Prevention of skeletal muscle insulin resistance by dietary cod protein in high fat-fed rats

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1Nutraceuticals and Functional Foods Institute, 2Department of Food Science and Nutrition, 3Department of Anatomy and Physiology, and 4Center for Research on Energy Metabolism, Laval University, Ste-Foy, Quebec, Canada G1K 7P4; and 5Lipid Research Unit, Laval University Hospital Research Center, Ste-Foy, Quebec, Canada G1V 4G2

Received 28 August 2000; accepted in final form 21 February 2001

Lavigne, Charles, Frédéric Tremblay, Geneviève Asselin, Hélène Jacques, and André Marette. Prevention of skeletal muscle insulin resistance by dietary cod protein in high fat-fed rats. Am J Physiol Endocrinol Metab 281: E62–E71, 2001.—In the present study, we tested the hypothesis that fish protein may represent a key constituent of fish with glucoregulatory activity. Three groups of rats were fed a high-fat diet in which the protein source was casein, fish (cod) protein, or soy protein; these groups were compared with a group of chow-fed controls. High-fat feeding led to severe whole body and skeletal muscle insulin resistance in casein- or soy protein-fed rats, as assessed by the euglycemic clamp technique coupled with measurements of 2-deoxy-D-[3H]glucose uptake rates by individual tissues. However, feeding cod protein fully prevented the development of insulin resistance in high fat-fed rats. These animals exhibited higher rates of insulin-stimulated glucose uptake and adipose tissue accretion, or expression of tumor necrosis factor-α in fat and muscle. Moreover, L6 myocytes exposed to cod protein-derived amino acids showed greater rates of insulin-stimulated glucose uptake compared with cells incubated with casein- or soy protein-derived amino acids. These data demonstrate that feeding cod protein prevents obesity-induced muscle insulin resistance in high fat-fed obese rats at least in part through a direct action of amino acids on insulin-stimulated glucose uptake in skeletal muscle cells.

interest in the role of fish and its constituents on glucose metabolism arose from the lower incidence of non-insulin-dependent diabetes mellitus (NIDDM) in populations consuming large amounts of fish (21). These effects have been primarily attributed to the presence of fish oil, because omega-3 fatty acids have been shown to improve insulin sensitivity in insulin-resistant animals (28, 39). However, the beneficial effects of omega-3 fatty acids on glucose tolerance in humans are still under debate (see Refs. 10 and 24 for reviews). Moreover, epidemiological studies suggest that another constituent in fish could protect against the development of impaired glucose tolerance and NIDDM in lean fish eaters (9).

Few studies have directly addressed the role of dietary proteins in the regulation of insulin sensitivity and glucose homeostasis. Hubbard and Sanchez (17) reported that a soy protein meal induced lower postprandial blood insulin concentrations in human subjects than a casein meal. Animal studies further showed that rats fed soy protein had lower plasma insulin concentrations than those fed casein (43). In untreated type 2 diabetic subjects, the insulin response curve was lower after ingestion of a meal containing fish or soy proteins compared with casein (11). We showed similarly, in rats fed a high-sucrose diet, that both cod and soy proteins reduced fasting plasma glucose concentrations compared with casein. Dietary cod and soy proteins were found to improve glucose tolerance and whole body insulin action on glucose disposal (22). However, it has been shown that a high-sucrose diet induces major impairment of insulin action in the liver, with a smaller contribution from peripheral tissues (40). It was, therefore, of significant interest to further evaluate the effects of those dietary proteins on insulin sensitivity and glucose utilization in another model of insulin resistance with a more severe defect in insulin-mediated peripheral glucose disposal.

The high-fat/sucrose-fed rat is a well established animal model of insulin resistance, reproducing the common form of the abdominal (visceral)-obese insulin-resistant syndrome seen in humans (4, 20, 37, 38). Rats fed this diet rapidly develop whole body and marked skeletal muscle insulin resistance (13, 42). Thus, in the present study, we tested the effect of casein, cod protein, and soy protein on whole body insulin action and glucose uptake in peripheral tissues of high fat/sucrose-fed rats. The effects of dietary proteins on basal and insulin-stimulated glucose uptake in individual tissues was investigated by measuring the uptake of...
2-deoxy-d-[3H]glucose in skeletal muscles, heart, and adipose tissues in vivo. Furthermore, we explored the possibility that dietary proteins modulate insulin sensitivity by a direct action of protein-derived amino acids on skeletal muscle cells.

