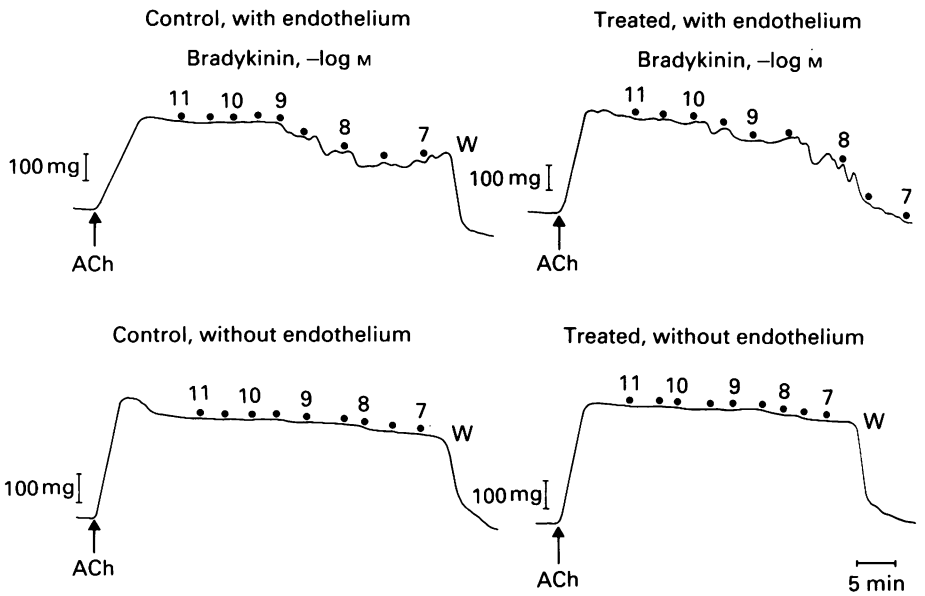

Figure 1 Isometric tension recording in coronary microvessels from control (left; diameter 0.312 mm) and treated (right; 0.306 mm) pigs. Endothelium-dependent relaxations to bradykinin during a contraction evoked by the individual EC_{40} of acetylcholine (ACh), in the presence of indomethacin (10^{-5} M). W = wash out with control solution.

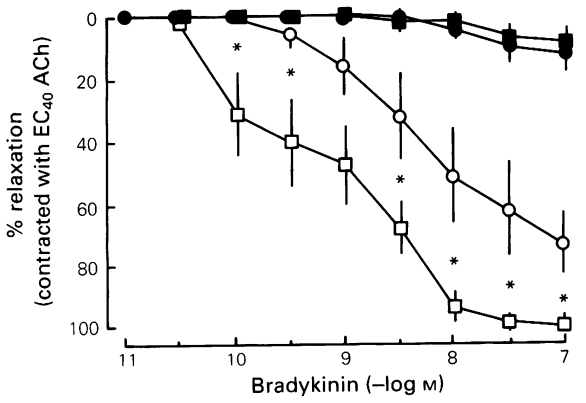

Figure 2 Cumulative concentration-response curves to bradykinin during contractions evoked by the individual EC_{40} of acetylcholine in coronary microvessels of control (○, ●) and treated (□, ■) pigs, in the presence of indomethacin (10^{-5} M) ($n = 7$, each); open symbols represent rings with endothelium and filled symbols represent those without endothelium. The responses are expressed as percentage changes in tension from the contraction evoked by acetylcholine (ACh). Data are shown as mean with s.e.mean indicated by vertical lines. Asterisks denote a significant difference between rings with endothelium from control and treated groups ($P < 0.05$).

respectively). No difference was noted between the control and treated groups in plasma concentrations (mg dl^{-1}) of cholesterol (114 ± 7 and 101 ± 7, respectively) or triglyceride (23 ± 4 and 27 ± 7); there was no difference between the two groups in low-density lipoprotein fractions (mg dl^{-1}) of plasma cholesterol (80 ± 6 and 75 ± 4) or triglyceride (5 ± 1 and 3 ± 1).

Fatty acid profiles of plasmid lipids

Among the major fatty acids, the plasma concentration of EPA significantly increased and that of arachidonic acid significantly decreased after four weeks of feeding (Table 1). As a result, the ratio of EPA to arachidonic acid significantly increased in the treated group. In contrast, the profiles of other fatty acids, including DHA, were unchanged (Table 1).

Organ chamber experiments

There was no difference in optimal point between control and treated groups in terms of tension (213 ± 11 mg and 225 ± 17 mg, respectively) or of effective transmural pressure (27 ± 2 mmHg and

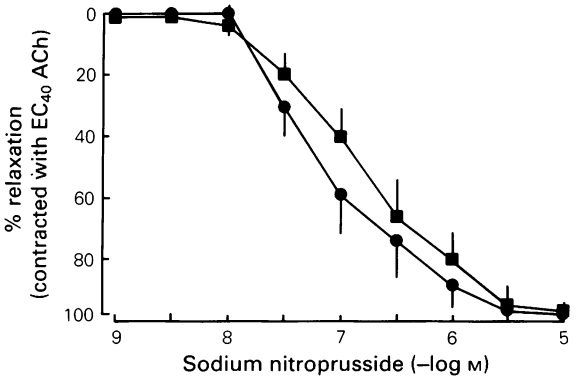

Figure 3 Cumulative concentration-response curves to sodium nitroprusside during contractions evoked by the individual EC_{40} of acetylcholine in coronary microvessels without endothelium from control (●) and treated (■) pigs, in the presence of indomethacin (10^{-5} M) ($n = 7$, each). The responses are expressed as percentage changes in tension from the contraction evoked by acetylcholine (ACh). Data are shown as mean with s.e.mean indicated by vertical lines.

