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Fish oil-enriched diet protects against ischemia by improving angiogenesis, endothelial progenitor cell function and postnatal neovascularization

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ABSTRACT

Background: Fish oil consumption has been associated with a reduced incidence of cardiovascular diseases. However, the precise mechanisms involved are not completely understood. Here we tested the hypothesis that a fish oil-enriched diet improves neovascularization in response to ischemia.
Methods and results: C57Bl/6 mice were fed a diet containing either 20% fish oil, rich in long-chain *n*-3 polyunsaturated fatty acids (PUFAs), or 20% corn oil, rich in *n*-6 PUFAs. After 4 weeks, hindlimb ischemia was surgically induced by femoral artery removal. We found that blood flow recovery was significantly improved in mice fed a fish oil diet compared to those fed a corn oil diet (Doppler flow ratio (DFR) at day 21 after surgery 78 ± 5 vs. 56 ± 4; $p < 0.01$). Clinically, this was associated with a significant reduction of ambulatory impairment and ischemic damage in the fish oil group. At the microvascular level, capillary density was significantly improved in ischemic muscles of mice fed a fish oil diet. This correlated with increased expression of VEGF and eNOS in ischemic muscles, and higher NO concentration in the plasma. Endothelial progenitor cells (EPCs) have been shown to have an important role for postnatal neovascularization. We found that the number of EPCs was significantly increased in mice fed a fish oil diet. In addition, oxidative stress levels (DCF-DA, DHE) were reduced in EPCs isolated from mice exposed to fish oil, and this was associated with improved EPC functional activities (migration and integration into tubules). In vitro, treatment of EPCs with fish oil resulted in a significant increase of cellular migration. In addition, the secretion of angiogenic growth factors including IL6 and leptin was significantly increased in EPCs exposed to fish oil.
Conclusion: Fish oil-enriched diet is associated with improved neovascularization in response to ischemia. Potential mechanisms involved include activation of VEGF/NO pathway in ischemic tissues together with an increase in the number and the functional activities of EPCs.

1. Introduction

Fish oil is a major source of *n*-3-polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Several epidemiologic studies have shown a reduced risk of cardiovascular events among persons who consume fish regularly or who take supplements containing *n*-3 fatty acids [1,2]. These data provided the stimulus for the elaboration of clinical trials evaluating the effect of such supplements on cardiovascular events and death. For example, the GISSI-P study was a large randomized trial demonstrating that in addition to medical therapy, daily supplementation with *n*-3 fatty acids could reduce cardiac and all-cause mortality in patients presenting with a recent myocardial infarction [3]. However, the exact mechanisms by which fish oil and *n*-3 PUFAs could have a favorable impact on cardiovascular events are not completely understood. Although fish oil has been shown to reduce atherosclerosis in some experimental animal models [4–6], other researchers reported no effect [7] or even negative effects [8]. Several alternative mechanisms have been proposed to explain the beneficial cardiovascular effects of fish oil, taking into account the pleiotropic actions of *n*-3 PUFAs on important physiological processes such as inflammation and oxidative stress [1]. However, one possibility that has not been investigated so far is that *n*-3 PUFAs could improve the ability of the organism to develop new blood vessels in response to ischemia (i.e. neovascularization).

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Neovascularization constitutes an important adaptive response to vascular occlusive diseases [9]. Postnatal neovascularization necessitates the activation, migration and proliferation of mature endothelial cells (angiogenesis) [10]. Vascular endothelial growth factor (VEGF), an endothelial cell specific mitogen, has been shown to be a critical limiting factor for the induction of angiogenesis [11]. The importance of nitric oxide (NO) for endothelial cell migration and VEGF-induced angiogenesis was also recently demonstrated [12,13]. However, it has been proposed that postnatal neovascularization relies not exclusively on sprouting of pre-existing vessels, but also involves the contribution of bone marrow-derived circulating endothelial progenitor cells (EPCs) [14]. Evidence suggests that these cells are mobilized from the bone marrow into the peripheral blood, migrate to sites of ischemia where they can promote neovascularization [15].

In young patients and in animal models with young and healthy animals, the neovascularization process is very effective so that blood flow restoration after ischemia is almost complete. Patients with atherosclerotic diseases however are usually of advanced age and also exhibit various risk factors that have been associated with impaired neovascularization such as diabetes, hypercholesterolemia, hypertension, and cigarette smoking [16–20]. In these patients, improving neovascularization could constitute a novel therapeutic strategy to restore blood flow and reduce tissue ischemia. In the present study, we tested the hypothesis that a fish oil-enriched diet could enhance neovascularization in response to ischemia. We also investigated potential mechanisms that could be involved, including the role of EPCs.

