Effects of n-3 fatty acids and acute exercise on endothelium-dependent vasorelaxation in healthy rat aorta

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The purpose of this study was to determine whether n-3 PUFA result in an effect on endothelial function that is in addition to that of acute exercise. For 4 weeks, male Sprague–Dawley rats were subjected to a diet based on n-3 PUFA or a standard diet. In each diet group, ten rats were submitted to an acute treadmill exercise while the remaining ten acted as sedentary controls. The running speed was progressively increased until the animals were exhausted. Endothelial function was then assessed by measuring isometric tension in rings of the thoracic aorta. In vessels precontracted with M-phenylephrine, responses to acetylcholine (ACh) were significantly improved following acute exercise in all diet groups. When PUFA supplementation was compared to the standard diet no significant difference was found in response to ACh, either at rest or after an acute exercise. Pretreatment of rings with N-nitro-L-arginine methyl ester (50 μM) inhibited the ACh-mediated vasorelaxation in all groups. Response to 10 μM nifedipine, an L-type Ca2+ channel antagonist, was similarly enhanced after acute exercise in both standard and PUFA diets. Furthermore, response to 0.01 μM nifedipine was significantly higher after acute exercise only in the PUFA diet. In conclusion, in our ‘healthy’ rat model with ‘normal’ baseline endothelial function, acute exercise improves response to ACh while PUFA supplementation alone or in combination with acute exercise has no effect on endothelium-dependent vasorelaxation. However, PUFA may potentiate the acute exercise effect on smooth muscle cell relaxation via L-type Ca2+ channel modifications.

Endothelium: Acute exercise: Fish oil: Acetylcholine: Nifedipine

Endothelium plays an important role in the regulation of vascular tone by releasing relaxing factors such as nitric oxide, endothelium-derived hyperpolarizing factor and prostacyclin(1). It also plays a protective role against development of atherosclerosis(2). Epidemiological studies suggest that n-3 PUFA have a beneficial effect on the incidence of atherosclerosis and its complications(3,4). Animal studies have demonstrated that ingestion of fish oil, a rich source in n-3 PUFA EPA and DHA, decreases platelet aggregation, reduces serum TAG levels and probably slows the impairment of endothelial function in atherosclerotic animals(5–9). Incorporation of PUFA into vascular membranes and the resulting changes in membrane fluidity may inhibit the expression of adhesion molecules(6) and proliferation of smooth muscle cells. It may also affect the activity of several ion channels, including voltage-dependent L-type Ca2+ channels(7). Although DHA seems to have a favourable effect on endothelium-derived mediators(9), both EPA and DHA have different effects on endothelium-dependent vasoreactivity depending on the mode of administration (diet v. in vitro incubation) and pathology. Moreover, physical exercise exerts beneficial effects on the release of vasoactive substances or on the endothelium-dependent control of vascular tone(8–12). Recently, Delarue et al. (13) showed that supplementation of the standard diet with fish oil reduced the stimulation of both rates of plasma glucose disappearance and hepatic glucose production during exercise in untrained males. The current study uses the same diet and a single exhaustive treadmill protocol on endothelium-dependent vasorelaxation in rats. The purpose of this study is to determine whether a diet enriched with n-3 PUFA increases the benefit of acute exercise on endothelial function evaluated in isolated rat aortic rings.

Methods

The present study was conducted in conformity with the procedures described in the Guide for the Care and Use of Laboratory Animals (14), and the procedures were in accordance with institutional guidelines.

Abbreviations: Ach, acetylcholine; EC50, half-maximal effective dose; Emax, maximal relaxation; l-NAME, N-nitro-L-arginine methyl ester; PE, phenylephrine.
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Forty male Sprague–Dawley rats, initially weighing 70–80 g, were housed individually in an environmentally controlled room (temperature 21 ± 1°C, 12 h light–dark cycle). Rats were randomly divided into two groups and placed on either a normolipidic diet containing n-6 PUFA (standard diet, n 20) or n-3 PUFA (n-3 PUFA diet, n 20) for 4 weeks. Rats were offered ad libitum food and water. Diets were manufactured by Atelier de Préparation Aliments Exper-imentaux (A.P.A.E., INRA, Jouy-en-Josas, France). The fatty acid content of each diet is described in Table 1. The standard diet contained 6.6 % (w/w) from fat as peanut–rape oil, 22 % from casein, 43 % from starch and 28.4 % from sucrose. The n-3 PUFA diet contained 4.4 % (w/w) from fat as peanut–rape oil, 2.2 % from fish oil, 22 % from casein, 43 % from starch and 28.4 % from sucrose.