RESEARCH DESIGN AND METHODS

Materials. 2-Deoxy-d-[1,2-3H]glucose (2-[3H]DG) and d-[14C]-sucrose were obtained from New England Nuclear Life Science Products (Boston, MA). Purified human insulin (Humulin R) was purchased from Eli Lilly (Indianapolis, IN). All diet components were from ICN Biochemicals (Cleveland, OH) except vitamin mix, which was from Harlan Teklad (Madison, WI), and cod protein, which was prepared in our laboratory as previously described (7).

Treatment of animals. Male Wistar rats (Charles River, Montreal, QC, Canada) weighing 200–250 g at the beginning of the study were placed on a high-fat diet for 4 wk, and food was available ad libitum. The high-fat diet contained 23% (wt/wt) protein (casein, soy protein, or cod protein as 15% of energy), 19.8% lard (33% of energy), 19.8% corn oil (33% of energy), 24.5% sucrose (20% of energy), and 5% cellulose. The high-fat diets were supplemented with 1.4% vitamin mixture, 6.7% AIN-76 mineral mix, 0.2% choline bitartrate, and 0.004% butylated hydroxytoluene. A control chow-fed group was also included in this study to assess the extent of insulin resistance induced by high-fat feeding. According to the manufacturer, the chow diet contained, in percentage of calories, 57.3% carbohydrate, 18.1% protein, 18.8% fiber, and 4.5% fat, and in energy 14.3 kJ/g (Charles River rodent chow 5075, Purina Mills, Strathroy, ON, Canada). High-fat diets were identical except for the amino acid composition of the dietary protein component, which differed as detailed previously (22). The amino acid composition of the chow diet was as follows: alanine, 6.2; arginine, 5.5; aspartic acid, 10.9; glutamic acid, 20.9; glycine, 4.6; histidine, 2.2; isoleucine, 3.9; leucine, 7.1; methionine, 2.2; lysine, 5.9; phenylalanine, 4.5; proline, 7.3; serine, 5.0; threonine, 3.1; tyrosine, 3.1; valine, 4.6 g/100 g of amino acids. The residual n-3 fatty acid content of cod protein has been measured, and although the amounts of 18:3 and 22:5 were not detectable, the amounts of 20:5 and 22:6 were 114 and 164 μg/100 g of diet, respectively. These very low amounts are considered negligible in this experiment. The energy content of the diet was measured in an automatic adiabatic calorimeter (model 1241; Parr Instruments, Moline, IL) and found to be isoenergetic: casein (25.5 kJ/g), soy protein (25.3 kJ/g), cod protein (25.4 kJ/g). The protein content (N × 6.25) was assayed by a Kjeldahl Foss autoanalyzer (model 1612; Foss, Hillerod, Denmark). The level of protein in the purified diets was adjusted to an isonitrogenous basis at the expense of carbohydrates. Food intake was estimated every day, and body weight was measured weekly.

Whole body glucose disposal and glucose uptake rates for individual tissues. Whole body insulin-mediated glucose disposal was assessed in unrestrained conscious animals by the hyperinsulinemic euglycemic clamp technique, as previously described (33). Briefly, catheters were inserted into the left jugular vein and into the right carotid artery. Rats were allowed to recover from surgery for 4–5 days before the clamp procedure was performed.

At that time, the food intake and body weights of all rats had returned to 80 and 90% of their presurgery values, respectively. Rats were fasted overnight (12–14 h) before receiving an infusion of saline (basal) or insulin at 4 mU·kg⁻¹·min⁻¹ for 140 min by means of a syringe pump (Razel, Stamford, CT). Venous catheters were used for the multiple infusions, and blood samples were obtained from the carotid artery.

In vivo basal and insulin-stimulated glucose uptake rates in individual tissues of clamped rats were determined by measuring the incorporation of 2-[3H]DG, as described in detail previously (33). Briefly, 2-[3H]DG and [14C]sucrose were administered together as an intravenous bolus 120 min after the start of the insulin infusion. Blood samples were obtained at 5, 7.5, 10, 12.5, 15, 17.5, and 20 min after bolus administration for determination of plasma disappearance rates of 2-[3H]DG and [14C]sucrose.

