Amelioration of Insulin Resistance and Hypertension in a Fructose-Fed Rat Model With Fish Oil Supplementation

Y.-J. Huang, V.S. Fang, C.-C. Juan, Y.-C. Chou, C.-F. Kwok, and L.-T. Ho

In type II diabetic patients, one can detect several pathologic changes including insulin resistance and hypertension. Sprague-Dawley rats fed a fructose-rich diet (group F) exhibited these characteristic abnormalities within 2 weeks and were an excellent laboratory animal model for research on insulin action and development of hypertension. Since fish oils containing omega-3 fatty acids have a beneficial effect in preventing atherosclerotic diseases, we performed repeated experiments to test the effects of fish oil supplementation in group F rats. Compared with control rats on a normal diet (group C), group F consistently developed hypertriglyceridemia without elevated plasma free fatty acid (FFA), fasting hyperinsulinemia together with fasting hyperglycemia (insulin resistance syndrome), and systolic hypertension within 3 weeks. Insulin-stimulated glucose uptake and insulin binding of adipocytes were significantly reduced. Rats fed the same high-fructose diet but supplemented with fish oil (group O) had alleviation of all of these metabolic defects and a normalized insulin sensitivity and blood pressure. β-Cell function as shown by plasma glucose and insulin responses to oral glucose remained intact in group F and group O. The plasma endothelin-1 (ET-1) level and ET-1 binding to adipocytes were not different among the three groups. Based on these results, we suggest that dietary high fructose induced hypertriglyceridemia and insulin resistance with normal islet function, and that the induced hypertension was not associated with plasma ET-1 abnormalities and was probably caused by other undefined pathologic changes that can be prevented by dietary omega-3 fatty acids.

COMPARED WITH THE general population, patients with non–insulin-dependent diabetes mellitus (NIDDM) have a much higher cardiovascular morbidity and mortality[1] because they manifest many of the contributory risk factors such as visceral obesity, hyperlipidemia, hyperinsulinemia, hypertension, etc. Although evidence for the interrelationship among these risk factors is so strong that they are now collectively called "syndrome X,"[2] the underlying biochemical mechanism(s) for the associations is not totally clear. To study these metabolic disorders in the laboratory, several animal models are available. For example, when Sprague-Dawley rats are fed fructose as the entire dietary carbohydrate, they become hyperinsulinemic and hypertensive within 2 weeks,[3] a pathologic status resembling human NIDDM.[4]

Fish oils rich in the long-chain polyunsaturated omega-3 fatty acids eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3) are known to have potentially antiatherosclerotic effects[5] and to reduce the mortality of human coronary heart disease[6] and the incidence of animal experimental myocardial and cerebral infarction.[7,8] Studies reviewed by Leaf and Weber[9] indicated the diverse actions of n-3 fatty acids, including hypotensive, hypotriglyceridemic, and antithrombotic actions, through various mechanisms. Sierra et al[10] observed that in euglycemic but hyperinsulinemic conditions, prefeeding with fish oil rich in n-3 fatty acids significantly improves muscular uptake of glucose without altering hepatic glucose production in rats treated with tumor necrosis factor. However, in human studies, fish oils given to diabetic patients may cause a mild degree of glucose intolerance or produce no effect on glucose homeostasis.[11-13] Thus, the beneficial effect of fish oil on glucose metabolism remains an unsettled issue.

Dietary fish oil supplementation has been shown to prevent hypertension by increasing the endothelium-dependent relaxation of porcine atherosclerotic coronary vasculature in response to pharmacological stimulation.[14] This vasodilating response is mediated principally through the increased release of endothelium-derived relaxing factors but not nitric oxide.[15] The potential role of endothelin-1 (ET-1) as a vasoconstrictor from the same endothelial and vascular system has not been explored. Since we have found that ET-1 suppressed insulin-mediated glucose uptake in the rat adipocyte,[16] the present study was designed to evaluate the effect of fish oil supplementation on insulin resistance, hypertension, and ET-1 binding in this fructose-fed rat model.