We chose this dose of n-3 PUFA and this length of diet because we previously demonstrated in our laboratory that it was correlated to an enrichment of n-3 PUFA content in phospholipids and neutral lipids in rat tissues. Food intake was checked daily and was similar for each group of rats (n-3 PUFA = 450.6 (SEM 32.17) kJ/d, standard = 428.98 (SEM 15.18) kJ/d). At the time of killing, no significant difference was observed in weight of rats fed with n-3 PUFA (259.1 (SEM 9.22) g) or the standard diet (263.15 (SEM 9.22) g).

In each group, ten rats acted as sedentary controls. The other ten were subjected to acute exercise. All rats were familiarized with the treadmill at 5 m/min (15° incline), 5 min/d for 3 d. At the end of this period, only the exercise-group rats were subjected to acute exercise as follows: the running speed started at 10 m/min and was progressively increased by 3 m/min increments every 5 min until the animals were exhausted. Standard and n-3 PUFA diet animals ran, respectively, 54.7 (SEM 3.38) and 53.2 (SEM 2.95) min at the maximal speed of 19.6 (SEM 0.88) and 19.3 (SEM 1.27) m/min. There was no statistical difference between groups.

### Table 1. Standard and n-3 PUFA diet compositions (%)

<table>
<thead>
<tr>
<th></th>
<th>Standard diet</th>
<th>n-3 PUFA diet</th>
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<tbody>
<tr>
<td>SFA</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>16.0</td>
<td>9.02</td>
</tr>
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<td></td>
<td>18.0</td>
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<td></td>
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<tr>
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<tr>
<td>MUFA</td>
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<tr>
<td></td>
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<td>30.57</td>
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<tr>
<td>Total fatty acids</td>
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### Preparation of vessels

Immediately after acute exercise, animals were anaesthetized using intraperitoneal injection of sodium pentobarbitone (50 mg/kg body weight). Rings of thoracic aortas were prepared as previously described. Briefly, thoracic aortas were gently removed and immediately stored in ice-cold Krebs–Henseleit–bicarbonate solution previously gassed with 95 % O2–5 % CO2. The composition of the Krebs–Henseleit–bicarbonate solution was (mm): 118.0 NaCl, 25.0 NaHCO3, 1.18 KH2PO4, 1.18 MgSO4, 2.5 CaCl2, 10.0 glucose (pH 7.4). Each aorta was carefully dissected to remove surrounding connective tissues and its mid-thoracic region was cut into rings of 2 mm length. One ring from each animal was then mounted in a jacketed 15 ml organ bath at 37°C and washed every 15 min. The tension was measured isometrically using a force transducer (EMKA Technologies, Paris, France) and recorded on a personal computer.

Passive tension was maintained at 2 g (determined as the optimal tension during preliminary investigation) throughout the experiment. Before each experiment was performed, rings were allowed to equilibrate for a 45 min period and washed every 15 min. Thereafter, aortic rings were contracted with 10−7 M-phenylephrine (PE). Between trials, the rings were allowed to recover for a 45 min period and washed every 5–10 min.

### Experimental protocol

To determine whether n-3 PUFA induced an additional effect to acute exercise on endothelial function in rats, dose–response curves for acetylcholine (ACh; 10−9 to 10−4 M) were constructed in rings precontracted with 10−7 M-PE. To evaluate the relative role of nitric oxide (NO) in endothelium-dependent response, vascular responses to 10−6 and 10−5 M-ACh were obtained in the presence of 5 × 10−5 M-Nω-nitro-L-arginine methyl ester (L-NAME), a relatively selective inhibitor of endothelial NO synthase. We used 10−6 and 10−5 M-ACh because in a previous study, they induced about 70 and 100 % of the maximal response to ACh.

