Effects of Dietary ω3 Fatty Acids on Vascular Contractility in Preanoxic and Postanoxic Aortic Rings

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Background. Vasomotor reactivity may contribute to the pathophysiology of ischemic injury. The atherosclerotic vessel may be particularly susceptible to vasoconstriction because of the damaged endothelial layer with resultant loss of vasodilatory factors. While dietary ω3 fatty acids have been proposed to protect against vascular occlusion, it is not clear to what extent this results from alterations in the function of platelets or from changes intrinsic to the blood vessel itself.

Methods and Results. The effects of dietary supplementation with fish oils on vascular contractility were examined in endothelialized and de-endothelialized aortic rings under pre- and postanoxic conditions. De-endothelialization was defined functionally by the loss of acetylcholine-induced vasodilation in norepinephrine-preconstricted aortic rings from rats fed normal rat chow. Three groups of rats were fed diets containing either 20% menhaden oil or 20% beef tallow, both supplemented with 3% corn oil or 23% corn oil for longer than 4 weeks. All animals received vitamin E. Under well-oxygenated conditions, de-endothelialized aortic rings from rats fed fish oil and corn oil contracted to similar extents with norepinephrine and vasopressin and less than rings from rats fed beef tallow. Endothelialized (intact) and de-endothelialized rings from rats fed fish oil relaxed more in response to acetylcholine than rings from rats fed beef tallow and corn oil. After anoxic exposure and reoxygenation, KCl-induced contraction of intact rings from rats fed fish oil and corn oil was similar and less than rings from rats fed beef tallow. Intact and de-endothelialized rings from rats fed fish oil relaxed more to acetylcholine than did rings from rats fed beef tallow and corn oil.

Conclusions. Under preanoxic or postanoxic conditions, rings from rats fed fish oil and corn oil contracted less than rings from rats fed beef tallow. The relaxation response to acetylcholine, however, was greater in rings from rats fed fish oil than from rats fed either corn oil or beef tallow. These vascular effects of fish oil feeding may result in increased blood flow to ischemic and reperfused tissues in vivo. (Circulation 1991;84:1393–1401)

There has been much interest in the possible contribution of vasomotor reactivity to the pathophysiology of ischemic injury. Vasos-
vasoactive agents such as catecholamines, angiotensin II, serotonin, and vasopressin, which might otherwise further compromise ischemic or postischemic blood flow. Incorporation of ω3 fatty acids may alter vascular responsiveness of subendothelial cells either directly or indirectly by altering the production of substances by the endothelial cell that affect endothelial cell function. For example, during ischemia and reperfusion, generation of reactive oxygen species by endothelial cells or by neutrophils that have infiltrated into the tissues can produce endothelial cell injury, inactivate endothelium-derived relaxing factor (EDRF), and impair endothelial cell regulation of vascular responses. ω3 fatty acids, by decreasing neutrophil infiltration, might result in fewer reactive oxygen species formed and more active EDRF present.

Previous studies have characterized the effects of ω3 incorporation into vascular tissue on hormone-induced vascular contractility under oxygenated conditions. Our study was designed to extend these findings by assessing the effects of dietary fish oils on vascular contractility in isolated endothelialized (intact) and de-endothelialized vessels under oxygenated conditions and subsequent to a period of anoxia and reoxygenation in vitro. We postulated that dietary fish oils may result in preservation of tissue perfusion in organs with ischemic injury by reducing postischemic vasoconstriction and promoting vascular relaxation. The effects of dietary fish oils on KCl- and hormone-induced contractility and relaxation were determined. Responses of aortic vascular rings from rats fed fish oil were compared with those observed in rings from rats fed either beef tallow or corn oil.