2. Methods

2.1. Experimental animals and diets

The protocol was approved by the Comité Institutionnel de Protection des Animaux (CIPA) of the Centre Hospitalier de l'Université de Montréal (CHUM). 6–8 weeks old female C57Bl/6 mice were purchased from Charles River (St. Constant, Canada). Mice were maintained in 12 h light–dark cycle and fed ad libitum. Two diets were compared: a 'Mediterranean-type' diet containing 20% fish oil, rich in long-chain *n*-3 polyunsaturated fatty acids (PUFAs), and a 'Western-type' diet containing 20% corn oil, rich in *n*-6 PUFAs (Table 1). The diets were started one month prior to surgery and maintained during the whole study. The mouse diets were based on the AIN-93M formulation and supplied by Harlan Teklad. The corn oil diet consisted of the following ingredients per kg: 175 g casein, 2.2 g L-cystine, 246.66 g corn starch, 155 g maltodextrin, 100 g sucrose, 210 g corn oil, 50 g cellulose, 43 g mineral mix (AIN-93M-MX), 2.5 g calcium phosphate (dibasic), 12.5 g vitamin mix (AIN-93-VX), 3 g choline bitartrate, 0.04 g TBHQ (antioxidant). For the fish oil diet, 200 g/kg of corn oil was replaced with 200 g/kg of menhaden fish oil. To prevent oxidation, the diets were vacuum packed and changed twice a week. The fatty acid composition of the dietary oils is described in Table 1. Blood cholesterol and triglyceride levels were measured at day 21 after surgery.

Table 1
Fatty acid composition of dietary oils.

	Corn oil	Fish oil
g/100 g Total fatty acids		
12:0 Lauric	ND	ND
14:0 Myristic	ND	8.4
16:0 Palmitic	12.2	15.2
16:1 Palmitoleic	ND	11.6
18:0 Stearic	2.2	2.7
18:1 (*n*-9) Oleic	27.5	9.5
18:2 (*n*-6) Linoleic	57.0	1.8
18:3 (*n*-3) Linolenic	1.0	1.8
20:5 (*n*-3) EPA	ND	16.0
22:5 (*n*-3) DPA	ND	3.9
22:6 (*n*-3) DHA	ND	10.8
P:M:S	58:27.5:14.4	41.9:22.7:28.1

P:M:S, PUFA:MUFA:SFA; ND, not detectable.

2.2. Murine ischemic hindlimb model and monitoring of blood flow

Unilateral hindlimb ischemia was surgically induced in mice as previously described [20]. Briefly, the animals were anesthetized with 2% isoflurane, after which an incision was performed in the skin overlying the middle portion of the left hindlimb. After ligation of the proximal end of the femoral artery, the distal portion of the saphenous artery was ligated, and the artery and all side branches were dissected free and excised. The skin was closed with a prolene monofilament (6-0) (Johnson & Johnson, ON, Canada). Hindlimb blood flow was monitored with a Laser Doppler perfusion imager (LDPI) system (Moor Instruments Ltd., Axminster, UK). The perfusion signal was split into six different intervals, each displayed in a separate color. Low and/or no perfusion were displayed in dark blue, whereas the highest perfusion interval was displayed in red. Color images were recorded and analysis was performed by calculating the average perfusion of the ischemic and nonischemic hindlimb. To account for variables such as ambient light and temperature, the results are expressed as the ratio of perfusion in the left (ischemic) vs. right (normal) hindlimb. Ambulatory impairment was evaluated using a scale from 1 (normal walking) to 4 (walking with the leg dragging behind). Evaluation of the ischemic damage of the leg and foot was evaluated using a scale from 0 (no necrosis) to 4 (amputation). The mice were killed at predetermined time points after surgery with an overdose of sodium pentobarbital.

2.3. Tissue preparation and immunochemistry

For immunohistochemistry, whole ischemic hindlimbs were immediately fixed in tissue-fix overnight. After bones had been carefully removed, 3-μm thick tissue transverse sections of the hindlimbs were cut at the level of the gastrocnemius muscle and paraffin-embedded so that the whole leg could be analyzed on each section. Identification of endothelial cells was performed by immunostaining for platelet endothelial cells adhesion molecule-1 (PECAM-1 or CD31) with a rat monoclonal antibody directed against mouse CD31 (Pharmigen, San Diego, CA).

2.4. Western blot analysis

For total protein extraction, isolated muscles from whole hindlimbs were rinsed in PBS to remove excess blood, snap-frozen in liquid nitrogen, and stored at −80 °C until use. Whole-cell protein extracts were obtained after homogenization of muscles from whole hindlimbs in ice-cold lysis buffer contaning 50 mM HEPES pH 7.6, 150 mM NaCl, 1 mM EDTA, 25 mM b-glycerophosphate, 1 mM sodium orthovanadate, 1 mM NaF, 0.1% tween 20, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mg/mL leupeptin, and 1 mg/mL aprotinin. 60 mg of protein per sample was separated in reducing 12% polyacrylamide gel and electroblotted on nitrocellulose membranes. The following antibodies were used: 1:200 VEGF (Santa Cruz Biotechnology), 1:1000 peNOS (Cell Signaling Technology, Boston, MA), 1:1000 eNOS (Santa Cruz Biotechnology, Santa Cruz, CA) and 1:500 alpha-tubulin (Santa Cruz Biotechnology). Protein expression was quantified by high-resolution optical densitometry (Alpha Imager 2000; Packard Instruments, Perkin Elmer, Boston, MA). Results are expressed as density values normalized to alpha-tubulin.