At the completion of the clamp, rats were quickly killed by decapitation, and tissues were rapidly removed, weighed, and frozen in liquid nitrogen and stored at −80°C for subsequent analysis. The accumulation of 2-[3H]DG in tissues, corrected for extracellular space with [14C]sucrose, was used as an index of glucose uptake rates, as described previously (33).

Cell culture and in vitro glucose uptake. L6 skeletal muscle cells (a kind gift of Dr. A. Klip, Hospital for Sick Children, Toronto, Ontario) were grown and differentiated as described previously (3). Fully differentiated myotubes were serum deprived for 5 h before glucose transport experiments. Cells were incubated for 1 h in Earle’s balanced salt solution containing mixtures of amino acids corresponding to those found in rats that consumed casein, cod protein, soy protein, or a standard chow diet. Insulin or medium alone (control) was added during the last 45 min of the treatment. The concentration of each amino acid was previously determined in rats fed one of the three purified diets varying in protein source, namely casein, cod protein, soy protein, and in rats fed the nonpurified diet (chow) for 28 days. Fasted rats received a 5-g meal of their assigned experimental diet for 30 min. This time point was selected on the basis of preliminary observations that, after exposure to the test meal, maximal plasma amino acid changes occur 30 min postmeal (unpublished data). We have also found that plasma insulin, C-peptide, and glucagon responses were maximal and statistically different between dietary protein groups at that time point (22). The mean concentrations of amino acids 30 min after a meal are shown in Table 1. After treatment with amino acids with or without

Table 1. Dietary protein-derived amino acid mixtures used for L6 culture conditions

<table>
<thead>
<tr>
<th></th>
<th>Casein</th>
<th>Cod Protein</th>
<th>Soy Protein</th>
<th>Chow</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>663</td>
<td>563</td>
<td>501</td>
<td>603</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>150</td>
<td>194</td>
<td>176</td>
<td>137</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>129</td>
<td>111</td>
<td>121</td>
<td>108</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>26</td>
<td>21</td>
<td>20</td>
<td>29</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>23</td>
<td>29</td>
<td>24</td>
<td>25</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>110</td>
<td>114</td>
<td>211</td>
<td>104</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>1263</td>
<td>925</td>
<td>1027</td>
<td>1370</td>
</tr>
<tr>
<td>L-Glycine</td>
<td>215</td>
<td>255</td>
<td>272</td>
<td>382</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>76</td>
<td>67</td>
<td>68</td>
<td>58</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>115</td>
<td>90</td>
<td>98</td>
<td>89</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>170</td>
<td>122</td>
<td>130</td>
<td>131</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>470</td>
<td>441</td>
<td>384</td>
<td>373</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>98</td>
<td>102</td>
<td>67</td>
<td>76</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>104</td>
<td>76</td>
<td>82</td>
<td>76</td>
</tr>
<tr>
<td>L-Proline</td>
<td>366</td>
<td>189</td>
<td>222</td>
<td>285</td>
</tr>
<tr>
<td>L-Serine</td>
<td>291</td>
<td>218</td>
<td>270</td>
<td>283</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>366</td>
<td>261</td>
<td>277</td>
<td>386</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>112</td>
<td>66</td>
<td>85</td>
<td>91</td>
</tr>
<tr>
<td>L-Valine</td>
<td>230</td>
<td>163</td>
<td>166</td>
<td>178</td>
</tr>
</tbody>
</table>

Amino acid mixtures are expressed in μM.
insulin, cells were rinsed once with glucose-free HEPES-buffered saline solution, pH 7.4 (in mM: 140 NaCl, 20 HEPES-Na, 5 KCl, 2.5 MgSO₄, 1 CaCl₂) and subsequently incubated for 8 min with 10 μM 2-deoxy-D-glucose containing 0.3 μCi/ml 2-[³H]DG in the same buffer, as described previously (3).

