EFFECTS OF DIETARY FISH OIL SUPPLEMENTATION ON THE SKELETAL MUSCLE BLOOD FLOW RESPONSE TO SUBMAXIMAL TREADMILL EXERCISE

by

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Abstract

Dietary supplementation with omega-3 polyunsaturated fatty acids (PUFAs) containing docosahexaenoic (DHA) and eicosapentaenoic acid (EPA) has been demonstrated to produce advantageous effects on vascular function. Specifically, PUFA supplementation has resulted in enhanced brachial artery blood flow ($\dot{Q}$), dilation, and vascular conductance (VC) during rhythmic handgrip exercise. The effects of fish oils (FO) on skeletal muscle blood flow ($\dot{Q}_m$) during dynamic whole body exercise, however, remain unknown. **PURPOSE:** To test our hypothesis that 6 weeks of dietary FO supplementation with DHA and EPA enhances regional $\dot{Q}_m$ and VC to the hindlimb musculature during submaximal treadmill exercise. **METHODS:** Following 6 weeks of dietary supplementation with safflower oil (SO) (control; n = 9) or FO (n = 8), heart rate (HR), mean arterial pressure (MAP), and $\dot{Q}_m$ to the hindlimb were measured at rest and during submaximal treadmill exercise (20 m/min, 10%, ~65% $\dot{V}O_2max$) via radiolabeled microspheres in adult male Sprague-Dawley rats. **RESULTS:** HR and MAP were not different between SO and FO at rest or exercise ($P<0.05$). $\dot{Q}_m$ and VC were not different between SO and FO at rest. During exercise, FO exhibited greater $\dot{Q}_m$ in 8 of the 28 muscle parts measured as well as greater VC in 11 of the 28 muscle parts measured. Additionally, FO exhibited greater $\dot{Q}_m$ (158±9) and VC (1.156±0.066) to the total hindlimb musculature than SO (128±10 ml/min/100g, 0.918±0.077 ml/min/100g/mmHg) ($P<0.05$). **CONCLUSION:** These results demonstrate that 6 weeks of dietary FO supplementation with DHA and EPA results in enhanced $\dot{Q}_m$ and VC to the hindlimb during submaximal exercise. Thus, supplementation with DHA and

EPA may have therapeutic effects on oxygen delivery and vascular function in patients with impaired vascular function and exercise tolerance (i.e., congestive heart failure, diabetes).

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CHAPTER 1 - Introduction

Healthy vascular function helps ensure the blood supply that is crucial for delivery of oxygen and substrates required to meet the energetic requirements of exercising skeletal muscle. In contrast, vascular dysfunction compromises skeletal muscle function and limits exercise tolerance. Dietary supplementation with fish oils containing the omega-3 polyunsaturated fatty acids (PUFAs) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) improves vascular function (24). In healthy individuals, dietary PUFA supplementation improves endothelium-dependent vasodilation and enhances skeletal muscle blood flow ($\dot{Q}_m$) during rhythmic handgrip exercise (24, 47). Therefore, it is reasonable to pose the question whether dietary supplementation with DHA and EPA might also augment $\dot{Q}_m$ during dynamic, locomotory exercise.

Pertinent to this issue, DHA and EPA have been demonstrated to enhance the production and release of nitric oxide (NO) from the vascular endothelium (28, 38). In the rat, dietary supplementation with DHA and EPA increases the content of eNOS (endothelial nitric oxide synthase) mRNA in the aorta as well as enhancing eNOS expression, eNOS activity, and overall NO bioavailability (28). Increased NO production and release from the vascular endothelium could be advantageous because of its potential to increase $\dot{Q}_m$ and O_2 delivery during exercise.

At exercise onset, $\dot{Q}_m$ increases rapidly to meet metabolic demands and in highly oxidative muscles can achieve values exceeding 40-fold those at rest (9). While the rapid vasodilation within the first contraction cycle is of uncertain origin, NO and other vasoactive substances released from the endothelium do contribute to the subsequent sustained elevations of $\dot{Q}_m$ by facilitating terminal arteriolar vasodilation (19, 30, 31, 34). This dilation ascends from

these arterioles to the larger feed arteries and conduit blood vessels located external to the skeletal muscle and consequent to this ascending vasodilation, $\dot{Q}_m$ will continue to increase to the contracting muscle (9, 44). This additional $\dot{Q}_m$ will produce further increases in shear stress on the vascular endothelial cells and thus will generate further increases in $\dot{Q}_m$ mediated by the NO regulatory pathway (41). Therefore, an overall increased bioavailability of NO subsequent to DHA and EPA supplementation could plausibly enhance $\dot{Q}_m$ and O_2 delivery to the contracting musculature during dynamic exercise.

Previous studies have assessed the $\dot{Q}_m$ response to dietary fish oil (FO) supplementation at rest, but we do not know whether PUFAs impact $\dot{Q}_m$ among and within different locomotor muscles during exercise. Following FO supplementation, enhanced endothelium-dependent vasodilation, reduced vascular oxidative stress, and augmented $\dot{Q}_m$ have been demonstrated at rest (6, 7, 24). While $\dot{Q}_m$ during exercise has also been increased following supplementation, the mode of exercise utilized is non-locomotory in nature (i.e., rhythmic handgripping) which employs a small muscle mass (47). Consequently, the effect of dietary PUFA supplementation on dynamic locomotory (large muscle mass) exercise remains unknown.

Given the substantial role of dynamic locomotory exercise to benefit health and quality of life, it is important to determine the effects of FO on $\dot{Q}_m$ and vascular function during whole body exercise. The Sprague-Dawley rat is an accepted model of the human vasculature and established microsphere techniques exist for the precise measurement of muscle and regional $\dot{Q}$ during running exercise. This model will allow us to determine the effects of dietary FO supplementation (DHA and EPA) on both $\dot{Q}_m$ and vascular conductance (VC) during dynamic locomotory exercise.

The primary objective of this study is to test the hypothesis that dietary supplementation with fish oils that contain the PUFAs DHA and EPA will enhance the $\dot{Q}_m$ response to dynamic submaximal treadmill exercise in the rat hindlimb. If dietary supplementation with fish oils containing DHA and EPA can enhance $\dot{Q}_m$ and vascular conductance in healthy individuals during exercise, it is plausible that these supplements would also enhance $\dot{Q}_m$ and VC in those patients afflicted with cardiovascular disease. This would establish a template for the use of DHA and EPA supplementation as a potential therapeutic treatment for individuals with cardiovascular disease as these diseases often result in a lifestyle-eroding reduction in exercise tolerance that may potentially result from a reduced $\dot{Q}_m$ (23).

CHAPTER 2 - Review of Literature

Regulation of $\dot{Q}_m$ During Exercise

During exercise, healthy $\dot{Q}_m$ and O_2 delivery are necessary to meet the metabolic demands of the exercising musculature. At the onset of dynamic exercise, $\dot{Q}_m$ is increased within the first second following contraction and, in healthy individuals, reaches steady-state levels within ~30-90 seconds (9). While this immediate rapid vasodilation within the first contraction cycle is of uncertain origin, it is likely that the factors that contribute to it are not the same as the ones that sustain $\dot{Q}_m$ during steady-state exercise. Therefore, this review will focus on the factors involved in the regulation of sustained elevations of $\dot{Q}_m$ during dynamic, steady-state exercise.

Metabolic Control

Muscular contractions result in the production of metabolites that diffuse out of the cell into the extracellular fluid (ECF). These metabolites include NO, prostaglandins (PG), adenosine, lactic acid, potassium, inorganic phosphate, and hydrogen ions (9). As muscle metabolism increases with increased exercise intensity, the concentration of these metabolites in the ECF also increases. The metabolites can then diffuse to the arterioles where they act upon the vascular smooth muscle resulting in a vasodilation of the arteriole that allows for increased blood flow (10). Therefore, $\dot{Q}_m$ is regulated metabolically as it is increased during exercise to allow the O_2 supply to meet the increased O_2 demand of the working musculature.

