Eicosapentaenoic Acid Inhibits Endothelium-Dependent Relaxation to Acetylcholine in Guinea Pig Coronary Resistance Vessels (43455)

LINDA LEE, R. CLINTON WEBB,[1] AND BERTRAM PITT
Department of Physiology, University of Michigan, Ann Arbor, Michigan 48109-0622

Abstract. **Dietary supplementation with eicosapentaenoic acid (EPA) alters arachidonate metabolism. This study characterizes the effect of dietary EPA on endothelium-dependent vasodilation to acetylcholine (ACH) and ATP in guinea pig coronary resistance vessels. Guinea pigs were fed standard chow (n = 6), standard chow + sesame seed oil (n = 6), or standard chow + menhaden fish oil (17% EPA; n = 6). Coronary vasodilations were examined in the isolated, potassium-arrested heart utilizing a modified Langendorff preparation. Coronary vessels were constricted with prostaglandin $F_{2\alpha}$ and relaxed with ACH (5.5×10^{-9}–10^{-6} moles) or ATP (10^{-10}–10^{-7} moles). Endothelium-dependent dilations to ACH, but not ATP, were attenuated by dietary supplementation with EPA. To assess the role of the endothelium in modulating vascular responses to agonists following dietary manipulation, the perfusate was stimulated by electrolysis (9 V, 4 Hz, 2 msec) in order to generate free radicals, which we have shown to preferentially damage the endothelium. After endothelial damage, responses to ACH, ATP, and nitroprusside were similar between the dietary groups.**

In an additional group of standard diet animals (n = 6) experiments were performed to assess the role of prostanoid metabolism in affecting coronary vascular reactivity. Perfusion of hearts with indomethacin (14 μM) reduced endothelium-dependent vasodilations to ACH (5.5×10^{-9}–10^{-6} moles), but not to ATP (10^{-10}–10^{-7} moles). After endothelial damage, infusion of ACH resulted in vasoconstriction, whereas vasodilation responses to ATP were absent. We conclude that dietary supplementation with EPA inhibits endothelium-dependent dilations to ACH in guinea pig coronary microvessels. These diet-related differences in vascular reactivity may be related to the fish-oil-induced alteration of vasodilator prostaglandin metabolism. In the coronary bed, different endothelial factors appear to mediate relaxation to ACH and ATP.

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Epidemiologic data suggest that populations consuming a diet rich in omega-3 marine oils have a lower rate of cardiovascular disease (1). The fatty acid components of some marine oils have been demonstrated to produce beneficial changes in plasma lipid levels, a decrease in platelet aggregation, and a lower production of thromboxane A_2, a potent vasoconstrictor (2). Studies have shown that dietary supplementation with cod liver oil augments endothelium-dependent relaxations in porcine coronary arteries and that eicosapentaenoic acid (EPA), a polyunsaturated fatty acid of the omega-3 series, is believed to be important in mediating this effect (3). Other studies have suggested that dietary supplementation with fish oil alters intrinsic prostaglandin synthesis and impairs the contractile effects of norepinephrine in isolated rat aortic strips (4).

The purpose of this study was to determine whether dietary supplementation with EPA alters endothelium-dependent vascular responses in the coronary resistance vessels of the perfused isolated guinea pig heart.

[1] To whom requests for reprints should be addressed at the Department of Physiology, University of Michigan, 7813 Medical Science Building, Ann Arbor, MI 48109-0622.