**Tumor necrosis factor-α protein expression in adipose tissue and skeletal muscle.** Enzyme-linked immunosorbent assays (ELISA) were used for the detection of tumor necrosis factor-α (TNF-α) in tissue extracts. Epididymal white adipose tissue was homogenized with a glass tissue grinder (Kontes, Vineland, NJ) in lysis buffer (20 mM imidazole, pH 6.8, 100 mM KCl, 1 mM EGTA, 10 mM NaF, 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail) and centrifuged at 2,500 g for 10 min. Mixed gastrocnemius muscle was homogenized with a polytron in 6 volumes of lysis buffer (20 mM Tris, pH 7.5, 140 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 10% glycerol, 1 mM PMSF, and protease inhibitor cocktail), solubilized with 1% NP-40 for 1 h at 4°C, and centrifuged at 14,000 g for 10 min. ELISA was carried out using a TNF-α antibody (Pharmingen, Mississauga, ON, Canada). Streptavidin-horseradish peroxidase conjugate and 3,3′,5,5′-tetramethylbenzidine were purchased from RDI (Flanders, NJ). Secondary antibodies were biotinylated with NHS-LC-biotin (Pierce, Rockford, IL). Recombinant TNF-α was used for the standard curves by use of an antibody against recombinant TNF-α (R&D Systems, Minneapolis, MN). Values for skeletal muscle were corrected for protein content determined by the bicinchoninic acid method with BSA as the standard. Adipose tissue TNF-α levels were corrected for DNA content obtained by the bicinchoninic acid method with BSA as the standard. Adipose tissue TNF-α levels were corrected for DNA content obtained as follows. Adipose tissue was homogenized in Tris buffer (150 mM NaCl, 0.1% Triton X-100, 10 mM Tris, pH 8.0) and incubated at 37°C for 2 h with 0.1% SDS, 100 μg/ml proteinase K, and 10 mM EDTA. DNA was extracted with 1 volume of phenol-CHCl₃ and precipitated in 2 volumes of ethanol and resuspended in water. DNA content was determined spectrophotometrically (260 nm).

**Analytical determinations.** Plasma glucose was measured using the glucose oxidase method, with a Beckman glucose analyzer (Beckman Instruments, Palo Alto, CA). Plasma insulin and leptin concentrations were measured by radioimmunoassay by use of a rat insulin- and rat leptin-specific kit from Linco (St. Louis, MO). Nonesterified fatty acids and triglycerides were determined enzymatically (Wako Chemicals, Richmond, VA, and Roche Diagnostics, Montreal, QC, Canada, respectively). The amino acid composition of protein sources (see Ref. 22) and of the chow diet were determined by ion-exchange chromatography with a Beckman amino acid analyzer model 6300. The residual n-3 fatty acid levels in cod protein-fed rats, a covariate analysis was performed to ensure that this could not in part have contributed to the increased glucose infusion rate and tissue 2-[³H]DG uptake in this group. No significant relationship between these variables (P values between 0.14 and 0.99) was found, and accordingly, the comparison between the adjusted and unadjusted treatments means gave similar statistical differences. Dunnnett’s procedure was used to compare the high-fat treatments and the control chow. P < 0.05 was accepted as statistically significant.

**RESULTS**

**Effect of dietary proteins on physiological parameters of high fat-fed rats.** Body weight, energy intake, and fasting and clamped plasma glucose and insulin concentrations were similar among high fat-fed dietary groups but were significantly higher compared with the control chow fed group (Table 2). Fasting plasma concentrations of nonesterified free fatty acids (casein: 0.6 ± 0.1, cod protein: 0.6 ± 0.1, soy protein: 0.6 ± 0.1, chow: 0.6 ± 0.1), triglycerides (casein: 0.6 ± 0.1, cod protein: 0.7 ± 0.1, soy protein: 0.8 ± 0.1, chow: 0.9 ± 0.1) were similar among dietary groups. Fasting plasma leptin concentration was significantly lower (P < 0.05) in the chow-fed animal groups (2.3 ± 0.3 ng/ml) compared with high fat-fed animals (casein: 3.3 ± 0.3, cod protein: 3.8 ± 0.4, soy protein: 4.3 ± 0.3).