Endothelial Control and Flow-Mediated Dilation

The vascular endothelium of the arteriole releases vasodilator substances in response to various stimuli. The endothelium possesses the ability to detect both forces on the vessel walls and chemical substances within the blood and respond via the release of vasoactive molecules such as NO and PG that initiate a vasodilation of the arterioles (40). This dilation can then progress to the feed arteries external to the muscle via ascending vasodilatory mechanisms that help to further regulate $\dot{Q}_m$ to the exercising muscle (9, 44, 49).

When $\dot{Q}$ is increased in the arterioles, it causes increased shear stress on the vascular endothelium that results in a further dilation in the vascular resistance vessels (25). This process has been termed flow-mediated dilation and it is currently believed that flow-mediated dilation is dependent, at least in part, on the production and release of NO (17). This concept is supported further by Rubanyi and colleagues who have found enhanced production of eNOS in response to increases in shear stress (41). Therefore, during dynamic exercise, the regulation of $\dot{Q}$ in both the terminal arterioles and the larger feed arteries is thought to be dependent, in part, upon the availability of NO.

Nitric Oxide

NO is an endogenous signaling molecule that is synthesized by nitric oxide synthase (NOS) from the vascular endothelium (eNOS) and sarcolemma and neuromuscular junction of skeletal muscle (neuronal NOS) (45). When released, eNOS initiates a cascade of reactions in smooth muscle via a cyclicGMP pathway that activates a cGMP-dependent protein kinase to facilitate a reduction in intracellular calcium concentration, decreased calcium-dependent phosphorylation of smooth muscle myosin regulatory light chain and thus, relaxation of the vascular smooth muscle (16, 26). The intramuscular location of neuronal NOS (nNOS) at the

sarcolemma and neuromuscular junction has led to speculation that upon contraction, it could diffuse to smooth muscle cells in the adjacent vasculature, bind to soluble guanylyl cyclase and initiate the cGMP relaxation cascade that is seen with eNOS, thus implicating both forms of NOS in exercise-induced vasodilation (16, 26). Arteriolar relaxation has been demonstrated to be decreased in contracting muscles of mice lacking either eNOS or nNOS, demarcating a potential role for both in the $\dot{Q}_m$ response seen in contracting muscle (26). Additionally, a follow-up study by Grange and colleagues in 2001 has further evidenced the contribution of NO derived from both eNOS and nNOS in the vasodilation of contracting skeletal muscle (16).

In studies performed in the rat, the $\dot{Q}_m$ response to NOS inhibition has been demonstrated to be dependent on both the muscle groups recruited by the exercise and the fiber type composition of the individual muscles (19, 30, 31, 34). When measured during dynamic treadmill exercise (20m/min, 10% grade, ~65%$\dot{V}O_2$ max) using the NOS inhibitor L-NAME, $\dot{Q}_m$ was decreased in 16 of 28 individual muscles or muscle parts measured, dependent upon their fiber type composition (19). Specifically, $\dot{Q}$ was decreased in the individual skeletal muscles that contained a large percentage of slow twitch oxidative (SO) and fast twitch oxidative glycolytic (FOG) fibers. This indicates that highly oxidative fibers exhibit greater NO induced-vasodilation during exercise (19, 30, 31, 34). Moreover, at running speeds that elicit $\dot{V}O_2$ max (60m/min, 10% grade), vascular conductance (VC; $\dot{Q}$ normalized to mean arterial pressure) is also reduced further in muscles composed of more FOG and SO fibers than those composed of FG fibers (34). Consequently, NO availability appears to contribute to the regulation of $\dot{Q}_m$ during dynamic treadmill exercise. Additionally, the role of NO in $\dot{Q}_m$ during exercise likely varies among muscles with differing fiber types.

It is important to note, however, that the effectiveness of L-NAME and other NOS inhibitors on NO produced by the skeletal muscles (nNOS) is currently unclear. A limitation of the previous studies that have utilized L-NAME to inhibit NOS and determine the role of NO in exercise-induced vasodilation is that L-NAME may inhibit NOS produced from the vascular endothelium (eNOS), but not the production or release of nNOS from the contracting skeletal muscle. As mentioned previously, eNOS and nNOS have both been implicated in the hyperemic blood flow response to exercise. Therefore, while L-NAME may inhibit NO formation and release from the vascular endothelium (eNOS), it is possible that the NO produced in the skeletal muscle (nNOS) is not inhibited and may therefore contribute to the exercise-induced vasodilation seen during eNOS inhibition thereby masking a larger overall effect of NO.

Dietary Fish Oil Supplementation

Dietary supplementation with fish oils containing the omega-3 polyunsaturated fatty acids DHA and EPA has been demonstrated to have advantageous effects on the cardiovascular system. These effects are numerous and include reduced vascular oxidative stress and enhanced dilation and $\dot{Q}_{m}$. While FO supplementation has been demonstrated to produce these beneficial effects, the mechanism or mechanisms behind these benefits have not been clearly identified. Therefore, this review will encompass both the beneficial effects of FO supplementation and the potential mechanisms behind these effects.

Nitric Oxide and Prostaglandins

While the previous literature has shown reductions in vascular oxidative stress, improved vascular reactivity, enhanced endothelial function, and enhanced $\dot{Q}$ subsequent to dietary FO supplementation, the mechanisms for this enhancement have not been clearly identified. Lawson

et al. examined the treatment of rat aortic rings with EPA and DHA and determined that it results in enhanced synthesis or release of EDRF (NO) and PG (27). In 1997, Okuda et al. investigated NO production from endothelial cells following treatment with EPA (38). Their results demonstrated an enhancement in NO production from endothelial cells following the EPA treatment (38). Hishinuma and colleagues later demonstrated that FO supplementation also increases bovine endothelial cells' release of PGI_2 (prostacyclin) and PGI_3 (prostaglandin I3)(20). Application of the results of these studies is somewhat limited, however, for the because the endothelial cells were treated directly with EPA and DHA and not by the use of dietary supplementation.

In 2000, Lopez et al. determined that dietary FO supplementation potentiated endothelial production of NO in the aorta of Sprague-Dawley rats (29). In 2004, they followed-up the previous study by demonstrating an upregulation of eNOS in the aorta of the rat subsequent to 8 weeks of supplementation of a diet rich in fish oils (28). Additionally, they were able to demonstrate an enhancement in both NO production and activity subsequent to the supplementation (28). As such, the enhancement of either NO bioavailability, or both NO and PG production and release subsequent to fish oil supplementation implicate both as potential mechanisms for the improved vascular reactivity, enhanced endothelial function, and augmented $\dot{Q}_m$. Furthermore, while the literature has shown enhancement of the bioavailability of eNOS subsequent to supplementation, it is not known whether there is a concomitant enhancement in nNOS bioavailability.

Oxidative Stress

In addition to enhanced $\dot{Q}_m$ and endothelial function, reductions in oxidative stress have also been observed subsequent to FO supplementation. Oxidative stress is a condition in which

reactive oxygen species (ROS) are elevated such that the cells' antioxidant systems are overwhelmed and produce deleterious effects on the vascular endothelium (46). In 1997, Carbonell et al. investigated the effects of 5 weeks of dietary FO supplementation on the production of superoxide (O_2-), a ROS, in the vasculature and found that O_2- production was 31% less in rats fed FO than in those on the control diet of corn oil (6). This finding is significant because O_2- can react with NO to generate other more detrimental ROS such as peroxynitrite (ONOO-) that can damage the vasculature (46). Dietary FO supplementation can also enhance superoxide dismutase (SOD) activity in the rat (7). An enhancement in SOD is important because it can react with O_2- to form hydrogen peroxide (H_2O_2), a more stable ROS that is enzymatically converted to H_2O by catalase or glutathione peroxidase (46). Similar results have been found in type-2 diabetic subjects who received 6 weeks of dietary supplementation with fish oils (33). Oxidative stress was shown to be significantly attenuated subsequent to PUFA supplementation (33) and similar results have been found in fructose-fed rats when supplemented with fish oils (37). The results of these studies have led Mori and colleagues to suggest that dietary supplementation with the PUFAs EPA and DHA may have a positive impact on the redox state in the vasculature of those with cardiovascular disease, thereby reducing the stress and endothelial damage caused by the ROS (33). This could potentially be responsible for the beneficial effects of fish oils on cardiovascular health.