Methods

Animals

Forty-two male Sprague-Dawley rats, 4 weeks old, were randomly divided into three groups. Thirty-two rats were fed chow supplemented with 20%, by weight, of either menhaden oil (16 rats) or beef tallow (16 rats). Both groups received 3% corn oil and vitamin E (10 units per rat per day). The third group (10 rats) received chow supplemented with 23% corn oil and vitamin E (10 units per rat per day). Body weights were recorded weekly and dietary intake was adjusted accordingly. Experiments were performed on rats maintained on the diet for more than 4 weeks. Using an identical feeding protocol, we have found marked enrichment of heart and kidney phospholipids with ω3 fatty acids. While we did not directly measure the fatty acid composition of the rat aortas, others have shown that ω3 dietary fatty acids readily incorporate into the rat aorta.

Organ Chamber Experiments

Rats were anesthetized intraperitoneally with a thiobarbiturate, Inactin (Byk Gulden Konstanz, 100 mg/kg). The aorta was rapidly excised and immersed in cold 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; Na-EDTA, 0.016; and glucose, 11.1. In our preliminary studies, we found that the inclusion of Na-EDTA was necessary to prevent diminished responses to agonists over time. EDTA chelates any contaminating iron in the bathing solution, thus preventing production of iron-catalyzed reactive oxygen species. This may be the mechanism of EDTA’s protective effect. The aorta was carefully cleaned of all loose connective tissue. Rings 3 to 4 mm in length were excised. Half of the rings were deliberately denuded of much of the endothelium by inserting forceps into the vessel lumen and carefully rolling the vessel on Whatman filter paper moistened with cold Krebs-Ringer bicarbonate solution in a Petri dish on ice. Two aortic rings from each animal were used. One ring was used intact, and the other paired ring was de-endothelialized. The rings were mounted in a 25-ml water-jacketed organ chamber filled with incubation solution gassed with either 95% O2 and 5% CO2 or 95% N2 and 5% CO2 and maintained at 37°C with a Haake pump. Isotonic force measurements were obtained with a Grass force displacement transducer, model FT03D, attached to a Grass model 79D poly-graph DC amplifier (Grass Instrument Co., Quincy, Mass.), and recorded with a two-channel Kipp Zonen model BD 41 recorder. In these studies, “de-endothelialization” is defined functionally by the absence of a vasodilating response to acetylcholine in norepinephrine- or PGF2α-preconstricted rings taken from animals fed normal rat chow (23.4% protein, 4.5% animal fat, Farmers Exchange, Framingham, Mass.) or beef tallow diets. De-endothelialized rings from rats fed normal rat chow or beef tallow lost the acetylcholine-induced relaxation response but maintained the contractile response to KCl, attesting to the preserved functional integrity of the smooth muscle.

Protocol

Preliminary studies revealed that aortic rings placed under 1.5 g tension generated a maximal contractile response after KCl depolarization. Thereafter, all rings were equilibrated for 45 minutes under 1.5 g tension before experiments were started. In sequence, rings were incubated with: increasing doses of KCl (5–20 mM); norepinephrine (10−9–10−7 M); vasopressin (10−9–10−7 M); norepinephrine (5×10−9 M), followed by acetylcholine (10−9–10−7 M); and KCl (5–20 mM). The incubation solution was then replaced with an identical solution pre-equilibrated with 95% N2 and 5% CO2. The rings were exposed for 35 minutes to this solution, which was constantly aerated with 95% N2 and 5% CO2. After 35 minutes, the solution was changed back to a well-oxygenated buffer gassed with 95% O2 and 5% CO2. After a 35-minute reoxygenation period, the rings were exposed to KCl, norepinephrine, norepinephrine (precontraction) followed by acetylcholine, and KCl at concentrations identical to those used above. After each KCl or hormonal response,
the rings were washed with fresh Krebs-Ringer bicarbonate solution. During the course of the experiments on a single ring, the contractile response to KCl was repeated four times to ensure stability of response over time. Stability was also established in initial studies where the response to norepinephrine was reexamined after prior exposure of the ring to norepinephrine, vasopressin, acetylcholine, and washout of the drugs. The response to the second exposure to norepinephrine was 117% of that observed with the initial norepinephrine addition.