29 ± 2 mmHg, respectively). Acetylcholine (10^{-9} to 10^{-5} M) caused comparable contractions in rings with and without endothelium in both groups (Table 2). Relaxations were then examined in rings contracted with acetylcholine to a comparable level between control and treated groups; contractions as a percentage of maximal responses were $42 \pm 5\%$ in control and $45 \pm 4\%$ in treated group. Bradykinin (10^{-11} to 10^{-7} M) caused endothelium-dependent relaxations in both groups and the relaxations were significantly augmented in the treated group compared with the controls (Figures 1 and 2, Table 2). In contrast, the relaxations to sodium nitroprusside (10^{-9} to 10^{-5} M) in rings without endothelium were comparable in both groups (Figure 3, Table 2); the relaxations were significantly inhibited in rings with endothelium intact in both groups (Table 2).

Discussion

The major effects of dietary $\omega 3$ polyunsaturated fatty acids on plasma fatty acids and vascular reactivity of coronary microvessels of the pig were as follows: (1) the plasma concentration of EPA increased but that of arachidonic acid decreased without significant changes in plasma cholesterol or triglyceride. (2) Bradykinin caused endothelium-dependent relaxations, which were significantly augmented with the dietary treatment. (3) This facilitation occurred at a time when the ability of vascular smooth muscle to relax or contract was unchanged.

In the present study, vascular responses were examined in the presence of indomethacin. Therefore, it is reasonable to consider that the augmented endothelium-dependent relaxations are caused by endothelium-derived relaxing factor and not by vasodilator prostaglandins. Thus, the present study reproduces in the coronary microvessels the facilitation of endothelium-dependent relaxations observed in the large coronary arteries of the same species (Shimokawa & Vanhoutte, 1987). Since only the fraction of EPA increased with the dietary treatment, EPA appears to be responsible for the augmented relaxations, together with the decrease in arachidonic acid.

Endothelium-dependent relaxations are achieved through several processes, that is, synthesis and release of relaxing factor by the endothelial cells, its diffusion from the endothelium to the underlying vascular smooth muscle, and relaxation of the smooth muscle mediated by activation of soluble guanylate cyclase (Furchgott 1983; Rapoport & Murad, 1983). Since there was no intimal thickening (as a possible diffusion barrier) and since the relaxations to sodium nitroprusside (which also activates soluble guanylate cyclase, Gruetter *et al.*, 1979; Rapoport & Murad, 1983) were unchanged, an augmented synthesis and/or release of endothelium-derived relaxing factor is the most likely cause for the observed facilitated response to bradykinin. This interpretation has been proved correct in large coronary arteries of the pig, by use of bioassay techniques (Shimokawa & Vanhoutte, 1987).

Polyunsaturated fatty acids cause endothelium-dependent relaxations, probably by changing the fluidity of the endothelial membrane (Furchgott *et al.*, 1984). EPA can cause endothelium-dependent relaxations in large coronary arteries of the pig (Shimokawa *et al.*, 1987). The ratio of plasma concentrations of EPA to arachidonic acid was dramatically increased with the dietary treatment. Plasma free fatty acids appear to be the main source of the fatty acids that were esterified and incorporated into membrane phospholipids (Srivastava, 1980). Therefore, EPA may be chronically incorporated into the endothelium and change the fluidity of cell membranes, resulting in an augmented release of endothelium-derived relaxing factor. In addition, the changes in fatty acid compositions of endothelial membrane phospholipids could alter the breakdown or inactivation of endothelium-derived relaxing factor. This possibility remains to be examined. Low-density lipoproteins inhibit endothelium-dependent relaxations in the rabbit aorta (Andrews *et al.*, 1987). Since the plasma concentrations of low-density lipoproteins were not significantly decreased by the dietary treatment with $\omega 3$ polyunsaturated fatty acids, they probably do not contribute to the aug-

mented relaxations observed in the present study.

The endothelium plays an important role in modulating the vascular tone not only in large arteries (Busse *et al.*, 1985; Rubanyi *et al.*, 1986) but also at the microcirculatory level (Griffith *et al.*, 1987). In adult spontaneously hypertensive rats, endothelium-dependent relaxations are impaired in mesenteric microvessels, suggesting that the impaired responses may contribute to the elevated peripheral vascular resistance in this experimental model of hypertension (DeMey & Gray, 1985). Conversely, in the present study endothelium-dependent relaxations were augmented by the dietary $\omega 3$ polyunsaturated fatty acids. In large coronary vessels the dietary treatment augmented receptor-operated endothelium-dependent relaxations, including bradykinin, 5-hydroxytryptamine, ADP and thus aggregating platelets (Shimokawa & Vanhoutte, 1987). If this augmentation can be applied to microvessels (as in the case of bradykinin), the present study could provide a new aspect of the beneficial effects of dietary supplementation with fish oil in the coronary circulation (Bang & Dyerberg, 1980).

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