MuscleBlood Flow ($\dot{Q}_m$)

In healthy populations, dietary FO supplementation has resulted in enhancements in vascular reactivity, endothelium-dependent vasodilation, and thus $\dot{Q}_m$ (8, 24, 47). In 1992, Chin and colleagues established that FO supplementation dose-dependently decreased the vasoconstrictive response to the potent constriction induced by norepinephrine (NE) and

angiotensin II (Ang II) allowing for improved $\dot{Q}$ (8). The significance of this observation lies in the fact both NE and Ang II are implicated in the development of hypertension, which results in increased systemic vascular resistance (22). This finding also reflects the previous literature that has shown populations with greater fish consumption tend to have lower incidences of hypertension (3). In a 2003 study, Khan et al. studied the effects of FO supplementation on endothelial function and microvascular $\dot{Q}$ in healthy subjects (24). The results of the study illustrated a significant improvement in the endothelial response to acetylcholine (ACh), which is a vasodilator of vascular smooth muscle that allows enhanced $\dot{Q}$. While these results indicate a role for FO supplementation in the enhancement of $\dot{Q}$ at rest, supplementation has also been evidenced to significantly enhance brachial artery $\dot{Q}$ and VC during rhythmic handgrip exercise in healthy subjects (47). Based on their results, the authors suggested that treatment with fish oils could potentially delay the onset of fatigue to the working muscles in healthy individuals allowing them to work at a higher workload for a longer period of time (47). Altogether, these results demonstrate the beneficial effects of FO supplementation on $\dot{Q}_{m}$ and vascular function in healthy subjects.

Cardiovascular diseases such as hypertension, hypercholesterolemia, type-II diabetes mellitus, coronary artery disease, and congestive heart failure (CHF) often result in endothelial dysfunction (1, 5, 12, 13, 39, 48). This endothelial dysfunction may be attributable, in part, to increased oxidative stress and reductions in the bioavailability of NO in the vasculature and manifests itself as impaired endothelium-dependent vasodilation, increased systemic vascular resistance, and decreased vascular reactivity. These vascular derangements can lead to a decreased exercise tolerance and reduced functional capacity in affected individuals. This underscores the importance of resolving the potential for FO supplementation to improve

endothelial function. Consequently, if dietary PUFA supplementation could improve vascular endothelial function, active $\dot{Q}$, and O_2 delivery to the skeletal muscle during dynamic exercise conditions, then it could plausibly improve both the exercise tolerance and quality of life of individuals afflicted with various types of cardiovascular disease.

CHAPTER 3 - Methods

Animal Selection and Care

Seventeen adult male Sprague-Dawley rats were used in this study. Rats were maintained on a 12-12 hour light-dark cycle and received food and water *ad libitum*. All experiments were approved under the guidelines established by the Kansas State University Institutional Animal Care and Use Committee (IACUC) and conducted under the guiding principles of the American Physiological Society (APS) and the National Institutes of Health (NIH).

Dietary Supplementation

Two groups of rats were studied in the present investigation. Both groups were fed the following diet (expressed as grams/kg diet): Casein 225, cornstarch 446, sucrose 223, cellulose 31, DL-Methionine 1, standard mineral mix 14, standard vitamin mix 10, oil 50, and alpha-tocopherol acetate 0.09. Both diets consisted of 5% oil. In the control group of rats (Safflower Oil, n = 9) 50% of the oil consisted of safflower oil (SO) and in the experimental group of rats (Fish Oil, n= 8), 50% of the oil consisted of FO high in docosahexaenoic (~20%) and eicosapentaenoic acids (~30%). Both groups of animals followed their isocaloric dietary regimens for a duration of 6 weeks. A similar diet containing these levels of DHA and EPA has previously been reported to increase acetylcholine-induced dilation in the rat aorta (29).

Treadmill Acclimatization

All animals were familiarized with running on a motor-driven treadmill according to the guidelines described in the Resource Book for the Design of Animal Exercise Protocols as

published by the APS (Bethesda, MD). The animals were brought to the laboratory and placed into a lane on the treadmill belt. The animals were then acclimated to walking on the treadmill. During the protocol of familiarization (1-2 weeks), animals exercised for 5-10 minutes/day at a speed of 20 meters/minute and a grade of 10%. The treadmill is equipped with an electric eye that, when tripped by the animal, initiates a burst of high-pressured air towards the animal's hindquarters. This burst of air is considered to be a non-noxious stimulus and is used to encourage the animal to stay at the front of the treadmill lane. If they continue to the back of the treadmill lane, the animal will contact a grid designed to deliver an electric shock (20 volts) at a very low non-lethal current (0.2 mAmps). This stimulus is used during acclimatization to encourage the animal to keep pace on the treadmill and stay at the front of the lane and is typically not contacted by the animal following familiarization, if at all.

Surgical Procedure

Once it had been established that all of the rats were proficient runners, each animal was anesthetized with a 5% isoflurane and 95% oxygen mixture. Each animal was then maintained on a 3% isoflurane and 97% oxygen mixture during acute surgical instrumentation. While the animals were maintained at a non-reflexive anesthetic state, as demonstrated by the lack of response to painful stimuli (i.e. toe pinch, foot clamp, or incision), the right carotid artery and caudal (tail) artery were isolated and cannulated with polyethylene catheters (PE-10 connected to PE-50) (Intra-Medic polyethylene tubing; Clay Adams, Sparks, MD). The carotid artery catheter was advanced 2–3 mm rostral to the aortic valve. The caudal artery catheter was advanced toward the bifurcation of the descending aorta. The carotid and caudal catheters were then tunneled subcutaneously to the dorsal aspect of the thorax and exteriorized through a small puncture wound in the skin. This incision was closed with 3-0 silk suture. All wounds were

infiltrated with a 0.5% bupivacaine solution to maintain local anesthesia at the surgical sites. Isoflurane anesthesia was then terminated and each animal was given $\geq$ 2 hours to recover. This period of recovery was selected based on a previous study that has shown that hemodynamic, arterial blood gas, and acid-base parameters are stable in the awake rat during 1-6 hours of recovery from gas anesthesia (14).

Experimental Protocol

Subsequent to the recovery period, the final experimental protocol was initiated. Each animal was placed on the treadmill, and following a period of stabilization (~15 minutes), the caudal artery catheter was attached to a 1ml plastic syringe connected to a Harvard infusion/withdrawal pump (model 907). The carotid artery catheter was then connected to a pressure transducer to measure heart rate and blood pressure. Exercise was then initiated at a speed of 20 meters/minute up a 10% incline. After ~3 minutes of treadmill exercise, the carotid artery catheter was disconnected from the pressure transducer and $0.5x10^6$ radiolabeled microspheres (^{46}Sc or ^{85}Sr; 15 microns in diameter; Perkin-Elmer Life Sciences Incorporated, Boston, MA) were injected into the aortic arch to determine regional blood flow. Simultaneously, blood withdrawal from the caudal artery catheter was initiated at a rate of 0.25 ml/min. Approximately 30 seconds following the infusion of microspheres, blood withdrawal from the caudal artery catheter was stopped, exercise was terminated, and the animal was allowed ~60 minutes to recover on the treadmill lane.

Following the 60 minutes of recovery, the heart rate and blood pressure measurements were repeated and a second microsphere infusion was performed following the same procedures as above to determine regional blood flow under resting conditions. This procedure of measuring blood flow at rest following exercise minimizes the potential for blood loss to affect

the exercise response and allows for "resting" measurements that do not reflect the pre-exercise anticipatory state of the animal (2).