MATERIALS AND METHODS

Animal Preparation

Male Sprague-Dawley rats weighing 200 to 250 g were purchased from a local breeder and housed two per cage in an air-conditioned room (22° ± 2°C) with a 12-hour light cycle (6:00 AM to 6:00 PM). Before dietary manipulation, all rats were fed standard rat chow (Purina, St Louis, MO) composed of 60% vegetable starch, 12% fat, and 28% protein. Rats were acclimated to a mock blood pressure measurement between 10:00 AM and 4:00 PM three times for 1 week. Then, they were divided into three groups: the control (C) group continued on regular Purina chow while two groups of rats were fed a diet of 66% fructose, 12% fat, and 22% protein (Teklad, Madison, WI). In one of these fructose groups, 0.4 mL fish oil containing eicosapentaenoic acid (38.6%) and docosahexaenoic acid (25.8%) was administered by gavage at 6:00 PM every day. Rats on the fructose diet alone were designated as group F, and the other group receiving the fish oil supplement was group O. The dietary manipulation lasted for 3 weeks. This amount of fish oil constituted approximately 5% of the total energy supply to the rats. Experiments were performed according to the Taiwan Government Guide for the Care and Use of Laboratory Animals. The

From the Departments of Medical Research and Education and Internal Medicine, Veterans General Hospital-Taipei, Taipei; Department of Physiology, National Yang-Ming University, Taipei; and Department of Clinical Pathology, Military 803 General Hospital, Taipei, Taiwan, Republic of China.

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Address reprint requests to L.-T. Ho, MD, Department of Medical Research and Education, Veterans General Hospital-Taipei, 201 Shih-Pai Rd, Taipei, Taiwan, Republic of china.

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protocol was approved by the animal welfare committees of the Veterans General Hospital-Taipei and National Yang-Ming University.

Blood Pressure Measurement

Rats were transported to a quiet environment at 8:00 AM and kept in cages with free access to water. Blood pressure was measured after 10:00 AM by a tail-cuff method using Narco Bio-System Physiograph (Houston, TX) equipment.[17] The small-animal study unit of the equipment has a rat-holder base with a built-in warming element for increasing the ambient temperature to 37°C and maintaining an adequate circulation in the rat's tail to measure systolic blood pressure reliably. Since blood pressure was measured in conscious animals, the results are identical to those determined by arterial cannulation. The mean of five consecutive readings represents the final result of each blood pressure measurement.

Oral Glucose Tolerance Test

After 2 weeks on diet manipulation, the rats were subjected to an oral glucose tolerance test (OGTT) following the procedure described by Whittington et al.[18] After overnight fasting, a 0-hour blood sample (0.6 mL) was taken by cutting the tail tip. Without delay, a glucose solution (2 g/mL/kg body weight) was administered by gavage. Four more tail vein blood samples were taken at 30, 60, 90, and 120 minutes after glucose administration. All blood samples were collected in Eppendorf vials prerinsed with heparin solution (20 IU/mL) and kept on ice until centrifugation (3,500 × *g* at 4°C for 30 minutes) to separate plasma. The isolated plasma specimens were frozen at −20°C until later assays of glucose and insulin.

Isolation of Adipocytes

At the end of 3 weeks on diet manipulation, rats were killed by decapitation after an overnight fast, and the blood was collected in heparinized tubes with added aprotinin (400 KIU/mL blood). Plasma was separated by centrifugation and stored at −20°C until assays of triglyceride (TG), free fatty acid (FFA), ET-1, glucose, and insulin. The epididymal fat pads excised from each group of rats were pooled to isolate adipocytes by the Rodbell method[19] with minor modifications.[16] Briefly, fat tissue was minced and incubated in Krebs-Ringer bicarbonate (KRB) buffer solution containing pyruvate (1 mmol/L), bovine serum albumin (1%), and collagenase (0.1%) in an oxygen-rich shaking chamber (CO_2:O_2, 5:95; 50 strokes/min) for 1 hour at 37°C. The digested tissue suspension was filtered through nylon mesh (400 μm) and centrifuged at 100 rpm for 1 minute. The supernatant layer of fat cells was harvested and washed twice in the same KRB buffer containing pyruvate and albumin. The cell number of the adipocyte preparation in KRB buffer solution was determined after fixation with 2% osmium tetraoxide. The lipocrit was checked and used for normalization of the fat cell number before, during, and after each experiment.

