Dietary Fish Oil Prevents Vascular Dysfunction and Oxidative Stress in Hyperinsulinemic Rats


Background: Fish oil has been shown to improve blood pressure (BP) in some disease states by an unknown mechanism. We tested the ability of fish oil to prevent vascular dysfunction in fructose-fed rats, a model of insulin resistance and hypertension.

Methods: Rats were placed on three diets: 1) regular rat diet (control); 2) diet containing 60% fructose (FFR); or 3) diet containing 60% fructose and 4.4% fish oil (FFR + FO). After 8 weeks, blood, heart, aorta, and mesenteric artery tissue were collected from each animal. Secondary branch segments of mesenteric arteries were isolated for vascular reactivity studies.

Results: Systolic BP increased significantly in the FFR but was reduced to control levels by the addition of fish oil to the diet. In the mesenteric artery segments from FFR, the dose–response curves to acetylcholine were significantly shifted to the right compared with those of control rats and on the fish oil diet. Expression of endothelial nitric oxide synthase (eNOS) protein and mRNA was reduced in the FFR aortas and hearts, and this reduction was reversed by the fish oil. Dietary fish oil prevented the hyperlipidemia that occurred in the FFR but did not prevent hyperinsulinemia. Plasma concentrations of hydrogen peroxide, 8-isoprostane, and monocyte chemoattractant protein–1 were significantly elevated in the FFR and were significantly lower in the FFR treated with fish oil.

Conclusions: These results demonstrate that dietary fish oil prevents vascular dysfunction in FFR and that this effect of fish oil is associated with increased eNOS expression and decreased oxidative stress. Am J Hypertens 2005;18:213–219 © 2005 American Journal of Hypertension, Ltd.

Key Words: Omega-3 fatty acids, insulin, nitric oxide, hypertension, oxidative stress.

It is well established that feeding normal rats a high-fructose diet induces insulin resistance, hypertension, dyslipidemia, and vascular dysfunction. Although there are many metabolic disorders exhibited by the fructose-fed rat (FFR) model, the cause of hypertension in these animals is not known. Studies show that reactive oxygen species (ROS) are elevated in FFR and can interfere with the production of nitric oxide (NO), which maintains vascular relaxation in resistance arteries. Nitric oxide synthase (NOS) activity itself is also reduced in aortas from FFR. Thus, both a reduction of NOS activity and increased ROS production in FFR could lead to decreased NO bioavailability, resulting in increased vascular contraction and cardiovascular risk seen in this model of the metabolic syndrome.

Dietary fish oil can reduce oxidative stress and production of ROS in animals and humans, and oxidative stress is often associated with hypertension. Fish oil contains omega-3 fatty acids, also referred to as n-3 polyunsaturated fatty acids (n-3 PUFA), which have been associated with improved cardiovascular function in animal and human studies. Huang et al. showed that feeding rats a diet high in fish oil while on a high-fructose diet prevented hyperinsulinemia, hypertriglyceridemia, and hypertension in this model. Dietary eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the two n-3 PUFA found in fish oil, can also prevent hypertension in fructose-fed rats. However, the mechanism by which n-3 PUFA or fish oil prevent hypertension in FFR needs further elucidation. Reduction of oxidative stress is a potential mechanism by which fish oil could prevent hypertension in FFR. The present study determines to what extent...
magnitude dietary fish oil can reduce oxidative stress and improve vascular function in the FFR. The results clearly show that dietary fish oil not only prevents oxidative stress in FFR but that FO also lowers blood pressure (BP) and plasma lipids, improves vascular reactivity, and increases the expression of mRNA for eNOS in vascular tissue. Thus, FO is an ideal therapeutic agent to ameliorate the multiple vascular risks associated with the metabolic syndrome.