After the blood flow determinations were completed, each animal was removed from the treadmill lane and anesthetized with pentobarbital (40 mg/kg i.a.). An arterial blood sample (0.3 ml) was taken, and the animal was euthanized with an overdose of pentobarbitol anesthesia (100 mg/kg i.a.). Upon confirmation of death, the thorax was opened and placement of the carotid artery catheter into the aortic arch was confirmed by anatomical dissection. The kidneys, visceral organs, and muscles of both hindlimbs of the animal were identified, removed, weighed, and placed immediately into counting vials. The radioactivity of each tissue was then determined by a gamma-scintillation counter (Packard Auto Gamma Spectrometer, model 5230, Downers Grove, IL). Taking into account the cross-talk fraction between isotopes, blood flow to each tissue was determined using the reference sample method and expressed in ml/min/100g tissue (35). Adequate mixing of the microspheres was verified by the demonstration of <15% difference in right and left kidney blood flow. Blood flow measurements were then normalized to mean arterial pressure and expressed as vascular conductance (ml/min/100g/mmHg).

Statistical Analysis

Heart rate, mean arterial blood pressure, tissue $\dot{Q}$, and tissue VC were compared between groups using unpaired Student's t-tests. All values are expressed as means ± SEM. Statistical significance was set at $P \leq 0.05$.

CHAPTER 4 - Results

Fish Oil Effects on Heart Rate and Mean Arterial Pressure

Heart rate (HR) and mean arterial pressure (MAP) measured at rest were not different between FO and SO rats. While SO and FO rats both exhibited a significantly greater HR during exercise than at rest, MAP did not increase above resting values nor did it differ between SO and FO rats during the submaximal treadmill exercise (Table 4-1).

Fish Oil Effects on Blood Flow ($\dot{Q}$)

The effects of FO supplementation on the $\dot{Q}$ at rest and in response to exercise for the splanchnic organs are shown in Figure 4-1. At rest, SO rats exhibited significantly greater $\dot{Q}$ to the large intestine and pancreas than FO rats while there was no difference between SO and FO rats in the rest of the organs examined or in total splanchnic $\dot{Q}$. $\dot{Q}$ to the splanchnic organs was not different between the SO and FO rats during exercise (lower panel Figure 4-1).

$\dot{Q}_m$ to the total hindlimb musculature measured at rest was not significantly different between the SO and FO rats (upper panel Figure 4-2) nor was it significantly different for any of the 28 hindlimb muscles or muscle parts examined (Table 4-2). However, during exercise, the FO rats exhibited a 23% greater $\dot{Q}_m$ to the total hindlimb musculature (FO, 158±9: SO, 128±10 ml/min/100g, $P \leq 0.05$) (lower panel Figure 4-2). This reflected, in part, a significantly higher $\dot{Q}_m$ in 8 of the 28 muscles or muscle parts in the FO compared with the SO rats (Table 4-3, upper panel Figure 4-3).

Fish Oil Effects on Vascular Conductance (VC)

Although there was no significant difference in MAP between SO and FO rats, results were also examined and expressed as VC. The effects of FO supplementation on the VC during exercise for the splanchnic organs are shown in Figure 4-4. At rest, SO rats exhibited significantly greater VC to the large intestine and pancreas than FO rats while there was no difference between SO and FO rats in the rest of the organs examined (upper panel Figure 4-4). VC to the splanchnic organs was not different between the SO and FO rats during exercise (lower panel Figure 4-4).

VC to the total hindlimb musculature measured at rest was not significantly different between the SO and FO rats, nor was it significantly different for any of the 28 hindlimb muscles or muscle parts examined (Table 4-4, upper panel Figure 4-5). During exercise, however, the FO rats demonstrated 26% greater VC to the total hindlimb musculature than SO rats (FO, 1.156±0.066; SO, 0.918±0.077 ml/min/100g/mmHg, $P \leq 0.05$) (lower panel Figure 4-5). Additionally, VC was significantly higher than SO in 11 of the 28 hindlimb muscles or muscle parts in the FO compared to the SO rats (Table 4-5, lower panel Figure 4-3).

Table 4-1 Heart Rate and Mean Arterial Pressure

	HR (bpm)	MAP (mmHg)
Safflower Oil		
Rest	417±5	140±3
Exercise	500±6†	141±3
Fish Oil		
Rest	412±11	137±3
Exercise	492±7†	136±1

Heart rate measured at rest and during submaximal treadmill exercise in rats fed a diet supplemented with Safflower Oil (Control) and Fish Oil. Values are means ± SE, † $P \leq 0.05$ vs. Rest

Table 4-2 Resting Muscle Blood Flow

	Safflower Oil	Fish Oil
Ankle extensors		
Soleus	120±19	94±24
Plantaris	12±2	21±9
Gastrocnemius		
Red	24±3	37±16
White	17±4	17±3
Middle	12±2	17±7
Tibialis posterior	29±8	23±7
Flexor digitorum longus	49±17	51±7
Flexor hallicus longus	13±3	20±8
Ankle flexors		
Tibialis anterior		
Red	50±14	59±19
White	23±5	27±9
Extensor digitorum longus	22±5	32±13
Peroneals	16±4	23±8
Knee extensors		
Vastus intermedius	81±18	93±26
Vastus medialis	13±2	26±10
Vastus lateralis		
Red	85±18	89±35
White	16±5	18±7
Middle	25±5	27±11
Rectus Femoris		
Red	19±5	49±18
White	16±4	24±8
Knee flexors		
Biceps femoris		
Anterior	10±2	15±7
Posterior	12±3	13±5
Semitendinosus	11±2	14±4
Semimembranosus		
Red	15±3	19±8
White	12±2	15±5
Thigh adductors		
Adductor longus	123±20	143±20
Adductor magnus & brevis	14±2	21±8
Gracilis	14±2	15±3
Pectineus	35±11	38±15

Blood flow (ml/min/100g) measured at rest in all hindlimb muscles of rats fed a diet supplemented with Safflower Oil and Fish Oil for which there was no effect of Fish Oil. Values are means ± SE.

Table 4-3 Exercise Muscle Blood Flow

	Safflower Oil	Fish Oil
Ankle extensors		
Soleus	250±34	330±28*
Plantaris	222±31	263±26
Gastrocnemius		
Red	453±67	515±54
White	45±7	70±12*
Middle	184±25	232±20
Tibialis posterior	198±23	263±33
Flexor digitorum longus	83±18	122±12
Flexor hallicus longus	116±16	158±15*
Ankle flexors		
Tibialis anterior		
Red	289±32	373±38
White	110±18	124±13
Extensor digitorum longus	76±14	92±11
Peroneals	109±16	141±16
Knee extensors		
Vastus intermedius	418±52	500±47
Vastus medialis	152±17	225±37*
Vastus lateralis		
Red	407±53	414±44
White	92±42	85±16
Middle	242±29	251±24
Rectus Femoris		
Red	277±35	360±30*
White	131±14	171±16*
Knee flexors		
Biceps femoris		
Anterior	76±10	97±13
Posterior	94±10	117±10
Semitendinosus	46±5	59±9
Semimembranosus		
Red	175±19	215±22
White	48±5	74±9*
Thigh adductors		
Adductor longus	247±58	450±74*
Adductor magnus & brevis	102±10	126±14
Gracilis	38±6	69±17
Pectineus	36±8	51±13

Blood flow (ml/min/100g) measured during submaximal treadmill exercise in all hindlimb muscles of rats fed a diet supplemented with Safflower Oil and Fish Oil. Values are means $\pm$ SE. *$P \leq 0.05$ compared to SO.