Insulin Binding of Adipocytes

Binding of insulin to adipocytes was performed according to the procedure previously described.[16,20] Briefly, to aliquots of fat cells (2×10^5 cells/400 μL), 50 μL [^{125}I]insulin (to a final concentration of 0.25 nmol/L, or ~5×10^5 cpm/tube) and an increasing concentration of unlabeled insulin (0.1 pmol/L to 100 nmol/L/50 μL) were added. The cells were incubated in a 95% oxygen chamber at 37°C with gentle shaking (50 strokes/min) for 30 minutes. Then, 300 μL cell suspension was transferred to a new centrifuge tube containing 200 μL silicon oil. The mixture was centrifuged (1,000 × *g*) at room temperature for 1 minute. The cellular layer was taken for radioactivity counting as a measure of insulin binding. The nonspecific binding tube contained 1 μmol/L unlabeled insulin. A Scatchard plot was used to determine insulin binding sites (B_{max}) and binding affinity (K_d) of the cells.

ET-1 Binding of Adipocytes

ET-1 binding to fat cells was performed as reported previously,[16] using [^{125}I]ET-1 as tracer and unlabeled ET-1 as competitive binding ligand. Nonspecific binding was determined using 1 μmol/L unlabeled ET-1 in the reaction mixture. A Scatchard plot was used to determine ET-1 binding sites (B_{max}) and binding affinity (K_d) of the cells.

Glucose Transport Into Adipocytes

Insulin-stimulated glucose uptake by adipocytes was determined by measuring the transport of 2-deoxyglucose (2-DG) into the cells as described by Garvey et al[21] with some modifications.[16] Briefly, 400 μL fat cell suspension was mixed with an increasing concentration of insulin (0 to 100 nmol/L/50 μL) and incubated as before for 30 minutes. [^{3}H]2-DG (50 μL to a final concentration of 50 μmol/L) was added, and the incubation continued for 3 more minutes. The incubation was terminated by adding 200 μL unlabeled 2-DG (0.5 mol/L) to the mixture. Then, 300 μL cell suspension was transferred to a new vial containing 200 μL silicon oil. After centrifugation at 1,000 × *g* for 1 minute, the cellular layer was transferred to a vial containing 4 mL cocktail (Aquasol; New England Nuclear, Boston, MA) for counting radioactivity in a liquid scintillation counter.

Measurements for Glucose, Insulin, and ET-1

Plasma glucose concentration was measured by a glucose analyzer (Model 23A; Yellow Springs Instruments, Yellow Springs, OH). Plasma insulin was determined by a radioimmunoassay technique developed in our laboratory,[22] which can be used for assaying both human and rat insulin. Plasma TG and FFA content were determined by colorimetric methods.[23,24] ET-1 in plasma samples was extracted with a C18 Sep-Pak cartridge according to the procedure described by Xuan et al[25] and then assayed with a radioimmunoassay kit supplied by Peninsula Laboratories (Belmont, CA).

Statistical Analyses

There were three groups with six rats each, for a total of 18 rats in each experiment. We performed the same designed experiment three times (n = 3). Individual data for systolic blood pressure and plasma TG, FFA, ET-1, glucose, and insulin, including the incremental areas under the curve for glucose and insulin, were pooled in each experiment to calculate group means. These group means were further pooled, expressed as the grand mean ± SD, and compared by ANOVA. Since adipocytes were isolated from pooled fat tissue for each group of rats before measurements were made in duplicate or triplicate, the mean insulin- and ET-1-binding and glucose-transport data were calculated, expressed directly as the mean ± SD (n = 3), and compared by ANOVA. Statistical analyses were made using a computer program provided by the Microsoft Excel Kit (GreyMatter International, Cambridge, MA). The difference between any two groups is considered statistically significant at P less than .05.

RESULTS

The effects of a fructose-enriched diet and fish oil supplement on rat body weight and systolic blood pressure are shown in Fig 1. Dietary manipulation did not affect the normal growth of the rats, since the growing weight of the three groups was not statistically different even though fish oil seemed to retard growth slightly. Compared with the stable blood pressure of group C, group F showed significantly elevated blood pressure on day 13, which increased further hence as seen on day 15. Since blood pressure in group O was normal, fish oil supplementation apparently prevented the development of hypertension that occurred in group F.