Reagents

All chemicals and hormones were of highest purity available from Sigma (St. Louis, Mo.). Inactin was obtained from Promonta (Hamburg, FRG).

Data Analysis

Results are expressed as mean±SEM, with n referring to the number of animals used in the experiments. All vasoconstrictive hormonal responses are normalized to the mean response to 10 mM KCl alone, obtained before and after the hormonal exposure under either oxygenated or postanoxic conditions. To determine the relaxation response to acetylcholine, rings were first maximally contracted with norepinephrine. The response to acetylcholine was determined as the reduction in tension factored for the initial tension and is reported as relaxation. Statistical evaluation of the data was performed by a stepwise multiple regression analysis using a BMDP program (BMDP Statistical Software, Inc., Los Angeles, 1988). Contractile responses in aortic rings obtained from rats fed fish oil (FO) and beef tallow (BT) (hereafter referred to as FO and BT rings, respectively), under pre- and postanoxic, intact and de-endothelialized conditions, were collectively analyzed by a stepwise multiple regression. A change in the slope of the regression curve indicates a change in the responsiveness of the aortas to KCl or hormone. For example, a more positive slope indicates greater responsiveness to a vasoconstrictor hormone. In contrast, a change in intercept of the regression curve indicates an intrinsic change in the responsiveness of the vascular ring due to factors (e.g., Ca2+, prostanoids, etc.) that are distinct from the hormone under evaluation.

Grouping factors, such as diet, anoxic conditions, or de-endothelialization, were embodied as binary variables in the multiple regression analyses according to the method of dummy variables.10 The interactions of these grouping variables with each other and with dose were included in the stepwise regression analyses as simple crossmultiples of the variables. All two-way, three-way, and the single four-way interaction variables were used. Regressions for norepinephrine and vasopressin were performed on log-transformed constrictor responses versus log dose of hormone to linearize the plots and to obtain more nearly Gaussian residuals. For these log–log analyses, "intercepts" refer to the displacements of log constrictor response from the log of unit constrictor response (0) at unit dose of hormone [log (unit dose)=0]. Such intercepts are displacements of the entire log-of-constrictor–response curve upward or downward across the entire log–dose axis (i.e., without rotation of the curve). Comparisons among BT, corn oil (CO), and FO diets were performed as grouped regression analyses with analysis of variance on the estimated group slopes and intercepts to detect differences in dose response among the three diet groups under specific selected conditions (e.g., anoxic, de-endothelialized). These comparisons were performed with the program BMDP1R. Values for differences in slopes and intercepts were considered to be statistically significant at p<0.05.

Results

Baseline Data

At the initiation of the study, there were no differences in body weight of rats randomized to the FO, BT, or CO groups, respectively [98.0±3.1 g (n=16) versus 103±2.7 g (n=16) versus 98.8±4.1 g (n=10)]. Mean body weight increased by over 100% after 4 weeks of feeding for rats in all three treatment groups. After 9 weeks of feeding, mean body weights for rats fed FO, BT, and CO were 262±3.5 g (n=9), 258±4.3 g (n=11), and 321±3.4 g (n=7), respectively. Rats fed FO and BT had equivalent body weights, which, however, were significantly lower than the weights of rats fed CO (p<0.01). Using an identical feeding protocol, we have found no differences in systolic or diastolic blood pressures among the groups.6

Contraction and Relaxation in Aortic Rings Derived From Rats Fed Fish Oil and Rats Fed Beef Tallow

The contraction and relaxation data for oxygenated and anoxic aortic rings were analyzed by a stepwise multiple regression. The regression equations for the KCl or hormonal responses are reported in the figure legends. For KCl contraction, there were no differences in slopes preanoxia (Figure 1A). In postanoxic rings the slope of the dose–response curve in BT aortas exceeded the slope of the dose–response curve in FO aortas by +2 mg tension/mM KCl (p<0.025, Figure 1B). Anoxia and de-endothelialization were interacting variables and together reduced the slope of the KCl dose–response curve by 4.7 mg tension/mM irrespective of the dietary group (p<0.005, Figure 1B, right panel).