Table 4-4 Resting Muscle Vascular Conductance

	Safflower Oil	Fish Oil
Ankle extensors		
Soleus	0.857±0.139	0.696±0.182
Plantaris	0.087±0.019	0.154±0.071
Gastrocnemius		
Red	0.171±0.025	0.272±0.124
White	0.123±0.027	0.124±0.021
Middle	0.084±0.015	0.124±0.051
Tibialis posterior	0.207±0.057	0.168±.051
Flexor digitorum longus	0.357±0.128	0.373±0.057
Flexor hallicus longus	0.095±0.022	0.147±0.062
Ankle flexors		
Tibialis anterior		
Red	0.363±0.099	0.433±0.144
White	0.163±0.037	0.197±0.065
Extensor digitorum longus	0.156±0.037	0.239±0.098
Peroneals	0.116±0.032	0.166±0.061
Knee extensors		
Vastus intermedius	0.580±0.132	0.685±0.195
Vastus medialis	0.091±0.017	0.195±0.077
Vastus lateralis		
Red	0.613±0.135	0.649±0.261
White	0.112±0.034	0.133±0.052
Middle	0.178±0.038	0.200±0.085
Rectus Femoris		
Red	0.138±0.037	0.357±0.138
White	0.114±0.029	0.178±0.060
Knee flexors		
Biceps femoris		
Anterior	0.568±0.059	0.651±0.081
Posterior	0.083±0.020	0.097±0.035
Semitendinosus	0.078±0.014	0.101±0.032
Semimembranosus		
Red	0.110±0.027	0.140±0.063
White	0.084±0.012	0.108±0.041
Thigh adductors		
Adductor longus	0.873±0.146	1.053±0.153
Adductor magnus & brevis	0.102±0.018	0.156±0.059
Gracilis	0.100±0.015	0.113±0.026
Pectineus	0.256±0.079	0.284±0.115

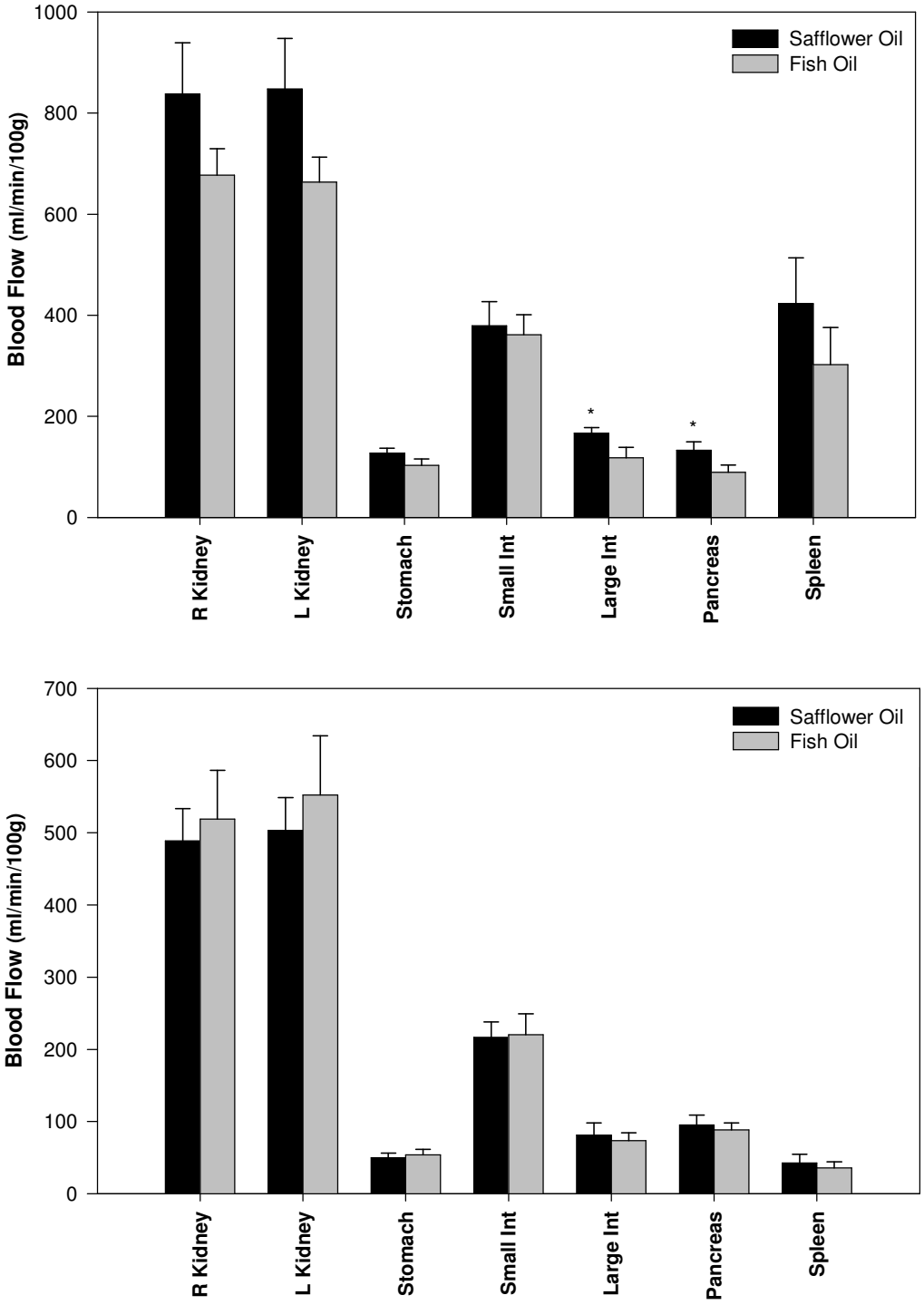
Vascular conductance (ml/min/100g/mmHg) measured at rest in all hindlimb muscles of rats fed a diet supplemented with Safflower Oil and Fish Oil for which there was no effect of Fish Oil. Values are means ± SE.

Table 4-5 Exercise Muscle Vascular Conductance

	Safflower Oil	Fish Oil
Ankle extensors		
Soleus	1.791±0.248	2.432±0.220*
Plantaris	1.584±0.221	1.925±0.185
Gastrocnemius		
Red	3.223±0.128	3.785±0.410
White	0.326±0.058	0.513±0.081*
Middle	1.311±0.185	1.705±0.145
Tibialis posterior	1.418±0.173	1.920±0.2331
Flexor digitorum longus	0.593±0.130	0.890±0.089*
Flexor hallicus longus	0.832±0.121	1.160±0.114*
Ankle flexors		
Tibialis anterior		
Red	2.074±0.258	2.749±0.300
White	0.794±0.143	0.911±0.098
Extensor digitorum longus	0.551±0.107	0.672±0.078
Peroneals	0.779±0.120	1.041±0.127
Knee extensors		
Vastus intermedius	2.986±0.382	3.670±.0351
Vastus medialis	1.085±0.128	1.639±0.264*
Vastus lateralis		
Red	2.909±0.390	3.041±.0333
White	0.634±0.273	0.616±0.112
Middle	1.732±0.219	1.835±0.171
Rectus Femoris		
Red	1.986±0.268	2.649±0.232*
White	0.939±0.102	1.256±0.118*
Knee flexors		
Biceps femoris		
Anterior	0.609±0.033	0.712±0.093
Posterior	0.673±0.085	0.857±0.079
Semitendinosus	0.327±0.040	0.433±0.065
Semimembranosus		
Red	1.253±0.148	1.579±0.162*
White	0.343±0.043	0.542±0.063*
Thigh adductors		
Adductor longus	1.757±0.406	3.325±0.576*
Adductor magnus & brevis	0.727±0.078	0.926±0.105
Gracilis	0.269±0.042	0.506±0.122*
Pectineus	0.262±0.063	0.376±0.092

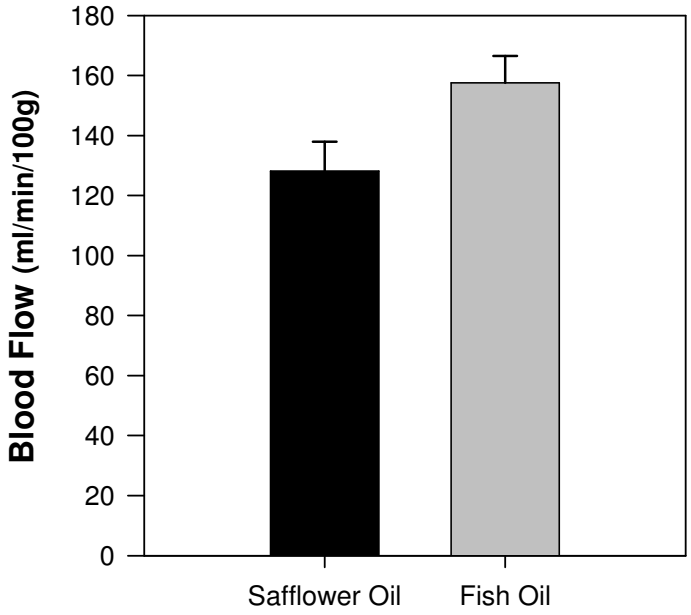
Vascular conductance (ml/min/100g/mmHg) measured during submaximal treadmill exercise in all hindlimb muscles of rats fed a diet supplemented with Safflower Oil and Fish Oil. Values are means $\pm$ SE. *$P \leq 0.05$ compared to SO.