Finally, vascular responses to nifedipine (10−6 M threshold response) and 10−7 M (maximum response), an L-type Ca2+ channel antagonist, were examined in aortic rings precontracted with PE (10−7 M).

All drugs were purchased from Sigma Chemicals (St Louis, MO, USA). PE, ACh and L-NAME were prepared in distilled, deionized water and frozen for later use. Nifedipine was dissolved in dimethyl sulphoxide (99.9 %).

### Statistical analysis

Data are expressed as means and their standard errors; n indicates the number of experiments conducted. The dilating responses were expressed as percentages of the PE-induced precontractile force. The logarithm of the concentration of ACh that elicited 50 % of the maximal response (EC50) was designated as the EC50. These values were determined for each animal by logistic curves fitting of the semilogarithmic dose–response relationship with Statistica 7.1 software (StatSoft France, 2005). To assess the combined effects of acute exercise and PUFA diet on the various ACh concentrations...
used, two-way ANOVA test for repeated measures was performed with ACh concentrations as dependent variables, and both acute exercise/rest and PUFA/normal diet as independent variable. Changes among groups $E_{\text{max}}$ and $EC_{50}$ values for ACh, as well as to a single dose of nifedipine were analysed by two-way ANOVA. Upon significant differences in the ANOVA, Student–Newman–Keuls post hoc tests were used to locate differences. Values of $P<0.05$ were considered significant.

Results

Contractile responses to phenylephrine

There were no statistically significant differences between rest and post-exercise groups, both in standard-fed animals (0.83 (SEM 0.081) g; 0.85 (SEM 0.134) g for rest and post-exercise, respectively) and n-3 PUFA diet (0.90 (SEM 0.075) g; 0.70 (SEM 0.071) g). However, in the presence of L-NAME, response to $10^{-7}\text{M}\text{PE}$ was significantly increased as compared to pre-contraction of the same ring in the absence of inhibitor, whatever the group (standard diet/rest, 1.46 (SEM 0.099) g; standard diet/exercise, 1.83 (SEM 0.064) g; n-3 PUFA diet/rest, 1.49 (SEM 0.080) g; n-3 PUFA diet/exercise, 1.44 (SEM 0.093) g).

Acetylcholine-induced relaxation

ACh elicited a dose-dependent relaxation of aortic rings from all groups of rats (Fig. 1).

Effects of acute exercise. Two-way ANOVA for repeated measures revealed a significant main effect of exercise ($P=0.0002$), indicating that ACh-induced relaxation of aortic rings was enhanced after acute exercise. To further assess the effect of acute exercise on ACh-induced relaxation, $E_{\text{max}}$ and $EC_{50}$ values were compared (Table 2). When diet-matched animals were compared, maximal ACh-induced relaxation was increased ($P=0.0004$) and $EC_{50}$ was decreased ($P=0.016$).

Effects of n-3 PUFA. Statistical analysis revealed no significant effect of n-3 PUFA supplementation on ACh-induced relaxation as compared to standard diet ($P=0.949$; Fig. 1). Neither $E_{\text{max}}$ nor $EC_{50}$ was significantly different, at rest or after acute exercise (Table 2).

Effects of a combination of acute exercise and n-3 PUFA. When compared to resting rats fed with standard diet, $E_{\text{max}}$ was significantly increased ($P=0.010$) but not $EC_{50}$ ($P=0.282$) in animals subjected to combined n-3 PUFA diet and acute exercise (Table 2). However, two-way ANOVA for repeated measures indicates no significant diet $\times$ exercise interaction (Fig. 1).

Effects of N$^\omega$-nitro-l-arginine methyl ester on the acetylcholine-induced vasodilation. ANOVA revealed that pretreatment of aortic rings with L-NAME resulted in an inhibition of ACh-mediated vasorelaxation. No differences were detected among groups regardless of the ACh concentration used.

Effects of nifedipine

Effects of acute exercise. In aortic rings precontracted with PE ($10^{-7}\text{M}$), acute exercise significantly modified the response to $10^{-5}\text{M}$ ($P=0.0009$) and $10^{-8}\text{M}$-nifedipine ($P=0.006$) (Fig. 2).