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Materials and Methods

Male guinea pigs (200 g) were placed on a control diet of Purina Guinea Pig Chow *ad libitum* (g/100 g diet; $n = 6$). The fish-oil-treated guinea pigs ($n = 6$) were fed the control diet supplemented with menhaden fish oil (16.4 g/100 g of basal diet, supplied by Dr. A. Bimbo, Zapata Haynie Co., Reedville, VA). During the same period an additional group of guinea pigs ($n = 6$) received standard chow supplemented with sesame oil (16 g/100 g of basal diet). After 4–6 weeks of either the control basal diet or the basal diet supplemented with fish oil or sesame oil, the animals were prepared for coronary perfusion experiments. Intraperitoneal heparin (300 units) was administered and the animals were sacrificed by a single blow to the head. The chest was opened and the heart was arrested *in situ* by a 4-ml injection of iced 16 m*M* KCl into the inferior vena cava. The heart was then rapidly excised and the ascending aorta was cannulated with polyethylene tubing. The left atrial appendage was carefully opened and a balloon catheter was placed in the left ventricle for the purpose of venting the left ventricle and to demonstrate persistence of the potassium-arrested state. The coronary arteries were perfused at a constant pressure of 70 cm of water with a salt solution containing the following (mmol/liter): NaCl (105.6), KCl (14.8), $MgSO_4 \cdot 7\ H_2O$ (1.1), KH_2PO_4 (1.2), $NaHCO_3$ (25.0), dextrose (11.0), $CaCl_2 \cdot 2\ H_2O$ (1.25), and $CaNa_2$ EDTA (0.03). The perfusion medium was maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2. Perfusate flow was measured with an electromagnetic flow probe located between the column of perfusate and the suspended heart. Flow was recorded via a meter recording on a Grass polygraph. The heart was allowed to equilibrate for 30 min before initiation of the experiment. Drugs were prepared daily and added directly to the perfusate at a known infusion rate in order to establish the amount of drug delivered.

Disruption of endothelial function was achieved by exposing the vessels to free radicals generated by electrolysis of the buffer solution. The perfusate was stimulated by platinum wire electrodes placed within the tubing cannulating the ascending aorta 2 cm from the suspended heart. The stimulus consisted of 9 V delivered as a train of square waves of 2-msec pulse duration at a frequency of 4 Hz for 2 min. In one series of experiments, in hearts from normal guinea pigs, it was observed that vasodilation responses to acetylcholine were not altered by the electrolysis procedure when 10^{-4} *M* ascorbate was added to the perfusate during the stimulation protocol. Scanning electron microscopy performed on similarly treated tail artery strips and mesenteric microvasculature of Sprague-Dawley rats revealed significant endothelial damage (cell membrane pitting) in tissues exposed to electrical stimulation without antioxidant protection (5).

Vessels were made to contract with prostaglandin (PG) $F_{2\alpha}$ (Sigma Chemical Co.) both before and after electrical stimulation of the buffer to achieve a flow of 3.5 ml/min (coronary vascular resistance of 15.3 mm Hg/ml/min). Endothelium-dependent dilation responses were elicited by acetylcholine (Michol; Cooper Vision Pharmaceuticals) and ATP (Sigma). Endothelium-independent dilations were produced using sodium nitroprusside (Nipride; Roche Laboratories). The drugs were dissolved and serially diluted in distilled water containing 16 m*M* KCl (identical to perfusate). Indomethacin (Sigma) was dissolved in ethanol and then added to the perfusate or the bathing medium. The final concentration of ethanol did not exceed 0.1%. Control experiments demonstrated that this concentration of ethanol had no effect on coronary tone.

Dilator responses in the coronary vasculature are presented as changes in coronary vascular resistance per gram of heart weight. All data are expressed as the mean ± SE. Statistical analysis of the data was performed by analysis of variance for repeated measures. In all cases, a *P*-value of less than 0.05 was considered to be statistically significant.