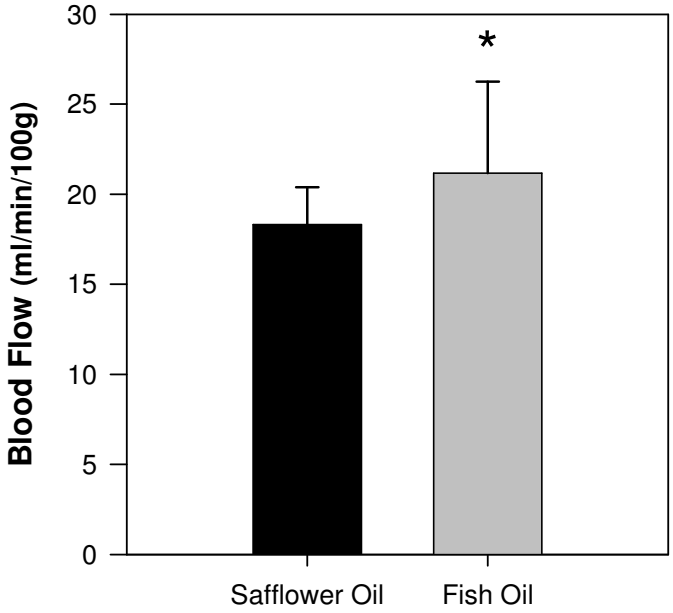
Figure 4-1 Splanchnic Blood Flow



Blood flow to the splanchnic organs measured at rest (upper panel) and during submaximal treadmill exercise (lower panel) in rats fed a diet supplemented with Safflower Oil (Control) and Fish Oil. *P≤ 0.05.