All responses to vasoconstrictive hormones were normalized to the contractile response of the ring to 10−2 M KCl, and the log of the ratio was analyzed. For both norepinephrine and vasopressin, there was a significant displacement of the dose–response curve in de-endothelialized BT aortas compared with FO aortas [+0.24 for preanoxic norepinephrine, p<0.005 (Figures 2A and 2B); +0.5 for vasopressin, p<0.005 (Figure 3)]. Because the slopes were not different between BT and FO responses, the vasoconstriction at a given dose of hormone was greater in the BT group.
The slope of the dose–response curve relating vascular relaxation to acetylcholine concentration is greater in intact than in de-endothelialized aortas in both FO and BT groups, whether preanoxic or postanoxic (1.23, \( p < 0.005 \), Figures 4A and 4B). The acetylcholine response is greater in FO aortas whether intact or de-endothelialized (+2.96, \( p < 0.005 \)), oxygenated or postanoxic. While the slopes of the dose–response curves are not different statistically when preanoxic and postanoxic aortas are compared, the percent relaxation in the postanoxic aortas is less than that of preanoxic aortas.

Removing endothelial cells had an effect on vascular reactivity to norepinephrine and vasopressin only in the BT aortas, which constricted to a greater degree at each concentration of agonist. This effect was apparent when all the data from Figures 2A and 2B were analyzed together as indicated in the legend to these figures. By contrast, removing endothelial cells resulted in a decreased relaxation response to acetylcholine in both BT and FO aortas, although FO rings remained more responsive than BT rings. Anoxia resulted in a reduction in the relaxation response to each dose of acetylcholine whether rings were BT or FO or whether intact or de-endothelialized.

When compared with rings from rats fed FO, rings from rats fed BT had an increased postanoxic dose response (greater slope) to KCl, and an increased vasoconstrictive response to norepinephrine and to vasopressin (preanoxic) when endothelial cells were removed. Furthermore, BT rings relaxed less to acetylcholine, regardless of the state of oxygenation or endothelialization of the vessel.

Observations on vessels from rats fed corn oil (CO rings) were made in parallel with those from animals fed BT or FO (Tables 1 and 2). When CO rings were compared with FO and BT rings (Tables 1 and 2), the following results were seen. 1) Under preanoxic de-endothelialized conditions, the contractile response to norepinephrine and vasopressin was equivalent in CO and FO rings (Table 1). 2) Under preanoxic conditions, intact CO rings relaxed to acetylcholine like BT rings and significantly less than FO rings (Table 1). 3) The relaxation response of de-endothelialized CO rings was equal to that of FO rings.

**FIGURE 1.** Graphs show KCl-induced contraction, expressed as milligrams of tension, as a function of KCl concentration in intact and de-endothelialized aortic rings under oxygenated (panel A) and anoxic (panel B) conditions. The regression equation is: KCl constriction = \(-100.5 + (22.6 \pm 1.8) - [(3.7 \pm 1.8) + (4.7 \pm 1.8) \times \text{de-endo}] - (5.7 \pm 1.8) \times \text{beef} \times \text{anoxia} \times \text{dose}\). A "no" answer to "?" equals 0; a "yes" answer to "?" equals 1. In the regression equation, \( \pm \) number equals standard error of regression coefficient. Dose is in millimolar units. Each data point represents mean \( \pm \)SEM of 11–17 rings, each from a different animal. Endo, intact endothelium; de-endo, de-endothelialized; Fish, aorta from rats fed fish oil; Beef, aorta from rats fed beef tallow. \( p < 0.05 \) for anoxia \times \text{dose} \times \text{effect}; \( p < 0.01 \) for de-endo \times \text{anoxia} \times \text{dose} \times \text{effect}; and \( p < 0.005 \) for beef \times \text{anoxia} \times \text{dose} \times \text{effect}. \( p < 0.0001 \) for pure dose effect.
rings and greater than that of BT rings (Table 1). 4) Postanoxia, the KCl-induced contractile response of intact CO rings was intermediate between that of FO and BT rings (Table 2). 5) Postanoxia, norepinephrine produced less contraction in intact CO rings than in BT rings, as indicated by a difference in slope (see legend, Table 2). 6) Postanoxia, both intact and de-endothelialized CO rings relaxed less to acetylcholine than did FO rings (Table 2). Thus, CO rings contracted in response to norepinephrine and vasopressin in a manner similar to FO rings and less than BT rings. CO rings, however, relaxed to acetylcholine in a manner similar to BT rings and less than FO rings.

