Dietary Cod Protein Restores Insulin-Induced Activation of Phosphatidylinositol 3-Kinase/Akt and GLUT4 Translocation to the T-Tubules in Skeletal Muscle of High-Fat–Fed Obese Rats

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Diet-induced obesity is known to cause peripheral insulin resistance in rodents. We have recently found that feeding cod protein to high-fat–fed rats prevents the development of insulin resistance in skeletal muscle. In the present study, we have further explored the cellular mechanisms behind this beneficial effect of cod protein on skeletal muscle insulin sensitivity. Rats were fed a standard chow diet or a high-fat diet in which the protein source was either casein, soy, or cod proteins for 4 weeks. Whole-body and muscle glucose disposal were reduced by ~50% in rats fed high-fat diets with casein or soy proteins, but these impairments were not observed in animals fed cod protein. Insulin-induced tyrosine phosphorylation of the insulin receptor and insulin receptor substrate (IRS) proteins were similar in muscle of chow- and high-fat–fed rats regardless of the dietary protein source. However, IRS-1–associated phosphatidylinositol (PI) 3-kinase activity was severely impaired (−60%) in muscle of high-fat–fed rats consuming casein or soy protein. In marked contrast, feeding rats with cod protein completely prevented the deleterious effect of fat feeding on insulin-stimulated PI 3-kinase activity. The activation of the downstream kinase Akt/PKB by insulin, assessed by in vitro kinase assay and phosphorylation of GSK-3β, were also impaired in muscle of high-fat–fed rats consuming casein or soy protein, but these defects were also fully prevented by dietary cod protein. However, no effect of cod protein was observed on atypical protein kinase C activity. Normalization of PI 3-kinase/Akt activation by insulin in rats fed high-fat diets with cod protein was associated with improved translocation of GLUT4 to the T-tubules but not to the plasma membrane. Taken together, these results show that dietary cod protein is a natural insulin-sensitizing agent that appears to prevent obesity-linked muscle insulin resistance by normalizing insulin activation of the PI 3-kinase/Akt pathway and by selectively improving GLUT4 translocation to the T-tubules. ***Diabetes*** **52:29–37, 2003**

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aPKC, atypical PKC; DHPr, dihydropyridine receptor; IRAP, insulin-responsive aminopeptidase; IRS, insulin receptor substrate; IM, intracellular membrane; PI, phosphatidylinositol; PKC, protein kinase C; PM, plasma membrane; PVDF, polyvinylidene difluoride; SR, sarcoplasmic reticulum; TNF, tumor necrosis factor; T-tubules, transverse tubules.

Development of peripheral insulin resistance is an important feature of type 2 diabetes (1,2). Skeletal muscle, accounting for >75% of glucose disposal in the postprandial state (1) represents an attractive therapeutical target for the prevention of this disease. The mechanism by which insulin increases glucose uptake in muscle involves the translocation of the insulin-sensitive glucose transporter GLUT4 from an intracellular storage site to the plasma membrane and the T-tubules (3–5). GLUT4 translocation has been shown to be defective in skeletal muscle of insulin-resistant and type 2 diabetic subjects (6–8). Insulin stimulates GLUT4 translocation by activating its receptor intrinsic tyrosine kinase activity toward intracellular substrates (5). In skeletal muscle, this leads to the tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2 leading to the recruitment and activation of phosphatidylinositol (PI) 3-kinase, a key enzyme in the regulation of insulin-stimulated glucose transport (9).

The identification of downstream effectors of PI 3-kinase involved in the regulation of glucose transport is the subject of intense investigation. These include the serine/threonine kinases Akt (also termed protein kinase B [PKB]) and atypical protein kinase C (aPKC). Evidence for the implication of both Akt and aPKC in the insulin-dependent regulation of glucose transport in muscle cells arises from transfection studies using either kinase-inactive or overexpression/constitutively active forms of the kinases (10–13). Whereas a defect in insulin activation of PI 3-kinase is well established in muscle of insulin-resistant animals (14–17), the potential role of either Akt of aPKC in the pathogenesis of insulin resistance remains poorly defined. Using the high-fat–fed rat as a model of obesity-linked insulin resistance, we recently found that insulin action on both Akt and aPKC kinase activities are blunted in skeletal muscle of these rats (14). These impairments in insulin signaling were associated with a complete abrogation of insulin-stimulated GLUT4 translocation to both the plasma membrane and the T-tubule domains of the muscle cell surface (14).