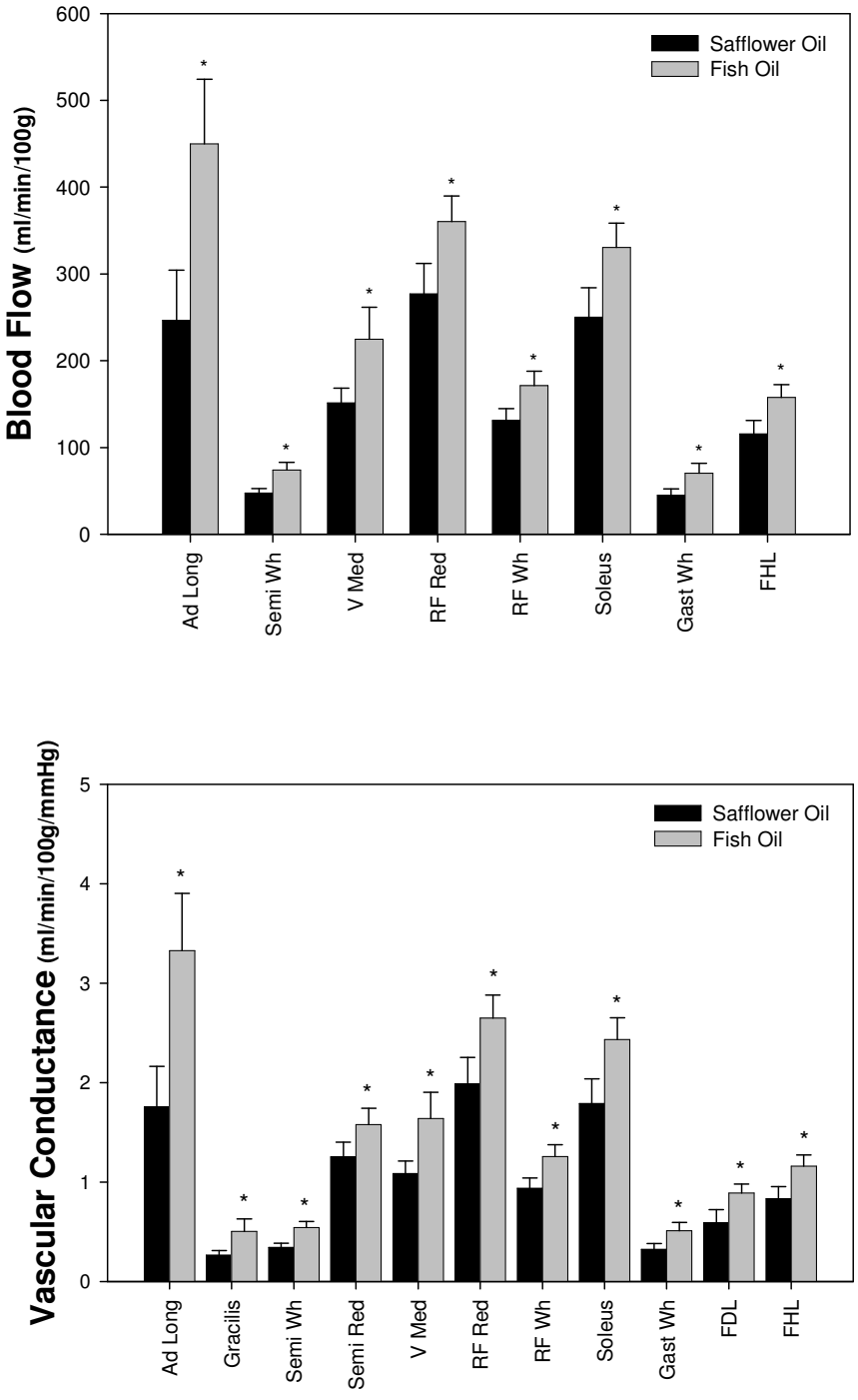
Figure 4-2 Bulk Hindlimb Blood Flow





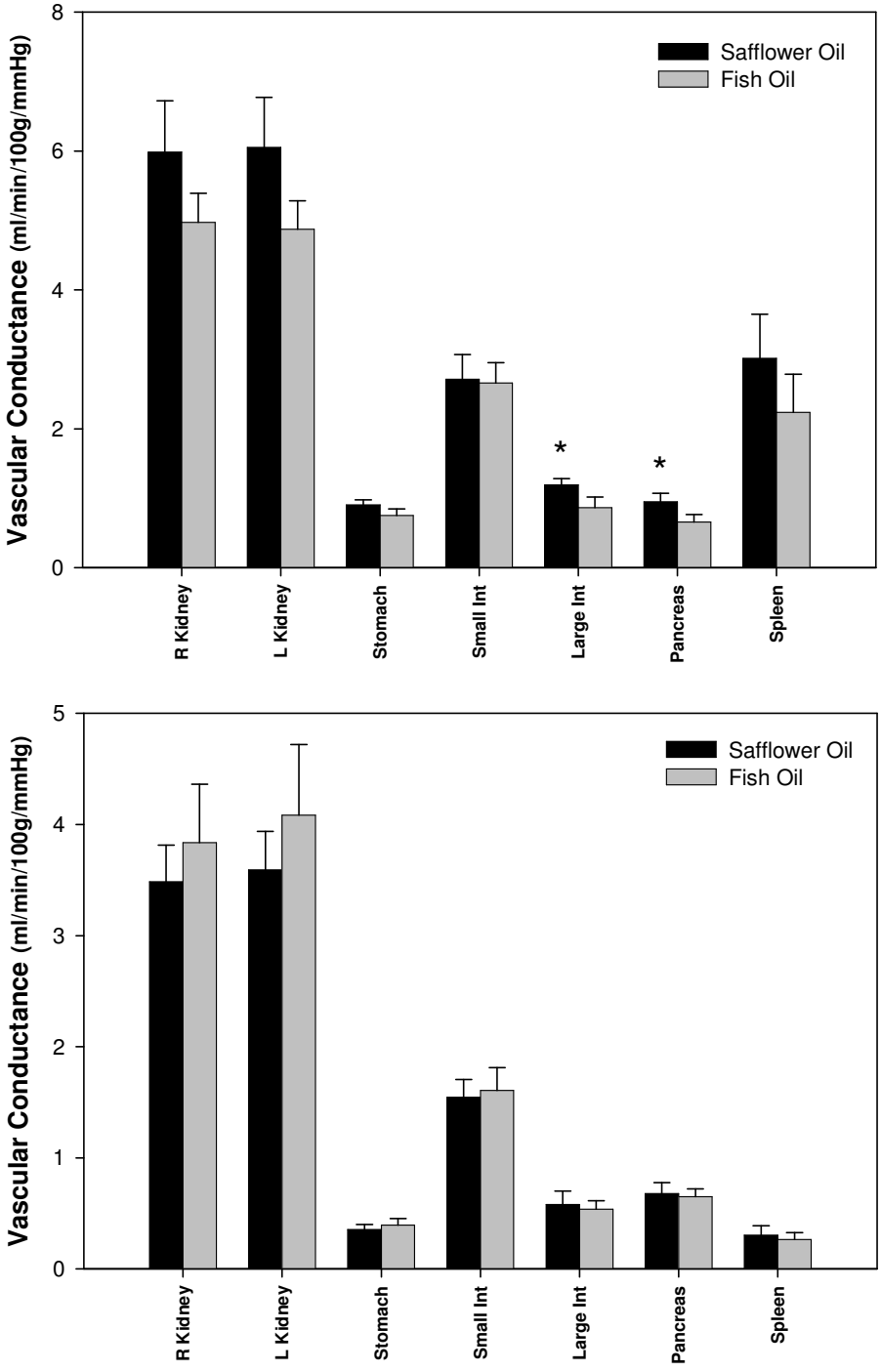
Blood flow to the total hindlimb musculature measured at rest (upper panel) and during submaximal treadmill exercise (lower panel) in rats fed a diet supplemented with Safflower Oil (Control) and Fish Oil. *$P \leq 0.05$.

Figure 4-3 Muscle Blood Flow and Vascular Conductance



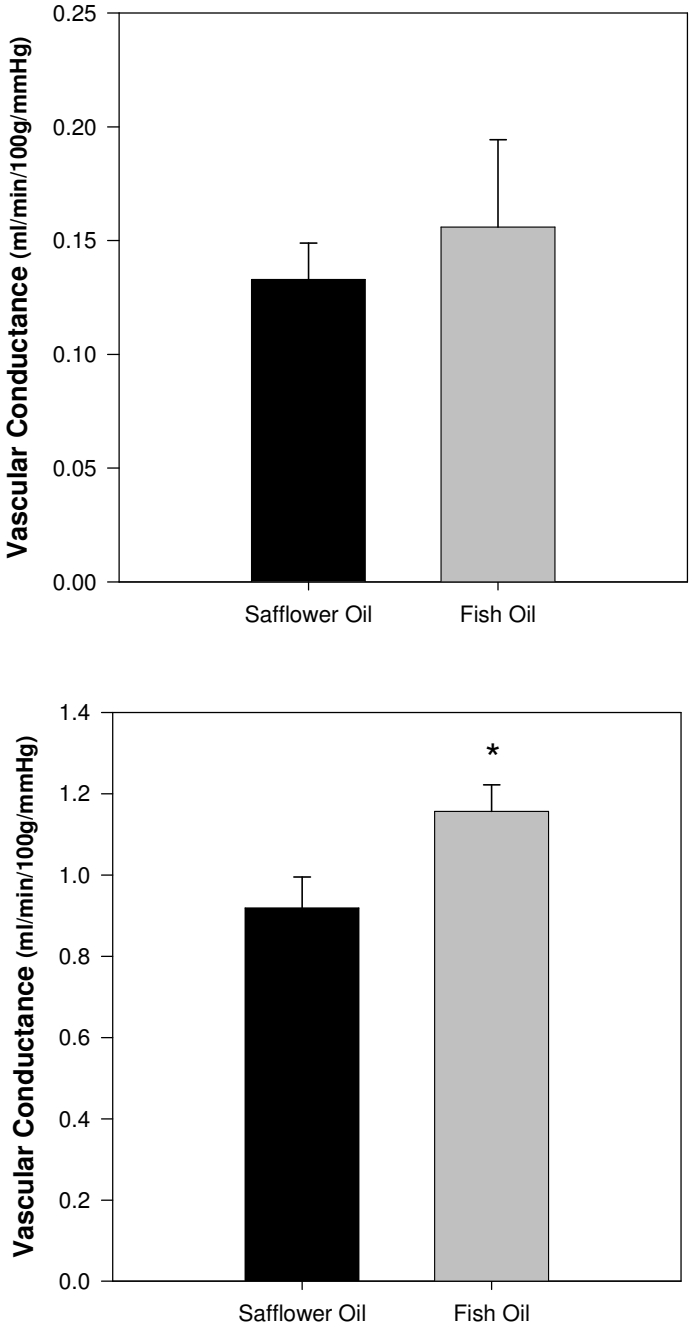
Blood flow (upper panel) and vascular conductance (lower panel) measured during submaximal treadmill exercise in muscles for which there was an effect of Fish Oil. *P< 0.05.

Figure 4-4 Splanchnic Vascular Conductance



Vascular conductance to the splanchnic organs measured at rest (upper panel) and during submaximal treadmill exercise (lower panel) in rats fed a diet supplemented with Safflower Oil (Control) and Fish Oil. *P≤ 0.05.

Figure 4-5 Bulk Hindlimb Vascular Conductance



Vascular conductance to the total hindlimb musculature measured at rest (upper panel) and during submaximal treadmill exercise (lower panel) in rats fed a diet supplemented with Safflower Oil (Control) and Fish Oil. *$P \leq 0.05$.

CHAPTER 5 - Discussion

The principal original finding of the present investigation is that 6 weeks of dietary FO supplementation with DHA and EPA enhances $\dot{Q}_m$ to the total hindlimb during locomotory treadmill exercise and that this enhancement occurs in both oxidative and glycolytic muscle fiber types. Furthermore, this effect occurred in the absence of changes in mean arterial pressure, as demonstrated by the increased VC, suggesting that the effects of PUFAs were manifested in the peripheral vasculature. Our findings agree with previous findings in healthy humans which demonstrated that dietary FO supplementation with DHA and EPA enhanced both $\dot{Q}$ and conductance in the conduit brachial artery during rhythmic handgrip exercise (47). Our investigation is the first, however, to demonstrate that dietary FO supplementation with DHA and EPA enhances $\dot{Q}$ and VC within and among exercising locomotory skeletal muscles. This is significant because enhanced $\dot{Q}$ in the conduit arteries would not be anticipated to enhance $\dot{Q}_m$ without a concurrent increase in conductance in the resistance arteries and arterioles, which was demonstrated conclusively in the present investigation.