Discussion

Vasospasm is well recognized as a consequence of endothelial injury in a variety of clinical conditions, including atherosclerosis and ischemia. Our study used intact and de-endothelialized aortic rings under preanoxic and postanoxic conditions to evaluate the effects of diets enriched in ω3 fatty acids on vascular reactivity. The major findings in this study are 1) a decrease in the preanoxic contractile response to norepinephrine and vasopressin in the de-endothelialized FO rings compared with BT rings, and 2) greater relaxation in response to acetylcholine in both intact and de-endothelialized FO rings compared with BT rings under both pre- and postanoxic conditions.

The first finding, the decrease in contractile response to norepinephrine and vasopressin in the preanoxic de-endothelialized FO rings, suggests a possible direct effect of FO on the contractility of vascular smooth muscle. The observation that postanoxic intact FO rings contracted less than BT rings to KCl provides further evidence for a direct action of FO on the arterial smooth muscle cells because increased extracellular potassium causes contraction by directly depolarizing muscle cells. FO also resulted in enhanced acetylcholine-induced vasodila-
tion in de-endothelialized rings. Yanagisawa and Lefer\(^{19}\) found, in the isolated perfused coronary artery from cats, that eicosapentaenoic acid exerted a vasodilator effect that was endothelium-independent and inhibited by a lipoxygenase antagonist.

Because vasoconstrictive agents are also derived from the endothelial cells (e.g., platelet-derived growth factor [PDGF]\(^{20}\) and endothelin\(^{21,22}\)), the augmented relaxation observed in FO endothelialized rings could be a result of inhibition by FO of release of a tonic vasoconstrictive agent or potentiation of release of a vasodilatory substance derived from endothelial cells. Indeed, Fox and DiCorleto\(^{23}\) have demonstrated that endothelial cells in culture, after incubation for 3 days with an emulsion of FO, develop an 85% inhibition in production of a PDGF-like factor. It has been demonstrated both in human coronary arteries\(^{24}\) and in rabbit aortas\(^{25}\) that vasodilation in response to acetylcholine is endothelium dependent. The increased relaxation to acetylcholine of rings from rats fed FO also is consistent with an enhanced release of endothelium-derived relaxing factor(s) (EDRF), and possibly an enhanced relaxation of vascular smooth muscle to the factor(s), as has been previously reported by Shimokawa and Vanhoutte.\(^{9,26}\) Our data in preanoxic and postanoxic de-endothelialized BT rings reveal a failure to relax with acetylcholine, consistent with a relaxation response to acetylcholine mediated by endothelium-derived factor(s). De Nucci et al\(^{27}\) have demonstrated that the release of prostacyclin and EDRF are coupled. In humans fed FO there is enhanced basal and arachidonate-stimulated prostacyclin production in saphenous vein and aortic and atrial tissues.\(^{28}\) Since FO feeding enhances prostacyclin biosynthesis,\(^{28,29}\) FO may augment EDRF release as well. We cannot discern from our study, however, whether FO may inhibit an endothelium-derived vasoconstrictive factor or augment the release or response to a relaxation factor.