Much effort is being spent to find new therapeutical

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approaches to overcome insulin resistance in humans. Dietary interventions have been proven to be a useful and efficient tactic to sensitize target tissues to insulin in animals (18). For instance, ω-3 polyunsaturated fatty acids derived from fish have been shown to improve insulin sensitivity in rats fed a high-fat diet (19). More recently, we have investigated the effects of dietary proteins on insulin sensitivity in diet-induced obesity. We found that dietary cod protein, but not casein or soy protein, prevented the development of whole-body insulin resistance by normalizing insulin-stimulated glucose uptake in muscle of high-fat–fed rats. This was observed despite similar body weight gain, adiposity, and TNF-α expression in both adipose tissue and skeletal muscle among the different dietary groups (20).

This study was therefore undertaken to clarify the cellular mechanisms behind the insulin-sensitizing action of cod protein in high-fat–fed obese rats. More specifically, we have tested the hypotheses that cod protein prevents skeletal muscle insulin resistance by *1*) enhancing insulin signaling to PI 3-kinase, Akt, and aPKC, *2*) increasing GLUT4 protein expression, and/or *3*) improving GLUT4 translocation to the muscle cell surface.

RESEARCH DESIGN AND METHODS

Materials. Reagents for SDS-PAGE and immunoblotting were from Bio-Rad (Mississauga, On, Canada). Enhanced chemiluminescence, 2-deoxy-D-[^{3}H]glucose, and D-^{14}C-sucrose were from NEN Life Science Products (Boston, MA). [γ-^{32}P]ATP, protein A- and G-Sepharose and anti-mouse or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase were purchased from Amersham Pharmacia Biotech (Baie d'Urfé, Quebec, Canada). Anti-goat immunoglobulin G conjugated to horseradish peroxidase, polyclonal antibodies against GLUT1 (raised against 20 C-terminal amino acids [C-20]), GLUT4 (C-20), IRS-1 (C-20), aPKCs (C-20), and Akt 1/2 (recognizing both Akt 1 and 2 [H-136]) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphospecific antibodies against Akt (Ser473 and Thr308) and GSK-3α/β (Ser21 and Ser9) were from New England Biolabs (Beverly, MA). Antibodies against phosphotyrosine (4G10 clone) and Akt/PKB substrate (Crosstide) were obtained from Upstate Biotechnology (Lake Placid, NY). The polyclonal antibody against gp160/insulin-responsive aminopeptidase (IRAP) was kindly provided by Drs. P.F. Pilch and K.V. Kandror (Boston University, Boston, MA). Monoclonal antibodies against the α_1-subunit of the dihydropyridine receptor (DHPr) and SR Ca^{2+}-stimulated adenosinetriphosphatase (Ca^{2+}-ATPase) were kindly supplied by Dr. K. Campbell (University of Iowa, Iowa City, IA). The monoclonal antibody against the α_1-subunit of the Na/K-ATPase was a generous gift from Dr. K. Sweadner (Massachusetts General Hospital, Charlestown, MA). Myelin basic protein was from Sigma (St. Louis, MO). Okadaic acid was purchased from Calbiochem (La Jolla, CA). L-α-PI was from Avanti Polar Lipids (Alabaster, AL). Oxalate-treated TLC silica gel H plates were obtained from Analtech (Newark, DE). All other chemicals were of the highest analytical grade.