Results

After 4–6 weeks of feeding standard chow or standard chow supplemented with fish oil, experiments were performed to compare coronary resistance vessel dilation with acetylcholine, ATP, and sodium nitroprusside. Guinea pigs tolerated fish oil or sesame seed oil supplementation without difficulty. At the time of sacrifice, standard diet animals weighed 328 ± 16 g, fish-oil-fed animals weighed 340 ± 19 g (P = NS), and sesame-seed-oil-supplemented animals weighed 335 ± 16 g (P = NS). Utilizing a modified Langendorff preparation, the initial resting coronary flow was similar among control, fish oil groups, and sesame-oil-supplemented groups (7.0 ± 0.06 ml/min, 7.3 ± 0.08 ml/min, and 7.35 ± 0.05 ml/min, respectively). Vessels were constricted with $PGF_{2\alpha}$ (1.5×10^{-7}–1.2×10^{-6} *M*) to achieve a flow of 3.5 ml/min (15.3 mm Hg^{-1} ml^{-1} min^{-1}). There was no difference in constrictor response to $PGF_{2\alpha}$ among control, fish oil, and sesame oil animal groups.

Infusion of acetylcholine (5.5×10^{-9}–10^{-6} moles) caused an endothelium-dependent, dose-dependent dilation in the standard chow diet group. Dilation responses to acetylcholine were significantly attenuated in the fish-oil-supplemented animals (Fig. 1) as compared with the standard chow group. Infusion of ATP (10^{-10}–10^{-7} moles) resulted in a similar endothelium-dependent, dose-dependent dilation in the coronary resistance vessels in both the standard chow and fish-oil-supplemented groups (Fig. 1). Endothelium-inde-

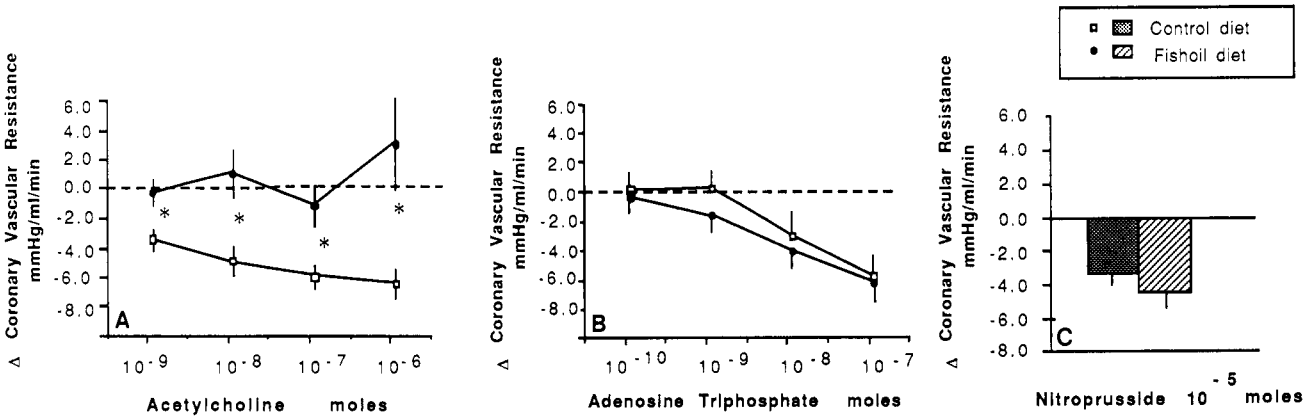

Figure 1. Effect of fish oil diet versus standard chow diet on coronary resistance vessel responsiveness. Perfusate flow responses to (A) acetylcholine, (B) ATP, and (C) nitroprusside in fish-oil-fed (n = 6; closed circles, shaded bar) versus standard chow guinea pigs (n = 6; open squares, hatched bar). Values are expressed as the mean ± SE. Asterisks denote statistical significance ($P < 0.05$) between control and fish-oil-treated guinea pigs.

pendent vascular responses to nitroprusside were similar among dietary groups (Fig. 1). Exposure of the coronary vascular bed to perfusate that had been electrically stimulated to disrupt endothelial cell function abolished dilatation responses to acetylcholine and ATP, but not to sodium nitroprusside in both animal groups (Fig. 2). Treatment with acetylcholine after this period of exposure to free radicals caused vasoconstriction in all preparations.