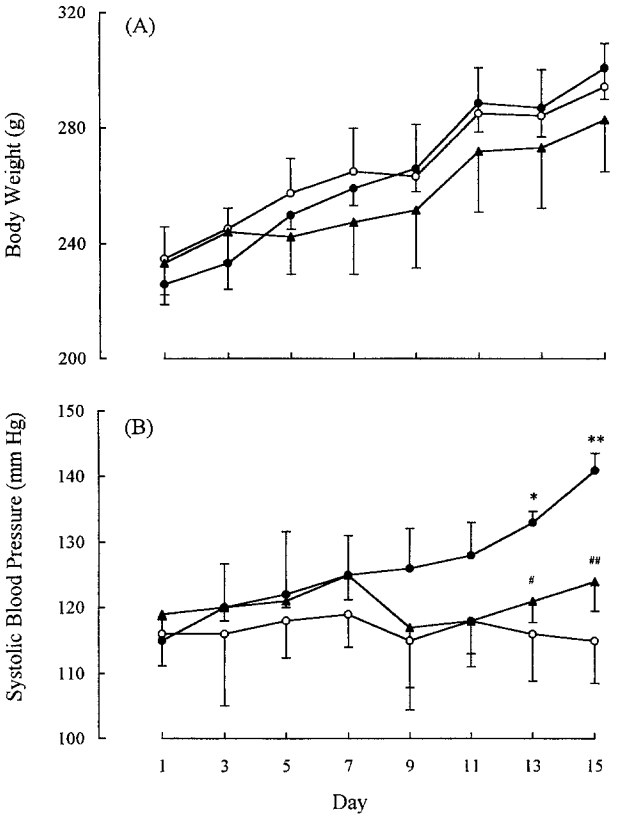

Fig 1. Body weight (A) and blood pressure (B) changes in rats since dietary manipulation on day 0. Rats of the C group (○) were fed regular Purina Chow, F group (●) high-fructose diet alone, and O group (▲) high-fructose diet with fish oil supplementation. Bar represents the SD from 3 experiments with 6 rats per group and 3 groups in each experiment. *$P < .05$, **$P < .01$: *v* group C. #$P < .05$, ##$P < .01$: *v* group F. (The same symbols are used for all figures.)

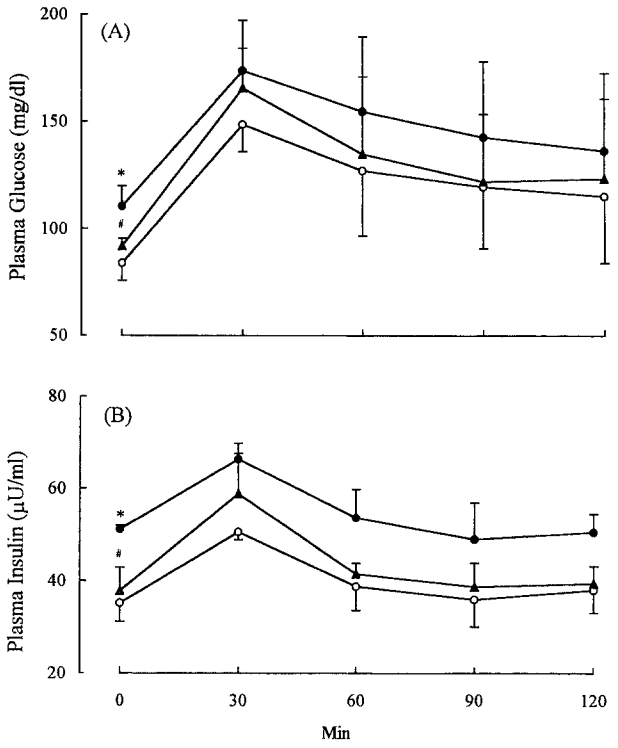

Fig 2. Plasma glucose (A) and insulin (B) responses in rats to an OGTT performed at the end of the second week of dietary manipulation.