The response to $10^{-5}\text{M}$-nifedipine was enhanced after acute exercise in vessels from animals fed with the standard diet (54.29 (SEM 5.26) v. 27.82 (SEM 5.56) %, $P=0.041$) but not the n-3 PUFA diet, as compared to the response in the diet-matched sedentary control. No statistically significant difference was found within diet-matched animals in response to $10^{-8}\text{M}$-nifedipine.

Effects of n-3 PUFA. When n-3 PUFA supplementation was compared to the standard diet, no significant change was found in response to nifedipine at rest or after acute exercise, whatever the concentration used (Fig. 2).

Effects of combined acute exercise and n-3 PUFA. Combined n-3 PUFA diet and acute exercise significantly enhanced the nifedipine-induced vasorelaxation (Fig. 2). Indeed, when compared to resting controls with standard diet, the response to nifedipine was significantly higher after acute exercise in n-3 PUFA diet, for both $10^{-7}\text{M}$ ($3.6$ (SEM 2.02) v. 5.11 (SEM 2.79) %, $P=0.01$) and $10^{-5}\text{M}$ (27.82 (SEM 5.56) v. 50.03 (SEM 3.78) %, $P=0.03$; Fig. 2).

Discussion

The present study shows that acute exercise resulted in a significant increase in endothelium-dependent vasorelaxation elicited by ACh in rats and that this effect was not amplified by dietary n-3 PUFA given at low dose over 4 weeks. Although in vitro studies showed that n-3 PUFA improve endothelial function$^{17,18}$, in vivo studies gave more conflicting results. On one hand, dietary n-3 PUFA increased endothelium-dependent vasorelaxation of mesenteric vasculature isolated from stroke-prone SHR rat$^{19}$ and of porcine arteries$^{20}$. They also restored endothelial function in atherosclerotic and hypercholesterolaemic animals$^{21}$. On the other hand, n-3 PUFA diet failed to modify endothelium-dependent...
Table 2. Maximal response and EC_{50} values for acetylcholine-induced relaxation of thoracic aorta from rat submitted to one bout of acute exercise or rest after standard or n-3 PUFA diet†‡

<table>
<thead>
<tr>
<th></th>
<th>Standard diet</th>
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<th>n-3 PUFA diet</th>
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<tr>
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<tr>
<td></td>
<td>Mean SE</td>
<td>Mean SE</td>
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<td>Mean SE</td>
</tr>
<tr>
<td>E_{max} (%)</td>
<td>95-97</td>
<td>7.22</td>
<td>130-82†</td>
<td>9-69</td>
</tr>
<tr>
<td>EC_{50} (log M)</td>
<td>7.26</td>
<td>0.18</td>
<td>7.40</td>
<td>0.12</td>
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</table>

EC_{50}, half-maximal effective dose; E_{max}, maximal relaxation.

Mean values were significantly different from those of the resting rats fed standard diet: *P<0.05.
Mean values were significantly different from those of the resting rats fed n-3 PUFA diet: †P<0.05.
‡For details of subjects and procedures, see Methods. Percentage relaxation was calculated as percentage reduction in force from 10^{-7} M-phenylephrine-induced tension.

Fig. 2: Effects of acute exercise and n-3 PUFA on vasorelaxation elicited by two doses of nifedipine in rings of rat thoracic aorta (○: rest; ●: exercise). Relaxation is expressed as percentage of the phenylephrine-induced precontractile force. Values are means with their standard errors depicted by vertical bars (n 10). Significant difference between rest and exercise for animals fed with the same diet: *P<0.05. Significant difference between n-3 PUFA/exercise and standard/rest animals: †P<0.05.