Methods

Animals

Animal protocols were approved by the Institutional Animal Care and Use Committee of the Veterans Affairs Greater Los Angeles Healthcare System, and the animals were housed in an AAALAC-approved animal research facility. Male CD:IGS rats (Charles River Laboratories, Wilmington, MA) weighing 250 to 300 g were divided into three groups. The control (CONT) group was fed normal rat chow (Purina, Richmond, IN). The fructose-fed (FFR) group was fed a high-fructose diet (60% fructose by weight, Harlan-Teklad, Madison, WI) and the FFR-fish oil group (FFR+FO) was fed a high-fructose diet for 1 week, then switched to a high-fructose diet that included 4.4% fish oil (Harlan-Teklad) for the remainder of the study. Systolic BP was measured weekly starting at week 2 of the study, using a tail-cuff method with an optical sensor (ITTC Inc., Woodland Hills, CA). After 8 weeks on the diets, the animals were euthanized and blood, heart, aorta, and mesenteric artery samples removed.

Vascular Reactivity Studies

Proximal sections of the duodenum and intact mesenteric vessel arcade were excised from euthanized rats and used as previously described. Briefly, segments of mesenteric resistance artery were transferred to a perfusion chamber (Living Systems, Burlington, VT). The perfusion chamber was mounted on an inverted microscope (Nikon TMS) and the video image of the superfused vessel was analyzed using a video imaging system (Living Systems). Lumen diameter measurements were recorded in response to serotonin or acetylcholine while the intraluminal pressure was maintained at 40 mm Hg. The vessels were contracted with $1 \times 10^{-6}$ mol/L serotonin (Sigma Chemical, St. Louis, MO). After 5 min, acetylcholine (Sigma) was added to the reservoir in cumulative amounts at 2-min intervals.

Plasma Analyses

Plasma glucose concentration was determined using a Glucose Analyzer 2 (Beckman Instruments, Fullerton, CA), and insulin concentration was determined by ELISA (Alpco Diagnostics, Windham, NH). Plasma nonesterified fatty acids (NEFA), triglycerides, and total cholesterol were determined with enzymatic assays (Wako Diagnostics, Richmond, VA). 8-Isoprostane and monocyte chemoattractant protein–1 (MCP-1) were determined in plasma using ELISA methods (Cayman Chemical, Ann Arbor, MI, and Assay Designs, Inc., Ann Arbor, MI).

FIG. 1 Time course of the systolic blood pressure (BP) changes during the dietary studies. Both the fructose-fed rats (FFR; ■) and the fish oil-treated FFR (FFR+FO; □) groups were started on the high-fructose diet at week 0. The FFR+FO group had the fish oil added to their diet at week 1. The BP measurements were started at week 2. After week 4, BP was consistently higher in FFR than in control (CONT; ●) or FFR+FO rats. At the end of the study period, systolic BP was significantly higher in the FFR group when compared with either CONT or FFR+FO. *$P < .01$ v CONT or FFR+FO; **$P < .001$ v CONT or FFR+FO.

FIG. 2 Cumulative dose–response curves to acetylcholine in mesenteric artery segments. Arteries were precontracted with serotonin and then exposed to increasing cumulative concentrations of acetylcholine. The curve from the fructose-fed rats (FFR; ■) is shifted to the right ($P < .05$ by two-way ANOVA) when compared with that of control rats (CONT; ●). Addition of fish oil to the high-fructose diet (FFR+FO; □) prevented this shift to the right.
respectively). Hydrogen peroxide in plasma samples was determined using an amplex red assay (Molecular Probes, Eugene, OR).