**Effect of dietary proteins on whole body and individual tissue insulin action in high fat-fed rats.** The effect of dietary proteins on insulin-mediated whole body glucose disposal and tissue glucose uptake was determined during euglycemic clamps in which plasma insulin was either kept at fasting levels (∼0.2–0.3 nM) (saline infusion) or raised to subphysiological concentrations (∼0.7–0.8 nM) by constant infusion of insulin (see Table 2). Figure 1 shows the insulin-mediated glucose infusion rates (GIR₆₀–₁₂₀) that were required to

| Table 2. Physiological parameters of experimental dietary groups |
|---|---|---|---|
| Dietary Groups | High Fat | |
| | Casein | Cod protein | Soy protein | Chow |
| Final body weight, g | 380 ± 20 | 400 ± 21 | 368 ± 23 | 351 ± 20 |
| Energy intake, kcal/day | 373 ± 18 | 378 ± 20 | 363 ± 12 | 320 ± 12 |
| Precap | | | | |
| Plasma glucose, mmol/l | 8.9 ± 0.2 | 8.3 ± 0.3 | 8.2 ± 0.3 | 7.2 ± 0.3 |
| Plasma insulin, nmol/l | 0.25 ± 0.03 | 0.26 ± 0.04 | 0.26 ± 0.05 | 0.13 ± 0.02 |
| Hyperinsulinemic clamp* | | | | |
| Plasma glucose, mmol/l | 8.5 ± 0.4 | 8.2 ± 0.6 | 8.0 ± 0.3 | 7.2 ± 0.4 |
| Plasma insulin, nmol/l | 0.68 ± 0.09 | 0.79 ± 0.19 | 0.69 ± 0.13 | 0.58 ± 0.08 |

Values are means ± SE; n = 7–9/group. Values for the chow-fed group are shown for comparison purposes and were significantly different (P < 0.05) from all high fat-fed groups, as determined by Dunnett’s procedure. *Steady-state plasma glucose and insulin during the last 60 min of the hyperinsulinemic euglycemic clamp. No significant differences were observed among high fat-fed dietary groups by ANOVA analysis.
GIR60–120 was found to be similar to that observed in protein-fed obese rats. In the latter group, the protein-fed groups, respectively, compared with cod and the high fat-fed groups. The effect of cod protein on significant differences between the chow-fed controls and soy protein-fed groups. However, there were no uptake rates in heart compared with high-fat casein-protein-fed groups exhibited higher 2-[3H]DG uptake and 3. Control chow- (dotted line) and high-fat cod protein-fed groups are shown in Figs. 2 and 3. The effect of cod protein on insulin-mediated 2-[3H]DG uptake in quadriceps was not reach the level of significance in these muscles. The high-fat cod protein-fed groups, but the differences did and red tibialis muscles were also higher in chow- and high-fat cod protein diet exhibited higher 2-[3H]DG levels observed in the chow-fed controls. Rats fed the high-fat casein or soy protein diets. 2-[3H]DG uptake rates in soleus compared with high-fat casein- and soy protein-fed rats compared with chow-fed controls. In adipose tissue of high fat-fed obese rats are shown in Figs. 2–3 and 3) and were not different from those measured in Chow-fed animals (data not shown).

We also examined the effects of dietary proteins on insulin-stimulated 2-[3H]DG uptake in brown and white adipose tissues (Fig. 3). It should be noted that glucose uptake values were much greater in brown adipose tissue (BAT) than in white adipose tissues (WAT) whatever the diet consumed (compare y-axes in Fig. 3, A vs. B), in accordance with previous studies (33). High-fat feeding was associated with impaired insulin action in brown fat of both casein- and soy protein-fed rats compared with Chow-fed controls. Insulin-mediated glucose uptake in BAT was marginally improved in cod protein-fed animals, but it failed to reach the level of significance compared with the other high fat-fed groups. Surprisingly, cod protein feeding failed to increase insulin-stimulated glucose uptake in WAT of high fat-fed obese rats (Fig. 3B). Compared with Chow-fed controls, insulin-mediated 2-[3H]DG uptake was comparably reduced in all high fat-fed groups. Basal 2-[3H]DG uptake rates in adipose tissues were not affected by dietary proteins (Fig. 3B) and were not different compared with Chow-fed rats (data not shown).

As expected, high fat-fed obese animals showed increased (2–3 times) epididymal, retroperitoneal, and interscapular BAT values compared with Chow-fed controls (Fig. 4A). However, no differences in adiposity were observed among dietary protein-fed groups. Moreover, the weights of the heart and skeletal muscles were not significantly different among obese rats on any of the protein sources and were similar to the weights of those tissues in Chow-fed rats (data not shown). In accord with previous studies (36), TNF-α protein levels were greater in adipose tissue of obese rats compared with Chow-fed rats (Fig. 4B). However, adipose tissue TNF-α levels were similar in casein-, cod protein-, and soy protein-fed obese rats. Skeletal muscle TNF-α concentrations were also similar among obese animals on the different protein sources and were not different from those found in muscle of Chow-fed rats (Fig. 4C).