The relaxation of SHR rat aortic rings\(^{(22)}\). These discrepancies raise the question of whether rat aorta could be resistant to n-3 PUFA. In this regard, evidence from in vitro studies\(^{(23,24)}\) indicates that the effect of n-3 PUFA is mediated partly by the activation of the PPAR-\(\gamma\) pathway. However, in vivo treatment with pioglitazone, a PPAR-\(\gamma\) agonist, failed to modify the ACh-induced relaxation of healthy rat aortic rings\(^{(25)}\), whereas, when present in the bath, pioglitazone enhanced endothelium-dependent vasorelaxation of the same vessels. Taken together, data obtained with both PPAR-\(\gamma\) agonists and n-3 PUFA suggest that, in healthy animals, the effect of n-3 fatty acids on aortic endothelium-dependent vasoreactivity may result from direct action only. Recently, Ceylan-Isik et al.\(^{(26)}\) reported that supplementation with cod liver oil (0.5 mg/kg), which comprises mainly an antioxidant vitamin A, DHA and EPA, over a period of 12 weeks completely prevented the impairment of endothelial-dependent aortic relaxation in streptozotocin-treated rat. Their result may be related to vitamin A rather than PUFA since Goirand et al.\(^{(27)}\) showed that in a rat model of streptozotocin-induced diabetes, supplementation of the diet over a period of 8 weeks with 17.2% DHA alone did not affect the decrease in the endothelial-dependent aortic relaxation induced by ACh. Moreover, Lucas et al.\(^{(28)}\) reported a lack of beneficial effect of dietary DHA or EPA alone against lysophosphatidylcholine-induced inhibition of ACh-induced aortic relaxation in Golden Syrian hamster. Taken together, data suggest that the effect of dietary n-3 PUFA may vary according to the experimental approach, i.e. the considered vessel, as well as the composition and duration of the diet.

In man, data from randomized trials indicate that doses of EPA and/or DHA ≥3 g/d prevent endothelial dysfunction and that these effects may be mediated through enhanced NO production\(^{(3)}\). In healthy subjects with normal blood lipid profile, 4 weeks of a Mediterranean-inspired diet did not modify vascular function or biomarkers of oxidative stress, as compared with a typical Swedish diet\(^{(29)}\). In a more recent study by Leeson et al.\(^{(30)}\), no relationship was found between circulating levels of n-3 PUFA (derived from normal dietary variation) and endothelial function in normal young adults. A positive association was present only in those subjects who had cardiovascular risk factors (smoking, high levels of insulin, glucose or TAG). It is possible that n-3 PUFA exert a significant effect only in the case of endothelial dysfunction.

Although the aim of the present study was not to investigate the precise mechanisms of n-3 PUFA effect on vasculature, other studies previously showed that DHA decreases expression of vascular cell adhesion molecule 1 and monocyte adhesion on the vascular endothelium\(^{(3)}\). They also indicate that EPA increases NO production in vitro\(^{(3)}\) and in vivo\(^{(5)}\) without altering vascular smooth muscle sensitivity to NO\(^{(22)}\).

Acetylcholine causes endothelium-dependent vasorelaxation through activation of the muscarinic receptors. The stimulation of the latter leads to an increase in cytosolic Ca^{2+} which is a prerequisite step for the production and release of NO, cyclo-oxygenase products (mainly prostacyclin, PGI\(_2\)) and endothelial-derived hyperpolarizing factor. Once released by endothelium, these factors trigger smooth muscle cell relaxation. Experimental as well as epidemiological studies have demonstrated that both regular and acute exercise improve vasodilation evoked by ACh and that this effect results mainly from increased NO bioavailability. As reported elsewhere\(^{(31,33)}\), exercise training up-regulates endothelial NO synthase gene.
expression and protein activity, and reinforces the antioxidant systems. These effects lead to increased NO-mediated vasodilation although smooth muscle cell sensitivity to NO donors is unchanged. On the other hand, enhancement of ACh-induced vasorelaxation by acute exercise results from increased NO production only (32). Previous studies indicate that this effect probably results from exercise-induced increase of either receptor number or affinity (9) and enhanced endothelial calcium influx and vasorelaxation without altering their relationship (33). Neither EC₅₀ values nor maximal dilatation in response to NO donors is modified after one bout of exercise (32). In the present study, the vasorelaxation induced by ACh was decreased by the inhibitor of endothelial NO synthase in all rings, which further confirms that an augmentation of NO availability is involved in exercise-induced endothelial-dependent improvement in vascular relaxation.