Treatment of animals. All experiments reported herein were approved by the Laval University Animal Care and Handling Commitee and comply with Canadian Council on Animal Care guidelines for the care and use of animals for research purposes. Male Wistar rats (Charles River, Montréal, Canada) weighing 200–250 g at the beginning of the study were housed individually in plastic cages in animal quarters maintained at 22°C with a 12:12-h dark-light schedule. Animals were fed a purified high-fat diet containing either casein, cod or soy proteins, or a low-fat diet (Charles River rodent chow 5,075, Purina Mills, St. Louis, MO) for 4 weeks as previously described (20). The high-fat diets were found to be isoenergetic: casein (25.5 kJ/g), soy protein (25.3 kJ/g), and cod protein (25.4 kJ/g). The high-fat diet consisted of 23% (wt/wt) protein (either casein, soy protein, or cod protein [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% BHT. 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 (Charles River rodent chow 5075, Purina Mills, St. Louis, MO), the chow low-fat diet provided 14.3 kJ/g as energy, of which 57.3% were from carbohydrate, 18.1% from protein, and 4.5% from fat.

Hyperinsulinemic-euglycemic clamp and tracer injection. The clamp procedure was essentially performed as previously described (21). Briefly, unrestrained conscious fasted animals were allowed to rest for 40 min before the initial blood sample (300 μl) was obtained. For hyperinsulinemic-euglycemic clamp, a continuous intravenous infusion of insulin was then started at the rate of 4 $mU \cdot kg^{-1} \cdot min^{-1}$ and continued for 2 h. The arterial blood glucose concentration was clamped using a variable-rate glucose infusion. Steady-state insulin levels (in nanomoles per liter) were as follows: (chow: 0.58 ± 0.08; casein: 0.68 ± 0.09; cod protein: 0.79 ± 0.19; soy protein: 0.69 ± 0.13). There were no statistically significant differences among high-fat–fed groups, but the insulin levels were lower in the chow versus all high-fat–fed groups. Control rats were infused with saline for the same period of time, and no exogenous glucose was necessary to maintain euglycemia. Tracer injection (2-deoxy-D-[^{3}H]glucose and ^{14}C-sucrose) was administered at the end of the clamp (saline- and insulin-infused) to determine individual basal and insulin-stimulated muscle glucose uptake as previously reported (20). Immediately after the clamp, the rats were killed and their hindlimb muscles (soleus, tibialis, gastrocnemius, and quadriceps) were rapidly excised, cleaned of extraneous tissues, and frozen in liquid nitrogen. The muscles were kept at −80°C until further processing.

Acute insulin stimulation. Overnight-fasted rats were injected with either saline or insulin (8 units/kg) for 4 min as described previously (4). Muscles were quickly excised and immediately frozen in liquid nitrogen. Gastrocnemius muscles were homogenized in six volumes of lysis buffer (20 mmol/l Tris, pH 7.5, 140 mmol/l NaCl, 1 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 10% glycerol, 10 mmol/l sodium pyrophosphate, 10 mmol/l NaF, 2 mmol/l Na_3VO_4, 2 mg/ml benzamidine, and 1 mmol/l PMSF) and protease inhibitors cocktail. Okadaic acid (100 nmol/l) was added in lysis buffer for Akt and aPKC kinase activities. Muscle homogenates were solubilized in 1% NP-40 for 1 h at 4°C and centrifuged at 14 000*g* for 10 min. Supernatant was used for insulin signaling studies as described below.

Tyrosine phosphorylation of the insulin receptor and IRSs. Muscle lysates (1 mg of protein) were immunoprecipitated with 2 μg of anti-phosphotyrosine (4G10) coupled to protein A-Sepharose overnight at 4°C. The immune complex was washed three times in PBS (pH 7.4) containing 1% NP-40 and 2 mmol/l Na_3VO_4, resuspended in Laemmli buffer, and boiled for 5 min. Proteins were resolved on SDS-PAGE (6% gel) and processed for Western blot analysis.