Semiquantitative Reverse Transcription–Polymerase Chain Reaction

Tissue samples for reverse transcription–polymerase chain reaction (RT-PCR) analysis were stored in RNA later (Ambion Inc., Austin, TX). The RNA was extracted from tissue samples using TRI Reagent (Sigma) and reverse-transcribed using an Omniscript RT kit (Qiagen, Valencia, CA). Gene-specific oligos were custom synthesized (Qiagen) and used with a hot-start Taq DNA polymerase (iTaq, Bio-Rad Laboratories, Hercules, CA). For endothelial nitric oxide synthase (eNOS), the oligos used were as follows: sense, TCCAGTAACACAGACACTGCA; antisense, CAGGAAGTAGTGAAGGC. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the oligos used were: sense, TCCCTCAAGATTGTCAGCAA; antisense, AGAGTCCACAACGGATACATT. Thermal cycling (iCycler, Bio-Rad Laboratories) was programmed for 94°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec (for 32 cycles). The PCR products were electrophoresed on ethidium bromide–containing agarose gels and then visualized, recorded, and analyzed using a Kodak 290 EDAS imaging system with 1D Analysis Software (Kodak, Rochester, NY). The RT-PCR results were calculated as eNOS/GAPDH densitometry ratios.

ELISA Determination of eNOS Protein Expression

Protein fractions from the TRI reagent heart tissue homogenates were treated per manufacturer’s instructions. The concentration of protein was measured using BCA reagents (Pierce, Rockford, IL) and the eNOS protein was quantitated using an eNOS ELISA kit (Quantikine, R & D Systems, Minneapolis, MN).

Statistical Analysis

Values shown are means ± SEM. The Student t test was used to test for significance of differences between groups. Two-way ANOVA was used to determine significance of differences in the dose–response curves. Values of P < .05 indicated significant difference.

Results

During the 8-week study, animal weight did not differ among the three diet groups. The high-fructose diet caused a significant increase in systolic BP within the first 2 weeks, when the first BP determinations were made. The inclusion of fish oil in the high-fructose diet after the first week of the study reduced systolic BP to a level not different from control (Fig. 1). The marked reduction in BP with fish oil occurred at week 4 and was sustained for the remainder of the study period.

Table 1. Plasma concentrations of metabolic parameters

<table>
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<tr>
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<th>Insulin (ng/mL)</th>
<th>Glucose (mg/dL)</th>
<th>TG (mg/dL)</th>
<th>T-Chol (mg/dL)</th>
<th>NEFA (mEq/mL)</th>
</tr>
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<tbody>
<tr>
<td>CONT</td>
<td>1.50 ± 0.23</td>
<td>371.5 ± 20.2</td>
<td>98.6 ± 10.9</td>
<td>40.4 ± 5.5</td>
<td>0.080 ± 0.008</td>
</tr>
<tr>
<td>FFR</td>
<td>2.71 ± 0.42*</td>
<td>405.5 ± 44.9</td>
<td>284.2 ± 32.2†</td>
<td>74.9 ± 8.8†</td>
<td>0.205 ± 0.035†</td>
</tr>
<tr>
<td>FFR+FO</td>
<td>3.42 ± 0.79*</td>
<td>422.4 ± 42.2</td>
<td>136.5 ± 21.8‡</td>
<td>55.6 ± 7.5§</td>
<td>0.124 ± 0.025§</td>
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CONT = control rats; FFR = fructose-fed rats; FFR+FO = fructose fed rats with added fish oil; NEFA = nonesterified fatty acids; TG = triglycerides; T-Chol = total Cholesterol.

Values are means ± SEM, n = 8 per group.

* P < .05 v CONT; † P < .01 v CONT; ‡ P < .01 v FFR; § P < .05 v FFR.

FIG. 3 Plasma concentrations of hydrogen peroxide in the different groups. The concentration of hydrogen peroxide in the plasma from the fructose-fed rats (FFR) was significantly elevated compared with that in control rats. Inclusion of fish oil in the high-fructose diet (FFR+FO) prevented this increase in plasma hydrogen peroxide. *P < .001 v Control; **P < .001 v FFR.
Vascular reactivity to acetylcholine in the mesenteric artery segments show that the dose–response curve was shifted to the right in FFR (P < .05 v control) (Fig. 2). Treatment with fish oil (FFR+FO) returned the dose–response curve toward the left (P < .05 v FFR). This finding signifies a decreased sensitivity to acetylcholine in the FFR group that was completely prevented by dietary fish oil.