In order to determine whether the reduced responsiveness to acetylcholine (ACH) noted in the fish oil group was a manifestation of nonspecific effects of a high fat diet, an additional group of guinea pigs (n = 6) was fed standard chow supplemented with sesame oil (16 g/100 g). After 4–6 weeks of feeding, the sesame-oil-supplemented group was sacrificed and vascular reactivity was examined in an identical manner to fish-oil-supplemented and standard chow animal groups. Vascular responses to ACH, ATP, and nitroprusside were similar in control and sesame-seed-oil-supplemented animals (Fig. 3). Dilation responses to acetylcholine were significantly attenuated in the fish-oil-supplemented animals as compared with the sesame-oil-supplemented group (Fig. 4). Infusion of ATP (10^{-10}–10^{-7} moles) resulted in a similar endothelium-dependent, dose-dependent dilation in the coronary resistance vessels of sesame-seed-oil-supplemented animals. These dilator responses to ATP in the sesame-seed-oil-treated group were not statistically different from those in the control or fish-oil-treated animals. Dilator responses to nitroprusside in hearts from sesame-seed-oil-treated animals were not statistically different from values obtained for fish-oil-treated guinea pigs.

Eicosapentaenoic acid has been demonstrated to alter prostaglandin metabolism; therefore, the possible role of prostanoids as mediators of coronary vasodilatation to acetylcholine, ATP, and nitroprusside was evaluated by exposure of coronary microvessels to indomethacin. In isolated, perfused hearts from standard chow guinea pigs, indomethacin (14 μM) was added to the perfusate after measurement of control responses

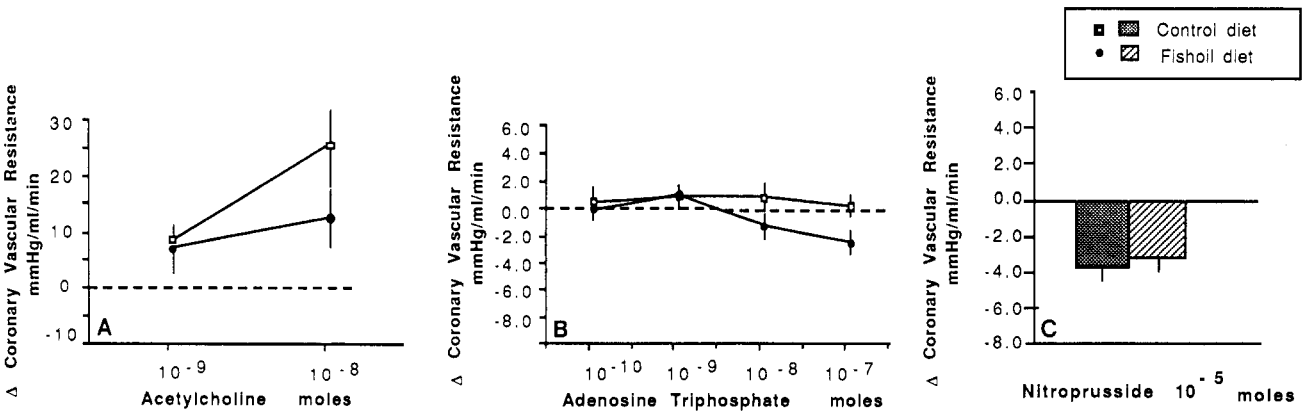

Figure 2. Effect of fish oil diet versus standard chow diet on coronary resistance vessel responsiveness after endothelial cell disruption. Perfusate flow responses to (A) acetylcholine, (B) ATP, and (C) nitroprusside in fish-oil-fed (n = 6; closed circles, shaded bar) versus standard chow guinea pigs (n = 6; open squares, hatched bar). Values are expressed as the mean ± SE.