At the end of the 3-week experiment, both fasting hyperinsulinemia and fasting hyperglycemia were obvious in group F (Table 1). OGTT results shown in Fig 2 confirmed these findings, which happened even before the third week. Basal fasting plasma glucose and insulin levels in group F were significantly higher than in group O and group C, but they were not different between groups O and C. During the 2 hours after glucose ingestion, all three groups had an elevated plasma glucose and insulin with almost the same response profiles—they peaked at 30 minutes and returned to basal levels within 90 minutes. The incremental area under the curve from 0 to 60 minutes representing the magnitude of insulin response was 518 ± 55.4, 494 ± 117.8, and 584 ± 76.2 μU · min/mL and the magnitude of the glucose response was 2,571 ± 110.8, 2,563 ± 90.1, and 2,842 ± 138.6 mg · min/dL for groups C, F, and O, respectively. These results were not statistically different among the three groups and therefore indicated that all rats had an intact physiological function of the β cells. However, the presence of fasting hyperinsulinemia together with fasting hyperglycemia observed in group F could be interpreted that these rats had developed insulin resistance, whereas in group O such an insulin defect was alleviated but not completely abolished by the fish oil supplement. If high-fructose ingestion was responsible for the defective insulin action on fasting glucose disposal, then the fish oil supplement was effective to counteract it.

The results of glucose uptake by adipocytes are shown in Fig 3. Insulin stimulated the glucose transport of fat cells in a dose-dependent manner. The basal glucose uptake of fat cells in group F (43.0 ± 8.0) was significantly less than in group C (80.2 ± 3.3) and group O (82.2 ± 1.6 fmol/10^5 cells/3 min, $P < .01$). The maximally stimulated glucose uptake (with 1 nmol/L insulin) by adipocytes from group F (160.8 ± 37.9) was also significantly less than from group C (366.0 ± 32.6, $P < .01$) and group O (338.7 ± 57.3 fmol/10^5 cells/3 min, $P < .05$).

Table 1. Fasting Plasma Glucose, Insulin, FFA, TG, and ET-1 Concentrations at the End of a 3-Week Dietary Manipulation

Group	Glucose (mg/dL)	Insulin (μU/mL)	FFA (μmol/L)	TG (mg/dL)	ET-1 (pg/mL)
C	90.6 ± 9.08	36.3 ± 4.62	593.1 ± 7.46	89.2 ± 21.0	3.34 ± 1.00
F	112.1 ± 2.93*	62.0 ± 7.93†	588.6 ± 9.73	212.6 ± 69.9†	6.84 ± 5.46
O	97.3 ± 10.76	36.6 ± 4.05§	587.7 ± 5.40	109.6 ± 49.4‡	4.43 ± 2.37

NOTE. Results are the mean ± SD from 3 experiments (n = 3). There were 3 groups and 6 rats per group in each experiment.
*$P < .05$, †$P < .01$: *v* group C.
‡$P < .05$, §$P < .01$: *v* group F.

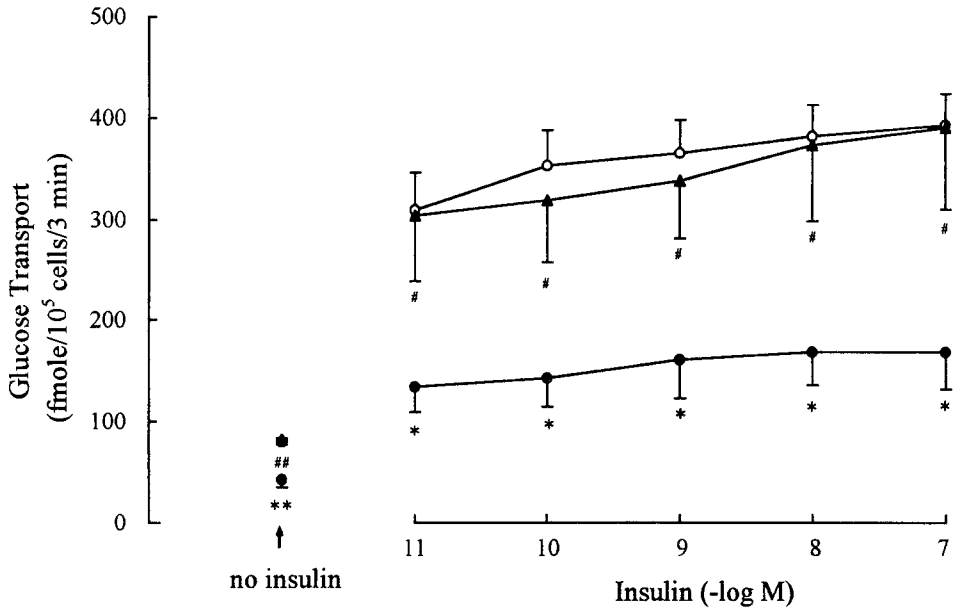

Fig 3. Insulin-stimulated glucose uptake (transport) by adipocytes isolated from rats after 3 weeks of dietary manipulation. Basal glucose transport is without insulin.