PI 3-kinase activity. Muscle lysate (1 mg of protein) was immunoprecipitated with 2 μg of anti–IRS-1 coupled to protein A-Sepharose overnight at 4°C. Immune complex was washed twice with Wash I (PBS, pH 7.4, 1% NP-40, and 2 mmol/l Na_3VO_4), twice with Wash II (100 mmol/l Tris, pH 7.5, 500 mmol/l LiCl, and 2 mmol/l Na_3VO_4) and twice with Wash III (10 mmol/l Tris, pH 7.5, 100 mmol/l NaCl, 1 mmol/l EDTA, and 2 mmol/l Na_3VO_4). Beads were resuspended in 70 μl of kinase buffer (8 mmol/l Tris, pH 7.5, 80 mmol/l NaCl, 0.8 mmol/l EDTA, 15 mmol/l $MgCl_2$, 180 μmol/l ATP, and 5 μCi [γ-^{32}P]ATP) and 10 μl of sonicated PI mixture (20 μg L-α-PI, 10 mmol/l Tris, pH 7.5, and 1 mmol/l EGTA) for 15 min at 30°C. Reaction was stopped by the addition of 20 μl 8 mol/l HCl, mixed with 160 μl $CHCl_3$:CH_3OH (1:1) and centrifuged. Lower organic phase were spotted on oxalate-treated silica gel TLC plate and developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (60:47:11.6:2). The plate was dried and visualized by autoradiography with intensifying screen at −80°C.

Akt/PKB activity. Muscle lysate (1 mg of protein) was immunoprecipitated with 4 μg of anti-Akt 1/2 coupled to protein G-Sepharose for 4 h at 4°C. Immune complex was washed twice with Wash I (PBS, pH 7.4, 1% NP-40, and 100 μmol/l Na_3VO_4) and twice with with Wash II (50 mmol/l tris, pH 7.5, 10 mmol/l $MgCl_2$, and 1 mmol/l DTT). Beads were resuspended in 30 μl of kinase buffer (50 mmol/l Tris, pH 7.5, 10 mmol/l $MgCl_2$, 1 mmol/l DTT, 8 μmol/l ATP, 2 μCi [γ-^{32}P]ATP, and 50 μmol/l Crosstide) for 30 min at 30°C. Reaction product was resolved on a 40% acrylamide gel and visualized by autoradiography with intensifying screen at −80°C.

Atypical PKC (ζ/λ) activity. Muscle lysate (1 mg of protein) was immunoprecipitated with 2 μg of anti-PKC (ζ/λ) overnight at 4°C, then immune complex was collected on protein A/G-Sepharose for 2 h. Beads were washed twice with Wash I (PBS, pH 7.4, 1% NP-40, and 2 mmol/l Na_3VO_4), twice with Wash II (100 mmol/l Tris, pH 7.5, 500 mmol/l LiCl, and 100 μmol/l Na_3VO_4) and twice with Wash III (50 mmol/l Tris, pH 7.5, 10 mmol/l $MgCl_2$, and 100 μmol/l Na_3VO_4). Beads were resuspended in 30 μl of kinase buffer (50 mmol/l Tris, pH 7.5, 10 mmol/l $MgCl_2$, 40 μmol/l ATP, 5 μCi [γ-^{32}P]ATP, and 5 μg myelin basic protein) for 12 min at 30°C. Reaction was stopped by the addition of Laemmli buffer and heated for 30 min at 37°C. Reaction product was resolved on 13% SDS-PAGE. Gel was dried and visualized by autoradiography with intensifying screen at −80°C.