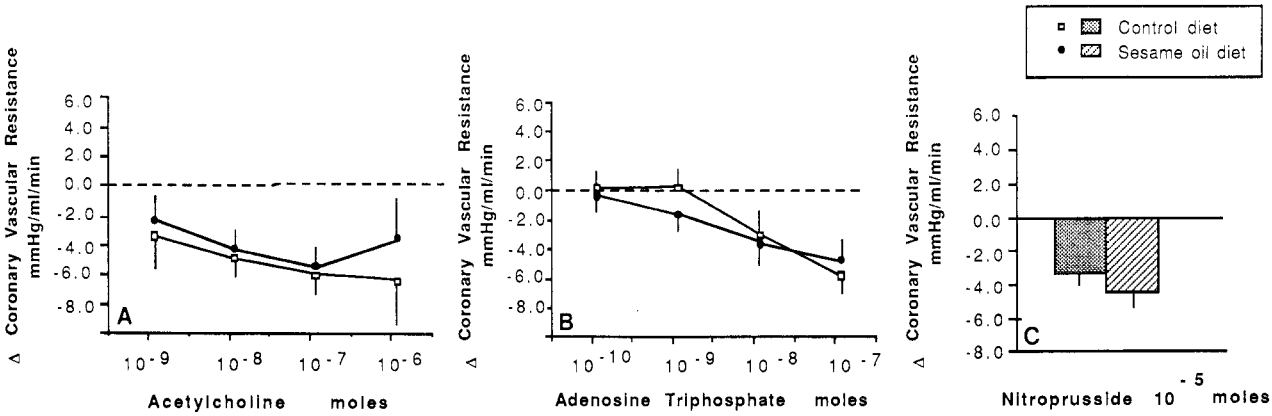

Figure 3. Effect of sesame seed oil diet versus standard chow diet on coronary resistance vessel responsiveness. Perfusate flow responses to (A) acetylcholine, (B) ATP, and (C) nitroprusside in sesame-seed-oil-fed (n = 6; closed circles, shaded bar) versus standard chow guinea pigs (n = 6; open squares, hatched bar). Values are expressed as the mean ± SE. Asterisks denote statistical significance ($P < 0.05$) between control and fish-oil-treated guinea pigs.

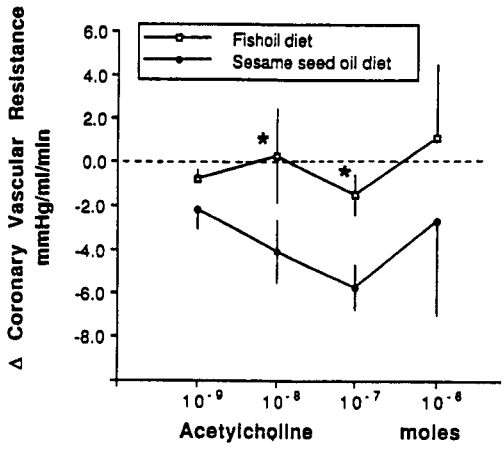

Figure 4. Effect of fish oil diet versus sesame seed oil diet on coronary resistance vessel responsiveness. Perfusate flow responses to acetylcholine in hearts from fish-oil-fed animals (n = 6; closed circles) were reduced compared with guinea pigs fed a diet supplemented with sesame seed oil (n = 6; open squares). Values are expressed as the mean ± SE. Asterisks denote statistical significance ($P < 0.05$) between groups.

to acetylcholine, ATP, and sodium nitroprusside. Indomethacin was infused for 60 min before and during the test period. Infusion with indomethacin did not affect the resting tone of the perfused coronary vessels or vasoconstrictor responses to $PGF_{2\alpha}$. Vasodilation responses to acetylcholine were attenuated in the presence of indomethacin, whereas vascular responses to ATP and nitroprusside were not affected (Fig. 5). In preparations that had been perfused with free radicals to damage the endothelium, an increase in coronary vascular resistance occurred in response to infusion of acetylcholine. This response was not affected by the presence of cyclo-oxygenase inhibition by indomethacin, which suggests that the increase in coronary tone was mediated by direct stimulation of the muscarinic receptors in the vascular smooth muscle wall. Furthermore, after endothelial disruption, vascular responses to ATP and nitroprusside were not affected by the presence of indomethacin (data not shown).