These results demonstrated that the fructose diet indeed impaired glucose transport of adipocytes, and the fish oil supplement could overcome this detriment caused by the fructose diet.

The insulin binding results for adipocytes (Fig 4) provided additional evidence demonstrating the detrimental effect of a fructose diet. Basal binding of insulin in adipocytes from group F (1.84% ± 0.28%) was significantly less than in group C (3.32% ± 0.43%, $P < .03$), but was not significantly different from group O (2.64% ± 0.97% bound/total, $P < .38$). The Scatchard plot of insulin-displacing curves produced the binding site and affinity results listed in Table 2. The fructose diet apparently led to a decrease in the affinity (with increased K_d) and a significant decrease in the number of binding sites (with decreased B_{max}) for the insulin receptor in the high-affinity category as seen in group F. There were no significant differences in the insulin receptor of the low-affinity category among the three groups.

Results for ET-1 binding to adipocytes are shown in Fig 5. A Scatchard plot of the data revealed no differences in K_d and B_{max} among the three groups: K_d values were 358.2 ± 101.7, 366.1 ± 125.6, and 381.5 ± 102.5 pmol/L and B_{max} values were 12.7 ± 5.20, 15.8 ± 11.26, and 13.0 ± 5.54 fmol/10^5 cells for groups C, F, and O, respectively. Furthermore, plasma ET-1 concentrations (Table 1) also indicate that there were no significant differences among the three groups.

Plasma FFA and TG concentrations of the three groups are shown in Table 1. Whereas FFA levels were almost identical, plasma TG of group F was significantly higher than that of group C and group O. Clearly, intake of the high-fructose diet resulted in hypertriglyceridemia, and fish oil supplementation could alleviate its development in the presence of high fructose.

DISCUSSION

In three identical experiments, we consistently found that when Sprague-Dawley rats were fed a diet in which fructose replaced carbohydrates, they developed hypertension, fasting

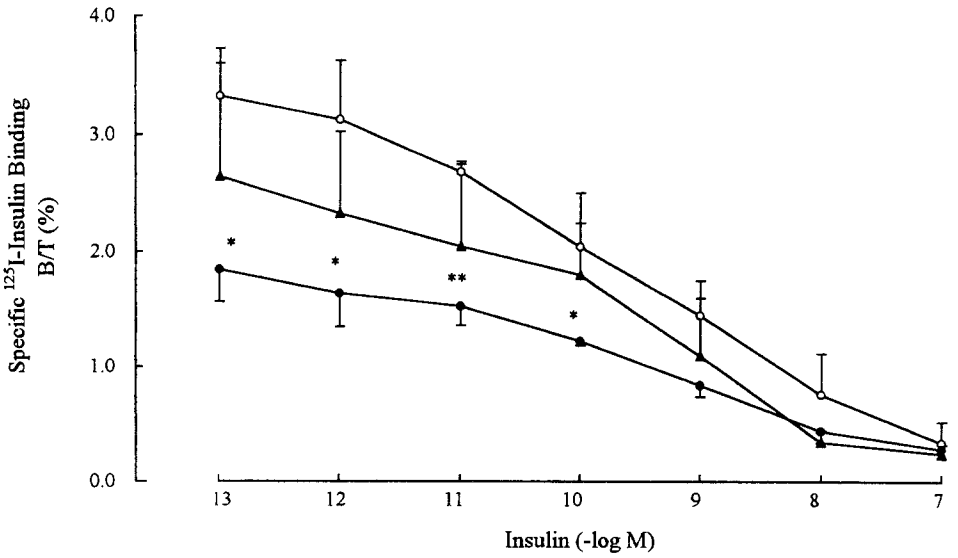

Fig 4. Insulin binding of adipocytes isolated from rats after 3 weeks of dietary manipulation.