Subcellular fractionation. Plasma membranes, transverse tubules (T-tubules), and GLUT4-enriched intracellular membranes were isolated from 8–10 g of muscles (mixed gactrocnemius and quadriceps) using a procedure

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TABLE 1
Characterization of membrane fractions from skeletal muscle

Fractions	Diet	Insulin	Protein recoveries (μg/g muscle)	5′-nucleotidase ($nmol \cdot mg^{-1} \cdot min^{-1}$)
PM				
	Chow	−	30 ± 5	557 ± 57
	Chow	+	30 ± 5	546 ± 74
	HF-Casein	−	38 ± 5	417 ± 138
	HF-Casein	+	31 ± 4	355 ± 77
	HF-Cod	−	37 ± 8	420 ± 70
	HF-Cod	+	38 ± 9	378 ± 95
	HF-Soy	−	34 ± 3	368 ± 36
	HF-Soy	+	38 ± 8	388 ± 45
TT				
	Chow	−	234 ± 24	90 ± 7
	Chow	+	279 ± 12	72 ± 8
	HF-Casein	−	258 ± 10	59 ± 14
	HF-Casein	+	265 ± 24	52 ± 14
	HF-Cod	−	267 ± 18	68 ± 21
	HF-Cod	+	305 ± 51	57 ± 17
	HF-Soy	−	220 ± 53	82 ± 19
	HF-Soy	+	280 ± 38	58 ± 14
IM				
	Chow	−	95 ± 21	ND
	Chow	+	99 ± 27	ND
	HF-Casein	−	136 ± 19	ND
	HF-Casein	+	219 ± 45	ND
	HF-Cod	−	190 ± 63	ND
	HF-Cod	+	181 ± 29	ND
	HF-Soy	−	207 ± 68	ND
	HF-Soy	+	193 ± 36	ND

Values are means ± SE. HF, high-fat; ND, nondetectable.

developed in our laboratory (4,22). This subcellular fractionation protocol has been extensively characterized with immunologic and enzymatic markers (4,22). In brief, this technique allows the simultaneous and separated isolation of plasma membrane, T-tubules, and intracellular membrane vesicles from the same muscle homogenate. GLUT4 content was determined in fractions obtained from saline- or insulin-infused rats by Western blotting as previously described (14). Characterization of membrane fractions are presented in Table 1 and Fig. 6*A* and are in good agreement with our previous studies (4,22).

Western blot analysis. Membranes (10 μg) or muscle homogenates (50 μg) were subjected to SDS-PAGE (7.5% gel) and electrophoretically transferred to polyvinylidene difluoride (PVDF) filter membranes for 2 h. PVDF membranes were then blocked for 1 h at room temperature with buffer I (50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl) containing 0.04% NP-40, 0.02% Tween-20, and 5% nonfat milk, followed by overnight incubation at 4°C with primary antibodies as described in figure legends. The PVDF membranes were then washed for 30 min, followed by a 1 h incubation with either anti-mouse or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase in buffer I containing 1% BSA. The PVDF membranes were washed for 30 min in buffer I, and the immunoreactive bands were detected by the enhanced chemiluminescence method. A muscle standard (an unrelated crude membrane fraction) was run on every gel for comparison of samples from different immunoblots.

Data analysis. Autoradiographs were analyzed by laser scanning densitometry using a tabletop Agfa scanner (Arcus II; Agfa-Gavaert, Morstel, Belgium) and quantified with the National Institutes of Health Image program (WEB: rsb.info.nih.gov/nih-image/). Data are presented as means ± SE. The effects of dietary proteins were compared by ANOVA. Differences were considered to be statistically significant at $P < 0.05$. Values for chow-fed animals were indicated as reference only and were thus not included in the statistical analysis.