Cod protein may exert its beneficial effect on insulin sensitivity by a direct action of cod protein-derived amino acids on insulin-stimulated glucose uptake in skeletal muscle cells. To test this possibility, we exposed cultured L6 myocytes to amino acid (AA) mixtures corresponding to the concentrations of plasma amino acids in rats fed Chow, casein, cod protein, or soy protein diets. Compared with AA mixtures corresponding to rats fed casein or soy protein, muscle cells exposed to the cod-derived AA mixture showed increased insulin action on glucose uptake (Fig. 5). The increasing effect of cod protein-derived AA was ob-
served at all doses of insulin tested and were statistically significant at 10 and 50 nM vs. soy protein-derived AA and at 10 and 500 nM vs. casein-derived AA. The stimulatory effects of insulin on glucose uptake in cells exposed to cod protein-derived AA mixture were similar to cells incubated with AA mixture corresponding to that of chow-fed rats (data not shown). On the other hand, calculation of the insulin sensitivity index (EC$_{50}$) from individual dose-response curves showed that insulin sensitivity was increased in L6 myocytes exposed to the cod protein-derived AA compared with the soy protein, but not to the casein-derived AA mixtures (see inset, Fig. 5). The similar insulin EC$_{50}$ in cod protein- and casein-derived AA-treated cells can be explained by the fact that the calculation of this index is dependent on the maximal insulin responsiveness (100% of response), which was not the same in these experimental groups.

DISCUSSION

The present study shows that the consumption of cod protein (but not casein or soy protein) fully prevented the development of skeletal muscle insulin resistance in diet-induced obesity. Indeed, in contrast to obese rats consuming either casein or soy protein, cod protein-fed obese rats exhibited higher insulin-mediated whole body glucose disposal rates that were comparable to the rate of nonobese chow-fed rats. It is well established that skeletal muscle is a major site of insulin resistance when rats are fed a high-fat/sucrose diet (42). Measurements of individual tissue glucose
uptake with 2-[^3]H]DG revealed that cod protein protects from the development of peripheral insulin resistance by improving insulin-stimulated glucose uptake in skeletal and cardiac muscles of high fat-fed animals. Similar results were obtained for muscles enriched with either oxidative (slow, type I), oxidative-glycolytic (fast, type IIa), or glycolytic (fast, type IIb) fibers, suggesting that the beneficial effect of cod protein is not dependent on the metabolic or contractile nature of the muscles. Furthermore, dietary proteins failed to affect basal 2-[^3]H]DG uptake rates in any of the muscles investigated, thus indicating that cod protein specifically modulates insulin action on glucose uptake rather than glucose uptake per se.

It has been reported previously that n-3 fatty acids derived from fish oil improve insulin sensitivity in insulin-resistant obese rats (27, 38, 39). However, it is very unlikely that the preventive effect of cod protein on insulin resistance could be attributed to the trace amounts of n-3 fatty acids found in the purified cod protein isolate. Indeed, we measured the amounts of n-3 fatty acids in our cod protein diet, and they are 150 times lower than the lowest dietary n-3 fatty acid levels that have been shown to improve insulin sensitivity in rats (6% of total calories) (38). Moreover, our finding that the cod protein-derived amino acid mixture increased insulin-stimulated glucose uptake in cultured L6 myocytes indicates that the beneficial effect of dietary cod protein on insulin sensitivity is mediated mainly by a direct action of cod protein-derived amino acids on the myocyte insulin-regulated glucose transport system and not by the trace amounts of n-3 fatty acids present in the cod protein diet.