Potential Mechanisms

A potential mechanism by which DHA and EPA may have exerted their effects on $\dot{Q}_m$ is by enhanced release of NO from the vascular endothelium. NO is an endogenous signaling molecule that is synthesized by NOS from the vascular endothelium (eNOS) and also the sarcolemma of skeletal muscle (nNOS) (45). When activated, eNOS initiates a cascade of reactions in smooth muscle via cyclic GMP (cGMP) and protein kinase which acts to reduce the intracellular calcium concentration and decrease calcium-dependent phosphorylization of smooth

muscle myosin regulatory light chain thereby relaxing vascular smooth muscle. DHA and EPA supplementation increase acetylcholine-induced NO-dependent relaxation, eNOS mRNA, eNOS expression, cGMP levels, and consequently the bioavailability of NO by ~90% in the aorta of Sprague-Dawley rats (28, 29). Therefore, it appears that these PUFAs can enhance dilation in the conduit vessels during exercise by means of the NO/cGMP system (21). The effects of PUFAs on the NO/cGMP system are pertinent to this investigation because endothelial NO production can be increased in response to the increased shear stress of the flowing blood on the vessel walls during exercise (36, 41). Moreover, previous work from our laboratory has shown regional hindlimb $\dot{Q}$ is attenuated in response to eNOS inhibition with L-NAME during submaximal treadmill exercise (20 m/min, 10% grade, ~65% $\dot{V}O_2max$)(19), thus indicating the important contribution of NO to $\dot{Q}_m$ during exercise in the rat. Therefore, given the important role of NO in the regulation of $\dot{Q}_m$ during exercise, enhanced bioavailability of NO is a likely candidate for the increased $\dot{Q}_m$ seen subsequent to PUFA supplementation in our investigation.

Interestingly, most of the literature has focused on the effects of PUFAs on NO derived from the endothelium (eNOS). However, the intramuscular location of the neuronal isoform of NOS (nNOS) at the sarcolemma and neuromuscular junction has led to speculation that it could diffuse out to smooth muscle cells in the adjacent vasculature, bind to soluble guanylyl cyclase and initiate the cGMP relaxation cascade that is seen with eNOS (16,26). In support of this notion, specific blockade of nNOS has been shown to attenuate exercise hyperemia in humans (18). Similar findings were reported in eNOS knockout mice, where nNOS was implicated in the vasodilation of the vasculature of contracting glycolytic skeletal muscle (16). However, to our knowledge, the effects of PUFAs specifically on nNOS has not been investigated. Therefore, if DHA and EPA exert similar effects on nNOS in the skeletal muscle as they do eNOS in the

endothelium, enhanced nNOS activity/expression could represent another potential mechanism by which DHA and EPA could elevate $\dot{Q}_m$ during exercise.

eNOS is expressed primarily in type I, red, oxidative skeletal muscle (19), whereas nNOS is expressed to a greater extent in type II, white, glycolytic muscle (45). eNOS inhibition causes a greater attenuation of $\dot{Q}$ in the individual muscles composed of oxidative versus glycolytic fibers during treadmill running (19). Therefore, it was expected that DHA and EPA would exert their effects primarily on the muscles composed of oxidative fibers, where eNOS is predominantly expressed in the vasculature, resulting in greater increases in $\dot{Q}$ within those muscles. Interestingly, the results of the present investigation revealed increased $\dot{Q}$ in muscles composed of both oxidative and glycolytic (i.e. low oxidative) fibers. Thus, if DHA and EPA do act to increase nNOS activity/expression, this may be responsible for the unexpected increase in $\dot{Q}_m$ that we found in muscles composed of glycolytic (low oxidative) fibers.

Another potential mechanism by which DHA and EPA may have enhanced $\dot{Q}_m$ and conductance is via the enhanced release of prostaglandins. Enhanced release of both prostacyclin (PGI_2) and prostaglandin I3 (PGI_3) has been demonstrated subsequent to PUFA supplementation (20). The vascular endothelium produces PGI_2 and PGI_3 in response to exercise-induced elevation of shear stress (15), and the release of prostaglandins does contribute to the regulation of $\dot{Q}_m$ at the onset of exercise (11, 50). Additionally, the combined effects of prostaglandins and NO have been demonstrated to contribute substantially to brachial artery $\dot{Q}$ during exercise in humans (43). Therefore, if PUFA supplementation can enhance endothelial release of both prostaglandins and NO, there exists the potential that both may have contributed to the enhanced $\dot{Q}_m$ and VC seen in this investigation.

Another potential factor that may have been responsible for the enhanced $\dot{Q}_m$ following DHA and EPA supplementation is a reduction in vascular oxidative stress. Oxidative stress is a condition in which excess reactive oxygen species (ROS) overwhelm the cells' antioxidant systems and exert deleterious effects on the vascular endothelium including impaired vasodilation (46). Recent evidence indicates that PUFAs can decrease the production of the ROS superoxide (O_2-) (6). Similar findings have been shown in type-2 diabetic and fructose-fed rats (33, 37). Additionally, DHA and EPA enhance superoxide dismutase (SOD) activity in the rat (7). These findings are significant because excess superoxide can react with endogenous NO to generate other ROS such as peroxynitrite ($ONOO^-$), which exerts pernicious effects on endothelial function. Conversely, SOD can also convert O_2- to hydrogen peroxide (H_2O_2), a more stable ROS, that can subsequently be converted to H_2O by catalase or glutathione peroxidase. Consequently, if DHA and EPA reduce O_2- production and increase SOD activity, they could potentially have a positive (lower oxidative state) impact on the redox state in the vasculature. Specifically, reductions in oxidative stress might well improve endothelium-dependent vasodilation in the exercising musculature and contribute to the mechanistic bases for the effects of DHA and EPA on $\dot{Q}_m$ in this investigation.

Dietary supplementation with DHA and EPA also increases muscle sympathetic nerve activity (MSNA) during exercise, at least in humans (32) which should cause a decrease in both $\dot{Q}_m$ and conductance in the exercising muscle. As previously mentioned, however, DHA and EPA supplementation have been able to enhance brachial artery diameter and $\dot{Q}$ during exercise (47). This contradiction may be explained by the ability of DHA and EPA to increase NO bioavailability as described above, thereby increasing endothelium-dependent vasodilation and simultaneously attenuating alpha-adrenergic vasoconstriction in exercising skeletal muscle, a

process termed sympatholysis (4, 8, 42). Therefore, increased NO production or availability subsequent to DHA and EPA supplementation may be responsible for the enhanced $\dot{Q}_m$ and VC seen in the face of increased MSNA.

Significance

Our results have shown that DHA and EPA significantly enhance bulk hindlimb $\dot{Q}_m$ and VC during submaximal treadmill exercise in the absence of significant changes in mean arterial pressure. Therefore, DHA and EPA increased O_2 delivery to the hindlimb musculature during a given level of submaximal exercise. The significance of these findings lies in the ability of increased O_2 delivery to raise microvascular and intracellular O_2 levels in the working muscles and in so doing reduce the degree of metabolic perturbation within the myocytes at a given level of exercise. This could be expected to delay the onset of fatigue and allow the individual to work at a given intensity for longer durations. The present investigation demonstrated that PUFAs can exert a beneficial effect on $\dot{Q}_m$ and VC even in a healthy population. The ability to offset fatigue may be more important, however, in a clinical condition such as cardiovascular disease. Cardiovascular diseases such as coronary artery disease, hypercholesterolemia, type II diabetes, and hypertension are all characterized by increased vascular resistance and endothelial dysfunction during exercise that contribute to a reduced exercise tolerance (23). Therefore, if DHA and EPA can enhance $\dot{Q}_m$ and VC during exercise in these populations, effective application may have profound effects on the quality of life of the affected individuals.

Limitations

The exercise intensity utilized in this investigation was approximately 65% of $\dot{V}O_2max$ (19). This workload was chosen because it limited increases in mean arterial pressure and permitted the rats to run without becoming fatigued. Higher workloads would cause greater shear stress and which would potentially enhance release of NO and/or prostaglandins. Therefore, the effects DHA and EPA may be even more marked at higher exercise intensities.

Conclusions

In conclusion, the present investigation demonstrated that 6 weeks of dietary FO supplementation with DHA and EPA significantly enhanced $\dot{Q}_m$ and VC to the total hindlimb musculature during submaximal treadmill exercise. Additionally, this effect was seen in both oxidative and glycolytic muscle fibers and was coincident without increases in mean arterial pressure. Consequently, these findings support the notion that DHA and EPA supplementation could enhance exercise tolerance by increasing O_2 and substrate delivery to the exercising musculature.

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