RESULTS

Effect of dietary proteins on whole-body and skeletal muscle insulin action in high-fat–fed rats. Physiological parameters of the same dietary groups have been previously described (20). In brief, body weight and energy intake were similar among high-fat–fed dietary groups but were significantly higher compared with the control chow-fed group (data not shown). The effect of dietary proteins on insulin-mediated whole-body glucose disposal and muscle glucose uptake was determined during an hyperinsulinemic-euglycemic clamp coupled with tracer 2-deoxy-D-[^{3}H]glucose injection. The insulin-mediated glucose infusion rates that were required to maintain euglycemia was significantly lower ($P < 0.05$) in casein- and soy protein–fed groups as compared with cod protein–fed obese rats (casein: 12.2 ± 1.1; soy protein: 7.9 ± 1.1; cod protein: 19.7 ± 2.7 $mg \cdot kg^{-1} \cdot min^{-1}$). Whole-body insulin action in the high-fat–fed obese rats consuming cod protein was found to be similar to that observed in the reference chow-fed lean group (16.9 ± 2.0 $mg \cdot kg^{-1} \cdot min^{-1}$). Insulin-stimulated glucose uptake in gastrocnemius and quadricep muscles of high-fat–fed obese rats was also significantly greater ($P < 0.05$) in high-fat–fed rats consuming cod protein, as compared with casein- and soy protein–fed groups (casein: 71.8 ± 8.4; soy protein: 63.1 ± 8.1; cod protein: 112.5 ± 17.4 $nmol \cdot g^{-1} \cdot min^{-1}$) and similar to that observed in chow-fed rats (112.8 ± 13.5 $nmol \cdot g^{-1} \cdot min^{-1}$). The glucose infusion rate and muscle 2-deoxy-D-[^{3}H]glucose uptake were not significantly different between casein- and soy protein–fed animals. Basal glucose uptake in muscles was measured following tracer injection at the end of the saline clamp and was found to be unaffected by the source of dietary proteins (data not shown).

Effect of dietary proteins on insulin receptor and insulin receptor substrate-1 tyrosine phosphorylation. We first examined whether cod protein exerts its

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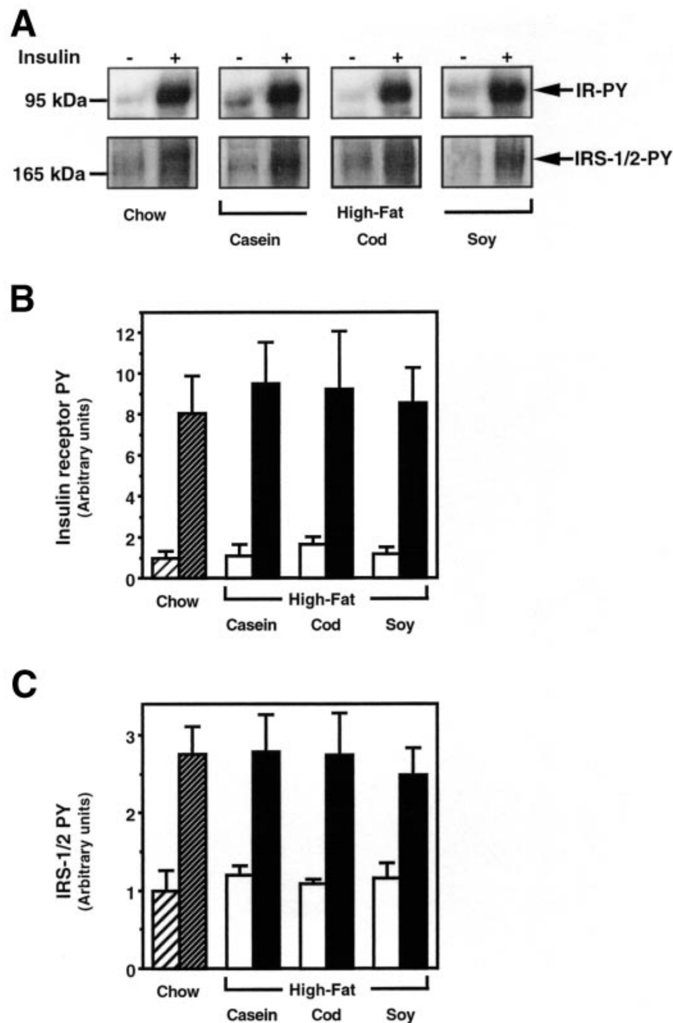

FIG. 1. Effect of dietary proteins on tyrosine phosphorylation of IR/IRS proteins. Overnight-fasted rats were injected with either saline or insulin (8 units/kg) for 4 min. Phosphoproteins were immunoprecipitated from muscle homogenates, resolved on 6% SDS-PAGE, and immunoblotted using anti-phosphotyrosine (4G10) as described under RESEARCH DESIGN AND METHODS. *A*: Representative immunoblots. *B*: Quantification of tyrosine phosphorylation of the insulin receptor. *C*: IRS proteins were expressed relative to chow-fed basal values. The means ± SE from 4–6 determinations from different animals are shown. □, basal; ■, insulin.