Table 2. Insulin Binding Kinetics of Adipocytes From Three Groups of Rats

	High-Affinity Binding Site		Low-Affinity Binding Site	
Group	K_d (nmol/L)	B_{max} (fmol/10^5 cells)	K_d (nmol/L)	B_{max} (fmol/10^5 cells)
C	0.19 ± 0.09	2.21 ± 0.83	17.1 ± 11.1	63.1 ± 33.3
F	0.23 ± 0.05	1.16 ± 0.54*	18.1 ± 10.3	53.1 ± 18.5
O	0.18 ± 0.05	2.26 ± 1.23	17.5 ± 10.8	80.7 ± 29.9

NOTE. Results are the mean ± SD (n = 3).
*$P < .05$ *v* group C.

hyperglycemia, fasting hyperinsulinemia, and hyperlipidemia, reproducing the observations originally reported by Zavaroni et al.[26] A similar cluster of metabolic abnormalities, the so-called "deadly quartet,"[4] including hypertension, hyperlipidemia, visceral obesity, and hyperinsulinemia, frequently diagnosed in NIDDM patients led Reaven[2] to group these clinical correlates collectively as syndrome X. Nevertheless, there was no definitive biochemical or metabolic mechanism explaining the etiology of this syndrome. The fructose rat model provides an ideal means for investigating the causes of a simulated syndrome X in animals. In addition, this model gives a convincing clue that dietary imbalance may initiate the development of syndrome X.

Dietary manipulation in the present study did not affect the animal's overall growth (Fig 1). Nevertheless, it is noteworthy that fish oil supplementation, despite an increased energy intake, tended to slow the rate of weight gain in the animals. This paradoxical finding is consistent with results reported by Rizkalla et al[27] in a similar experiment showing that fish oil substitution of a mixture of vegetable and animal oils in a control diet significantly reduced the body weight gain of normal and diabetic rats as soon as the first week.

Our results demonstrated that dietary fructose increased plasma TG to twice the normal level and confirmed the hypertriglyceridemic reaction to fructose or sucrose previously reported by many investigators. Recently, Sebokova et al[28] reported that the underlying molecular mechanism involves increased gene expression of several enzymes, including acetyl-coenzyme A carboxylase and fatty acid synthase, which are responsible for the enhanced synthesis of TG in liver and adipose tissue, and also malic enzyme, which supplies increased NADPH for enhanced synthesis of long-chain fatty acids. This mechanism provides a potential explanation for our results.

There are numerous studies suggesting a beneficial effect of fish oils in the prevention of human coronary heart disease. The low prevalence of atherosclerosis in Greenland Eskimos as compared with Danes and North Americans may be the result of the high content of long-chain polyunsaturated omega-3 fatty acids in fish oils consumed by the Eskimos.[29] Further study revealed that the principal effect of dietary omega-3 fatty acids is a reduction of TG and very–low-density lipoprotein cholesterol.[30] The results observed in the fructose rat model are consistent with the reported findings for omega-3 fatty acids in suppressing plasma TG in diabetic and normal subjects.[11,13,30]

The normal plasma FFA level in group F is an interesting finding. Lipids and carbohydrates can be interconverted in liver, skeletal muscle, and adipose tissue through the Randle cycle.[31] The conversion of food carbohydrates to glucose in humans has been established since the classic study by Jenkins et al.[32] Fructose with a glycemic index of 42.3 (compared with glucose with glycemic index of 100) stimulates a disproportionately high secretion of insulin.[33] Conceivably, an excessive intake of fructose, like glucose, leads to hypersecretion of insulin from normal β cells and also to simultaneous hyperglycemia. The sustained hyperglycemia further induces the compensatory secretion of insulin, which downregulates the insulin receptor in all affected tissues, including adipose tissue.[34] Our findings in group F of supranormal fasting plasma insulin and glucose and reduced insulin binding and insulin-stimulated glucose transport of adipocytes (Table 1 and Fig 3 and 4) are consistent with this suspected mechanism for the development of insulin resistance.[34] However, the normal plasma FFA of group F suggests that they had shifted their energy substrate by using FFA instead of glucose. The high plasma TG and normal FFA of group F suggest an insulin-resistant state existing only in terms of glucose, not FFA, metabolism. Omega-3 fatty acids, on the other hand, by stimulating TG metabolism, can reverse the