Plasma NEFA, triglycerides, and total cholesterol concentrations were all significantly elevated in the FFR group, but addition of fish oil significantly reduced these parameters (Table 1). Insulin was elevated in the FFR group and was not affected by the addition of fish oil (Table 1). Plasma glucose concentrations were similar in all three dietary groups.

The three indices of oxidative stress, hydrogen peroxide, MCP-1, and 8-isoprostane were all significantly elevated by fructose feeding, and the inclusion of fish oil in the diet prevented these increases (Figs. 3 to 5).

The expression of eNOS mRNA was significantly reduced in the aortas of the FFR group, and the addition of fish oil to the diet of high-fructose–fed rats increased eNOS expression (Fig. 6). Similar results for eNOS mRNA expression were also observed in the heart tissue from these groups (Fig. 7). In heart tissue, protein expression of eNOS was also decreased significantly in the FFR, and this reduced expression was prevented by dietary fish oil (Fig. 8).

**Discussion**

In this study, we demonstrate that dietary fish oil not only prevents many metabolic and cardiovascular dysfunctions induced by a high-fructose diet, but that fish oil also markedly attenuates oxidative stress observed in the FFR. The substance MCP-1 is an inflammatory chemokine, the production of which is upregulated by ROS and is associated with development of atherosclerosis. 8-Isoprostane (8-iso-prostaglandin F2α) is a nonenzymatic oxidative metabolite of arachidonic acid that is an indicator of oxidative stress. The observed increase of both of these parameters in the FFR indicates that oxidative stress is increased in this animal model of insulin resistance.

Plasma H2O2, a reactive oxygen species itself, was also elevated in the FFR and was normalized by fish oil intake. The ability of the dietary fish oil to prevent the increases of these three indicators of oxidative stress suggests that the fish oil fatty acids may prevent ROS-induced cellular dysfunction through direct or indirect mechanisms. One factor could be the elevated cholesterol concentration in FFR, as hypercholesterolemia has been shown to increase oxidative stress, so that the fish oil–induced prevention of oxidative stress may be due to the lowering of plasma cholesterol and triglycerides. Also, n-3 PUFA from fish oil can be readily oxidized themselves and could function as...
oxygen scavengers to directly prevent oxidative stress to the FFR tissue.

Dietary fish oil was also found to reverse dyslipidemia in FFR, as observed in studies in insulin-resistant rats.\(^{15,21}\) In the FFR we found that both plasma triglycerides and total cholesterol concentrations were significantly increased but were lower in the FFR\(^{-}/H_1\) FO group. Other workers have suggested that the n-3 PUFA compounds in fish oil are peroxisome proliferator–activated receptor-\(\alpha\) (PPAR\(\alpha\)) ligands\(^{22,23}\) and, by interacting with PPAR\(\alpha\) in the liver, could reduce the dyslipidemia found in the FFR. One of the omega-3 fatty acids found in fish oil, DHA, has been shown to reduce angiotensin II–induced oxidative stress and inflammation in the vascular walls of rats by activating PPAR\(\alpha\).\(^{24}\)

High-fructose diet also induces high BP and vascular dysfunction in FFR, as assessed by tail-cuff methods and vascular reactivity studies with acetylcholine. The increase in BP and the impaired vascular reactivity were completely prevented by dietary fish oil. Because the decreased vasodilatory response to acetylcholine in the FFR is a form of vascular dysfunction, its reversal or prevention by fish oil would be associated with the lowering of BP in the FFR\(^{-}/H_1\) FO rats. Vascular dysfunction of resistance arteries is often associated with hypertension; thus, this improvement in relaxation response to acetylcholine may decrease BP in these rats. Other investigators have suggested that DHA, one of the fatty acids in fish oil, improves BP in fructose-fed rats by affecting heart rate and cardiac adrenergic function.\(^{16}\)