In rabbit aorta, Howard et al. (34) reported decreased PE-induced contraction after a single bout of maximal acute exercise. Conversely, in the present study, the response of rat aorta to a lower dose of PE (10⁻⁷ m) remains unchanged after acute maximal exercise. Previously, Jen et al. reported the same result (33). This indicates that the changes we found in response to ACh are truly related to an effect on endothelium rather than an artefact of the response of vascular muscle to PE. Considering the interactions between physical exercise and dietary fat, data from previous studies demonstrated different effects. Quiles et al. (35) showed that both exercise and the interaction between the intake of virgin olive oil and exercise, but not diet alone, are effective in reducing plasma TAG and cholesterol in rats. In the same study, although the plasma lipid profile was modified by both diet and exercise, no interaction was found (35). Finally, another study (33) showed that fish oil supplementation reduced the stimulation of both rates of plasma glucose disappearance and hepatic glucose production during exercise. More recently, Hill et al. (36) investigated the effects of combined fish oil supplements and regular aerobic exercise. They reported an improvement in endothelium-dependent arterial vasodilation due to diet only, but no interaction with exercise training. However, in their study, the training programme alone (12 weeks, three 45 min walks per week at 75% age-predicted maximal heart rate) failed to alter endothelium-dependent vasodilation. Randomized controlled trials as well as uncontrolled studies reported a small non-significant improvement in exercise tolerance (3,37). Although dietary n-3 PUFA improved the 1 min heart rate recovery after exercise in patients with post-infarction history and ejection fraction lower than 40%, neither peak exercise heart rate nor test duration was modified by the diet. In the present study, large and small arteries compliance was not affected by n-3 PUFA supplementation, suggesting that endothelial function after exercise was not altered by diet (38). In patients with coronary artery disease, dietary EPA enhanced the exercise-induced increase in forearm blood flow and decrease of vascular resistance (39) without affecting the response to NO donors. This effect was abolished by L-NMMA treatment, indicated that EPA improved the NO bioavailability. No study has examined interactions between n-3 PUFA and acute exercise on endothelium-dependent vasodilation in rats. Nevertheless, in the present study, the combination of acute exercise and n-3 PUFA revealed no additional change in acetylcholine-mediated relaxation.

Dihydropyridine-type calcium antagonists are used for the treatment of hypertension and CHD. They induce their specific pharmacological effects by binding to L-type calcium channels (39,40), which results in a reduced Ca²⁺ influx with impaired electromechanical coupling both in vascular smooth muscle cells and in the heart. Nifedipine indirectly up-regulates endothelial superoxide dismutase expression by stimulating vascular endothelial growth factor production from adjacent vascular smooth muscle cells (41). Previous in vitro studies have shown that nifedipine enhances NO release via the up-regulation of endothelial NO synthase expression (42).

In the present study, vasorelaxation elicited by 10⁻⁵ M-nifedipine in PE-precontracted rings was similarly enhanced after acute exercise in both standard diet and n-3 PUFA-supplemented rats, while response to 10⁻⁸ M-nifedipine was altered after acute exercise in the n-3 PUFA diet rats only.

As discussed earlier, in these vessels, n-3 PUFA failed to alter the effect of acute exercise on the vasorelaxation elicited by both 10⁻⁵ and 10⁻⁶ M-ACh, which is related to NO as indicated by the lack of relaxation in the presence of L-NAME. Based on the present findings, we hypothesize that in the absence of effect on NO-mediated relaxation, PUFA may potentiate the acute exercise effect on smooth muscle cell relaxation via L-type Ca²⁺ channel modifications.

Several limitations of the present study should be mentioned. First, we investigated the effects of low doses of dietary n-3 PUFA on healthy animals. Thus, although this dose and length of diet have been previously reported to cause an enrichment of n-3 PUFA content in phospholipids and neutral lipids in rat tissues (15,16), we are unable to ascertain whether the lack of effect on endothelial function results from the absence of initial endothelial dysfunction. Furthermore, endothelial function was explored in vitro in rat aortic rings. With this artificial experimental approach, responses to pharmacological agents are assessed in the absence of n-3 PUFA. Prospective studies are needed to confirm the results in healthy as well as non-healthy human subjects.

In conclusion, the present study demonstrates that although acute exercise improves endothelium response to ACh, n-3 PUFA supplementation has no effect on endothelium-dependent vasorelaxation of aorta in healthy rats. However, it may potentiate an acute exercise effect on smooth muscle cell relaxation via L-type Ca²⁺ channel modifications.

Acknowledgements

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