Although consuming cod protein totally abrogated the insulin-resistant effect of the high-fat/sucrose diet in skeletal muscle, feeding soy protein under the same conditions failed to do so. This is in contrast to our previous observations of beneficial effects of both cod and soy proteins on glucose tolerance and insulin sensitivity in rats fed a high-sucrose diet (22). Our data suggest that the responsiveness of muscle insulin resistance to soy protein may be influenced by the con-
High-fat feeding caused marked insulin resistance in skeletal muscle, as described in RESEARCH DESIGN AND METHODS. Storlien et al. (40) demonstrated that sucrose feeding impairs whole body glucose disposal mainly because of a major impairment of insulin action in the liver with a smaller contribution from peripheral tissues. This is perhaps related to the fact that simple sugars such as sucrose and fructose increase triglyceride levels in the circulation but not in skeletal muscle, whereas high-fat feeding increased triglyceride concentrations in skeletal muscle (41) but not in the circulation (4, 41). Thus soy protein could reduce circulating triglyceride and improve liver insulin action (22). On the other hand, consuming cod protein could alleviate the metabolic defects associated with increases in both circulating and intramuscular triglycerides. Studies on the cellular mechanisms whereby cod and soy proteins incorporated in high-fat/sucrose vs. high-sucrose diets affect muscle insulin resistance would be helpful in explaining these divergent results.

High-fat feeding caused marked insulin resistance for glucose uptake in both muscle and adipose tissues compared with chow feeding. However, cod protein prevented the development of insulin resistance only in skeletal and cardiac muscles and not in white adipose tissues. Despite the lack of action of cod protein on white fat, insulin-mediated whole body glucose disposal was completely normalized in obese rats consuming cod protein. These results are in line with the fact that adipose tissue contributes to a minor fraction of total glucose disposal after a meal or during insulin stimulation (19). However, the lack of prevention of insulin resistance in adipose tissue of high-fat-fed rats may help to explain the finding that fasting insulin and glucose levels are still elevated in cod protein-fed animals despite improved insulin action in muscles. Furthermore, fasting hyperinsulinemia may also be explained by increased liver glucose output, reduced hepatic insulin extraction, and/or altered insulin secretion, because these defects have also been reported in high-fat-fed rats (1, 29).

The beneficial effect of dietary cod protein on skeletal muscle insulin sensitivity was observed even in the face of similar body weight gain and visceral adipose tissue accretion compared with casein- or soy protein-fed rats. These results strongly suggest that dietary cod protein prevented the causal link between visceral obesity and the development of peripheral insulin resistance. Several factors have been postulated to be responsible for the development of insulin resistance in obesity (see Ref. 8). One molecule that has received considerable attention is the cytokine TNF-α. There is accumulating evidence implicating TNF-α as a candidate mediator of obesity-associated insulin resistance (see Ref. 15 for a recent review). TNF-α is expressed at high levels in the enlarged adipose tissue from virtually all rodent models of obesity, as well as in obese humans (16). The cytokine has also been reported to be overexpressed in muscle cells isolated from NIDDM subjects (34). Moreover, genetic ablation of TNF-α or TNF-α function was reported to improve insulin sensitivity in various animal models of insulin resistance, including the high fat-fed mouse (46). However, there are also data to support a protective role for TNF-α receptors (p55 and p75) in obesity-linked diabetes, inasmuch as high fat-fed mice deficient in both receptors exhibited higher fasting hyperinsulinemia and glucose intolerance compared with high fat-fed wild-type mice (35). In the present study, we confirmed that TNF-α protein expression is increased in adipose tissue of high fat-fed animals. Nevertheless, our data indicate that TNF-α expression was not reduced in cod protein-fed obese rats compared with casein- or soy protein-fed obese animals. Moreover, we found no evidence for TNF-α overexpression in skeletal muscle of high fat-fed obese rats, whatever the dietary protein sources, compared with chow-fed animals. Taken together, these data indicate that the preventive effects of cod protein on skeletal muscle insulin resistance are unlikely to be explained by changes in adipose or muscle TNF-α expression in high fat-fed obese rats.

Another potential mechanism of obesity-associated insulin resistance is an increased availability of free...
fatty acids (FFA). Although we failed to observe any differences in fasting FFA levels between high fat-fed and chow-fed rats, it is likely that local lipid availability is increased in skeletal muscle of high fat-fed animals, as previously reported (29). Elevated concentrations of muscle FFA may inhibit insulin-stimulated glucose utilization through substrate competition for oxidation (the glucose-fatty acid cycle) (31, 32) and/or by increasing the flux of fructose 6-phosphate into the hexosamine pathway (14, 29). Ongoing studies are in progress to test the effects of cod protein on muscle lipid oxidation and its potential role in improving insulin sensitivity in high fat-fed obese rats.