Acetylcholine-induced vascular relaxation is a NO-dependent event. Thus, variables that can affect nitric oxide metabolism may be important determinants in how the high-fructose diet impairs vascular function. Reactive oxygen species such as \(\text{H}_2\text{O}_2\) can interact with NO very rapidly to produce peroxynitrite.\(^{4}\) The decreased eNOS mRNA expression observed in the cardiovascular tissues of the FFR group further emphasizes the impaired NO-dependent relaxation in FFR, which improves with fish oil added to the diet. Studies show that eNOS protein expression and eNOS activity is decreased in vascular tissue from FFR,\(^{3}\) and our results support these observations. The mechanism by which dietary fish oil increases expression of vascular eNOS mRNA may be related to the ability of fish oil to reduce oxidative stress or dyslipidemia or both. Some reports have failed to show decreased eNOS mRNA using an RNAase protection assay in aortic tissue from FFR.\(^{25}\) Our results demonstrate that the decrease of eNOS occurs at the transcriptional level, which would result in reduced production of the eNOS protein, thereby reducing eNOS activity. This decrease in eNOS expression in arterial tissue is most likely the cause of the

**FIG. 6** Analysis of aortic tissue for expression of endothelial nitric oxide synthase (eNOS) mRNA by reverse transcription–polymerase chain reaction (RT-PCR). The RT-PCR was conducted as described in the Methods section, and quantities were normalized with GAPDH values. Upper panels show the eNOS and GAPDH bands from representative samples from the three groups. Bar graph depicts the combined results from eight samples. The expression of eNOS mRNA was significantly lower in the aortas from the fructose-fed rats (FFR). Addition of fish oil to the FFR diet (FFR\(^{-}/H_1\) FO) prevented this downregulation and returned expression to control levels. *\(P < .05\) v Control; **\(P < .05\) v FFR.

**FIG. 7** Analysis of heart tissue for expression of eNOS mRNA by reverse transcription–polymerase chain reaction (RT-PCR). Upper panels show eNOS and GAPDH bands from representative samples from the three groups. Bar graph depicts combined results from four samples. The expression of eNOS mRNA was significantly decreased in the heart tissue from the fructose-fed rats (FFR). Addition of fish oil to the FFR diet (FFR\(^{-}/H_1\) FO) increased eNOS mRNA expression. *\(P < .02\) v Control; **\(P < .03\) v FFR.
vasorelaxation defect that we reported in our previous studies of FFR from this laboratory.17 The observation that cardiac expression of eNOS mRNA and protein also decreased suggests a more universal eNOS deficiency in FFR, as other workers have also found decreased renal eNOS protein expression in FFR.26

Several potential mechanisms of endothelial dysfunction–induced hypertension have been reported. One putative mechanism is the production of ROS via an endothelin/NADPH oxidant pathway in DOCA-salt hypertensive rat; and, interestingly, overexpression of eNOS destabilizes eNOS mRNA, thus resulting in lower expression of eNOS protein.31–33 Elevated cholesterol is associated with increased production of ROS.30,34 In addition, hypercholesterolemia is associated with increased production of ROS.30,34 Thus, in our study, endothelial dysfunction could be the result of oxidative stress or hyperlipidemia, both of which were corrected by the fish oil diet.

The mRNA for eNOS is constitutively expressed, but its expression can be regulated at both a transcriptional and post-transcriptional level.11–33 Elevated cholesterol is one factor present in the FFR that has been shown to destabilize eNOS mRNA, thus resulting in lower expression.34 Hypertriglyceridemia is another factor seen in the FFR, an effect that was prevented by the fish oil diet. The present study demonstrates that dietary fish oil can prevent the oxidative stress caused by a high-fructose diet, a result that is associated with reduction of hypertension, improved vascular reactivity, reduced dyslipidemia, and increased eNOS mRNA expression. Thus, fatty acids from fish oil are beneficial in reversing a large number of cardiovascular and metabolic disorders in the FFR, a model of the metabolic syndrome. Such therapy may have great potential in addressing multiple disorders with a single dietary treatment to reduce the high cardiovascular risk seen in patients with insulin resistance, such as those with the metabolic syndrome.

### References

FISH OIL PREVENTS VASCULAR DYSFUNCTION IN FRUCTOSE-FED RATS