Discussion

The present experiments demonstrated that endothelium-dependent dilator responses to acetylcholine are impaired in the coronary resistance bed of fish-oil-supplemented guinea pigs as compared with standard chow, control animals. Our study suggests that prostaglandin-mediated vasodilation via PGI_2 plays a crucial role in modulating tone in the coronary vascular resistance bed of the guinea pig. Animals fed standard chow demonstrated dose-dependent coronary vasodilation to acetylcholine. This dilation response was significantly attenuated in the coronary bed of animals fed a fish oil diet, which suggests that PGI_3, a produce of EPA metabolism via cyclo-oxygenase, may have fewer vasodilatory properties in the coronary bed. In addition, vascular responses in animals fed sesame-seed-oil-supplemented chow paralleled those of standard chow animals, which suggests that differences in vascular responses between fish-oil-supplemented animals and standard chow animals were not the result of nonspecific vascular changes associated with a high fat diet. Sesame oil comprises n-6 (43% polyunsaturated, 14% monosaturated) fatty acids as opposed to the n-3 fatty acids found in fish oils (6, 7). In animals and humans, n-6 fatty acids cannot be converted to n-3 fatty acids and, therefore, vascular effects seen in the supplemented groups are related to the specific fatty acid supplementation. Furthermore, incubation with indomethacin in control animals resulted in a significant attenuation of endothelium-dependent relaxation to acetylcholine, whereas the coronary bed vasodilatory response to ATP and sodium nitroprusside was unaffected by indomethacin, which supports the role of

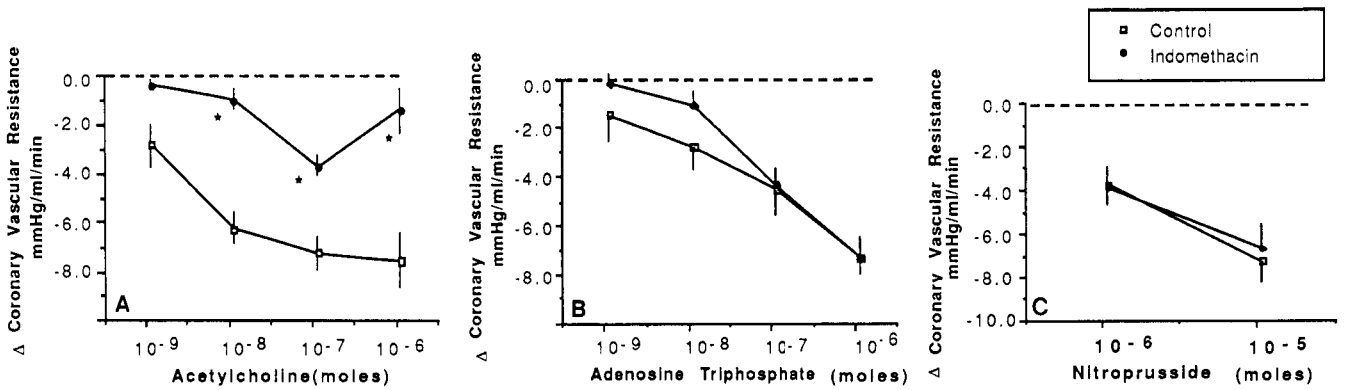

Figure 5. Effect of indomethacin in guinea pigs on coronary resistance vessel responsiveness. Perfusate flow responses to (A) acetylcholine, (B) ATP, and (C) nitroprusside in control group ($n = 6$; open squares) versus indomethacin-treated guinea pigs ($n = 6$; closed circles). Values are expressed as the mean ± SE. Asterisks denote statistical significance ($P < 0.05$) between control and fish-oil-treated guinea pigs.

prostanoid-mediated vasodilation in guinea pig coronary resistance vessels in response to acetylcholine.