Despite the possible implications of the aforementioned mechanisms in the prevention of obesity-induced insulin resistance, our finding that cod protein-derived amino acids can also increase insulin-stimulated glucose uptake in cultured L6 myocytes strongly suggests that a significant part of the beneficial effect of dietary cod protein on insulin-mediated glucose disposal in obese rats can be explained by a direct action of individual or groups of amino acids on skeletal muscle. Although data obtained with L6 muscle cells cannot be extrapolated to the high fat-fed animal model used in this study, these cells allowed us to test the effects of dietary protein-derived amino acids on insulin action without confounding factors present in vivo. The fact that different amino acid mixtures have a significant impact on insulin-mediated glucose uptake in these cells strongly supports our hypothesis of a direct action of individual or groups of amino acids on insulin-mediated glucose disposal on myocytes. To the best of our knowledge, this is the first observation that different pools of amino acids, at concentrations found in the plasma of rats fed physiological amounts of dietary proteins, can differently modulate insulin-stimulated glucose uptake in skeletal muscle cells.

The effects of the amino acid mixtures on myocyte glucose uptake were observed after only 1 h of treatment, strongly suggesting that transcriptional mechanisms are not involved in the modulatory actions of amino acids on insulin-stimulated glucose uptake. Rather, the effects of amino acids on glucose uptake may involve acute regulation of insulin-signaling events and translocation of GLUT-4 transporters. In support of this, Patti et al. (30) recently reported that exposure of cultured muscle and hepatic cells to a balanced mixture of amino acids downregulated early insulin-signaling steps critical for glucose transport and inhibition of gluconeogenesis. It will be interesting to test whether the amino acid mixtures corresponding to casein, cod protein, or soy protein differently modulate insulin signaling and GLUT-4 translocation in skeletal muscle cells.

It will be important to define the role of individual or groups of amino acids in mediating the effects of cod-derived amino acid mixture on insulin-mediated glucose uptake. Several amino acids are found in different concentrations in the plasma of rats fed casein, cod protein, or soy protein (see Research Design and Methods). Of those amino acids, a potentially interesting candidate is glutamine, because its concentration is selectively decreased in cod protein-fed compared with casein or soy protein-fed animals. Interest for glutamine as a potential modulator of insulin action arises from the pioneering work of Traxinger and Marshall (44), who observed a marked desensitization of the
glucose transport system in adipocytes incubated in a defined buffer containing high concentrations of both insulin and glucose, plus 15 amino acids found in DMEM. Of the 15 amino acids, glutamine was found to be fully effective in mediating loss of insulin action on glucose transport. It has been further reported that glutamine exposure also inhibits insulin-stimulated glucose transport in skeletal muscle and that it promotes insulin resistance by routing glucose through the hexosamine pathway (5, 25, 26, 44, 45). However, two observations made in this study argue against a role of the hexosamine pathway in the modulation of insulin action by dietary proteins. First, the hexosamine pathway is also operative in adipocytes, but we found no effect of cod protein on insulin-stimulated glucose uptake in white adipose tissues. Second, the amino acid-dependent modulation of insulin action in L6 myocytes was observed within 1 h of treatment, which is not likely to cause buildup of hexosamines in cells treated with casein- or soy protein-derived amino acid mixtures.

In summary, the present study shows that dietary cod protein prevents the development of skeletal muscle insulin resistance in high-fat-fed obese rats. The beneficial action of cod protein on insulin sensitivity occurred without reductions in body weight or adiposity, strongly suggesting that cod protein protects from obesity-induced insulin resistance. The effect of dietary cod protein appears to involve, at least in part, a direct action of cod protein-derived amino acids on insulin-stimulated glucose transport in skeletal muscle cells. Interest in the present data also arises from the fact that increased cod protein consumption is easily implementable in humans within guidelines of daily recommended allowances of essential nutrients (12, 18) and thus could represent a novel nutraceutical approach in preventing the development of insulin resistance in obesity. Because insulin resistance is a central factor in visceral obesity-associated complications such as hypertension, diabetes, and cardiovascular diseases (2, 6, 8), dietary cod protein may contribute to prevent the many metabolic aberrations that accompany the obese state.

We thank Nathalie Mercier for expert technical assistance in amino acid determination of plasma and dietary protein sources. This work was supported by grants from the Canadian Diabetes Association (H. Jacques and A. Marette). A. Marette was supported by scholarships from the Medical Research Council of Canada and Association (H. Jacques and A. Marette). A. Marette was supported by a studentship from the Quebec Hypertension Society.

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