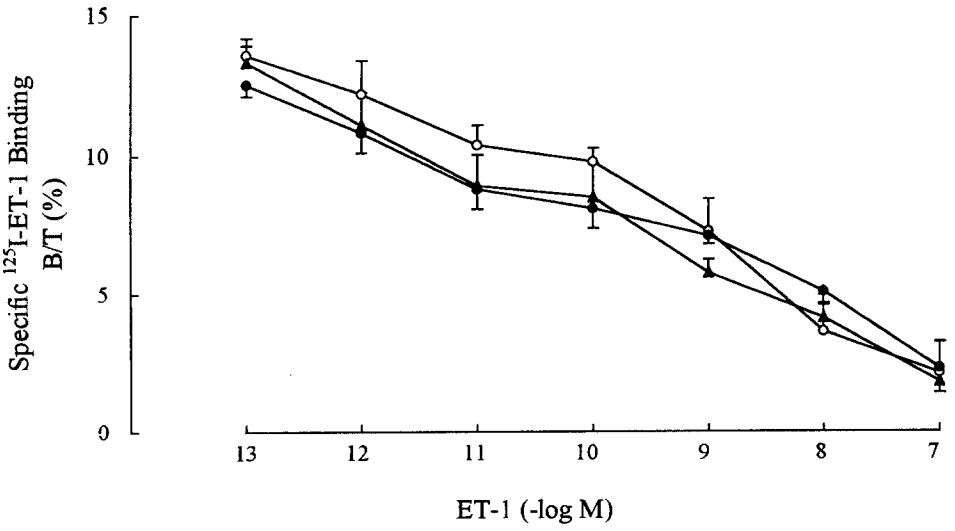

Fig 5. ET-1 binding of adipocytes isolated from rats after 3 weeks of dietary manipulation.

abnormal metabolic pathway and improve insulin sensitivity on glucose utilization.[30,35]

In the OGTT, the significantly higher basal fasting plasma glucose and insulin indicate insulin resistance in group F. The incremental area under the curve for insulin and glucose, which are not different among the three groups, suggest that group F maintained a normal β-cell function. Precisely why group F, with basal hyperinsulinemia, did not exhibit a high insulin response in the OGTT is not known, but it may indicate that some subtle subnormal β-cell function was associated with the fructose-rich diet.

With regard to the development of hypertension, it is puzzling as to why a high-fructose diet alone induces such a dramatic effect on blood pressure. This may be a consequence secondary to hyperinsulinemia, which leads to increased sympathetic activity[4] together with decreased adrenal medullary activity.[36] The usefulness of fish oil to counteract fructose effects on blood pressure is equally perplexing, because studies of large human populations show that the final results of using fish oil to control blood pressure are inconclusive.[37,38]

Omega-3 fatty acids possess some biochemical properties related to vascular relaxation. As reviewed by Leaf and Weber,[9] the most important action of eicosapentaenoic acid and docosahexaenoic acid in fish oils is to attenuate the cellular bioavailability of arachidonic acid (C20:4n-6) and to favor the synthesis of prostacyclin rather than thromboxane. Although we did not measure eicosanoid synthesis in our experiment, we speculate that the increased prostaglandins and decreased thromboxanes cause vasodilatation and may account for the antihypertensive results with the fish oil supplement in group O.

Since an increased production of nitric oxide is not the mechanism of the endothelium-derived relaxing factor(s) potentiated by eicosapentaenoic acid in a bioassay using canine coronary artery rings,[15] we were interested in examining the possible effect of plasma ET-1 as a potent vasoconstrictor, which has been increased in essential hypertension.[39] In another series of experiments, we found that intraperitoneal administration of ET-1 induced insulin resistance.[40] However, in the present study, we have not found any significant variance of ET-1 receptor binding in adipocytes and of plasma ET-1 levels among the three groups. It is possible that the fat pad is not a sensitive tissue for examining the relationship between ET-1 action and hypertension in the fructose-fed rat model. Since the action of ET-1 involving hypertension is primarily through a paracrine pathway, the plasma level of ET-1 bears no physiological consequence at all in our study. Then why did we study adipocytes? As suggested by Kahn,[41] the glucose transporter GLUT4 in adipose tissue plays the pivotal role in developing insulin resistance. Nevertheless, ET-1 receptors in adipocytes probably do not have the same influence on blood pressure as in the vascular system. Therefore, we now believe that fructose-induced hypertension is likely to be mediated by other mechanisms or ET-1 in vascular endothelial cells.

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