FIGURE 4. Graphs show acetylcholine (ACH)-induced relaxation after prior contraction with norepinephrine (5×10\(^{-9}\) M) expressed as percent relaxation. Intact and de-endothelialized aortic rings were studied under oxygenated (panel A) and anoxic (panel B) conditions. ACH relaxation=5−[(0.015±0.007)×KCl constriction]−[(4.1±0.35)−(2.96±0.27)×beef]−(1.23±0.28)×de-endo?]×dose. Dose is in nanomolar units. Each data point represents mean±SEM of six to 14 rings, each from a different animal. Endo, intact endothelium; de-endo, de-endothelialized; Fish, aorta from rats fed fish oil; Beef, aorta from rats fed beef tallow. p<0.0005 for independent effects of beef diet and de-endothelialization, p<0.025 for anoxia; p<0.05 for KCl constrictor response. p<0.0001 for the beef?×dose interaction.
FO has recently been reported to lower blood pressure significantly in hypertensive subjects.30,31 Experimentally, Ziemlanski et al.32 found that diets high in FO were more antihypertensive than diets high in sunflower seed oil, which provides larger amounts of linoleic acid. Both dietary groups had significant lowering of blood pressure compared with a third group fed BT. Our vascular effects of diets high in FO, CO (linoleic acid-enriched), and BT would predict the relative effectiveness of these diets to alter blood pressure. FO decreased vasoconstriction and enhanced relaxation in the rat aorta and might be predicted to have the most potent antihypertensive effect. CO diets, resulting in reduced vasoconstriction but not having as much of an effect on vascular relaxation, would produce an intermediate antihypertensive effect.

During reperfusion after ischemia, intense vasoconstriction further impairs blood flow.33,34 This vasoconstriction postanoxia may be related to endothelial release of constricting factors,35-38 which elevate cytosolic Ca++ ([Ca^2+]) and activate myosin light-chain kinase. Vanhoutte and associates36,39,40 have shown that anoxia produces endothelium-dependent contractions by a mediator that is not an arachidonic acid metabolite (possibly endothelin). These anoxic contractions are inhibited by calcium antagonists. Increased [Ca^2+], also can activate phospholipase A2 (PLA2), resulting in release of free arachidonate from phospholipids and generation of lipoxygenase products, which can activate K+ channels41 and depolarize the cellular membrane. PLA2, which is activated with ischemia,42 would release EPA and DHA (docosahexaenoic acid) when membranes are enriched with FO. Cells enriched in EPA and DHA may generate fewer 5-lipoxygenase products when stimulated.43 A reduction in lipoxygenase products would result in less membrane depolarization by K+.
channels, less Ca\(^{2+}\) influx, and less contraction of vascular smooth muscle.

In addition to enhanced vascular contraction, endothelium-dependent relaxations to acetylcholine are diminished with anoxia. Anoxia produced a reduction in the acetylcholine relaxation response, which is consistent with the data reported by De Mey and Vanhoutte. Our data demonstrate that FO feeding enhances the relaxation observed with acetylcholine in aortic rings. It is interesting to note that relaxation with acetylcholine was seen in rings from rats fed FO, even though de-endothelialized. This may result from an enhanced response to acetylcholine by the few remaining endothelial cells in the preparation. The absence of relaxation in the identically treated BT rings indicates that most of the functional endothelium must have been removed. It is possible that the few remaining endothelial cells release more vaso dilatory factors or that the vascular smooth muscle is more susceptible to these vasodilating substances in the 453 group.

Thus, de-endothelialized vascular rings from rats fed FO contracted less to vasoconstrictive agents and relaxed more to acetylcholine than did rings from rats fed BT. Postanoxic rings from rats fed FO exhibit a favorable balance in vascular reactivity with less contraction and enhanced relaxation. These effects may promote increased blood flow to ischemic tissues in vivo during the reperfusion period. If coronary arteries of human subjects fed FO respond to vasoactive agents as rat aorta does, vessels partially denuded of endothelium or exposed to anoxia with endothelium intact should be less vasospastic in vivo, and FO supplementation may prove therapeutically beneficial.

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