Eicosanoids are biologically active lipid compounds derived from the carbon-20 essential fatty acid, arachidonate. n-3 (Omega) fatty acids inhibit the synthesis of arachidonate from linoleic acid, reduce storage site availability for arachidonic acid, and compete with arachidonic acid for cyclo-oxygenase (8). In addition, n-3 fatty acids are proposed to inhibit the formation of specific hydroperoxidases necessary to maintain the activity of cyclo-oxygenase (9). In the presence of n-3 omega fatty acids, there is a reduction in the production of PGI_2, a potent vasodilator, and platelet thromboxane A_2, a proaggretory substance. EPA is a poor substrate for cyclo-oxygenase as compared with arachidonate. Fischer and co-workers (10) have demonstrated the primary metabolites of EPA to be PGI_3 and thromboxane A_3. PGI_3 has only modest vasodilatory and antiaggretory properties, as compared with PGI_2 (11). Thromboxane A_3 has been shown to have few platelet proaggretory effects (12). In contrast, Whitaker *et al.* (13) found that incorporation of ^{14}C-EPA into rat aortic rings did not lead to increased production of PGI_3, which suggests that the biologic activity of EPA may be related to a decreased production of dienoic compounds and not the formation of trienoic prostaglandins.

Shimokawa and co-workers (14) recently reported augmented endothelium-dependent relaxation in porcine coronary artery rings to bradykinin, serotonin, ATP, and thrombin in cod-liver-oil-supplemented animals. Similar data have been reported in atherosclerotic monkeys fed diets high in omega-3 fatty acids (15). Augmented vascular responses to endothelium-dependent dilators have been hypothesized to be the result of enhanced release of endothelin-derived relaxing factor or alteration of endothelial cell membrane characteristics resulting in augmented vasomotion (16). Differences between our findings and the results of Shimokawa *et al.* (14) may be explained by differences in the vascular responses of the small resistance vessel studied in the perfused heart model compared with the large epicardial arteries. PGI_2 may play a more prominent role mediating vasodilation in smaller resistance vessels as compared with larger vessels. Vasodilation to acetylcholine in the coronary resistance bed was markedly attenuated by incubation with indomethacin, confirming a vasodilatory role for prostaglandin metabolites in the guinea pig coronary resistance bed. Recently, we published data demonstrating that indomethacin did not affect relaxation responses in the abdominal aorta, which suggests that different factors mediate vasomotion between resistance and large conduit arteries (17).

The mechanism by which EPA alters vascular reactivity is unknown. Our data would suggest that contractile proteins are not altered because there were no differences in contractile responses to $PGF_{2\alpha}$ or relaxation responses to sodium nitroprusside in the perfused coronary bed of the control and fish-oil-treated guinea pigs. It is possible that dietary supplementation with EPA may alter endothelial muscarinic receptor function in the coronary circulation, resulting in altered endothelin-derived relaxing factor release. EPA, however, does not appear to alter the smooth muscle muscarinic receptor in this model, because EPA supplementation did not alter the smooth muscle vasoconstrictor response to ACH after endothelial disruption. Furthermore, it is possible that the vascular responses after dietary supplementation to menhaden fish oil might be secondary to some substance other than EPA in the oil. Previous work from our laboratory in rats supplemented with menhaden fish oil demonstrated an increase in plasma n-3 fatty acids, which suggests that this change in plasma lipid composition may be important in subsequent changes in vascular responsiveness (4).

In our model, dietary manipulation of fatty acids alters vascular responsiveness in the coronary bed to ACH. Prostaglandins appear to play a prominent role

in mediating vasomotion in the guinea pig coronary bed. High dietary EPA may alter prostaglandin metabolism, which results in an attenuated relaxation to acetylcholine in the guinea pig coronary bed. These findings are significant in that they demonstrate a change in vascular responsiveness to endothelium-dependent vasoactive stimuli after dietary manipulation of fatty acids.

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