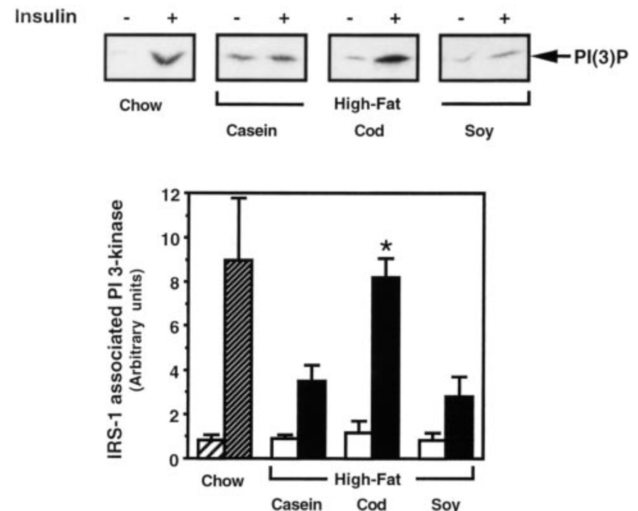

FIG. 2. Effect of dietary proteins on IRS-1–associated PI 3-kinase activity. PI 3-kinase was measured in anti–IRS-1 immunoprecipitates of muscle homogenate as described in RESEARCH DESIGN AND METHODS. Quantification of ^{32}P incorporated into PI 3-phosphate (PI 3P) was expressed relative to chow-fed basal values. A representative autoradiograph is shown at the top of the figure. The means ± SE from 4–6 determinations from different animals are shown. □, basal; ■, insulin. *$P < 0.05$ vs. casein- and soy protein–fed animals (insulin).

beneficial action on muscle insulin sensitivity by increasing tyrosine phosphorylation of the insulin receptor and IRS proteins, the more proximal steps in the propagation of the insulin signal to downstream effectors (5). An in vivo injection of insulin induced a large increase in the phosphotyrosine content of both the insulin receptor (Fig. 1*A* and *B*) and IRS proteins (Fig. 1*A* and *C*) in muscle of chow-fed rats. The stimulatory effect of insulin on IR/IRS tyrosine phosphorylation was similar in rats that were fed a high-fat diet irrespective of the dietary protein source (Fig. 1*A*–*C*).

Effect of dietary proteins on IRS-1–associated PI-3 kinase activity. Activation of PI 3-kinase represents an essential step in the stimulation of glucose transport by insulin (9). We therefore measured the impact of high-fat feeding and dietary proteins on insulin-stimulated PI 3-kinase activity in IRS-1 immunoprecipitates, as IRS-1 is the main isoform responsible for glucose uptake in muscle. In skeletal muscle of chow-fed rats, insulin strongly activated IRS-1–associated PI 3-kinase (Fig. 2). Feeding rats a high-fat diet with either casein or soy protein severely impaired the ability of insulin to activate the enzyme (~60% reduction versus chow-fed rats). Strikingly, dietary cod protein completely prevented the loss of insulin action on PI 3-kinase activity in high-fat–fed rats.

Effect of dietary proteins on Akt activation and GSK-3α/β phosphorylation. We next determined whether dietary proteins modulate Akt activation status by measuring its phosphorylation on both regulatory sites (Ser473 and Thr308) and its activity toward an exogenous substrate. Insulin stimulated the phosphorylation of Akt on both Ser473 and Thr308 in chow-fed animals (Fig. 3*A*). Insulin-induced Akt phosphorylation was not different in high-fat–fed rats consuming either casein, cod, or soy proteins. Akt kinase activity was then measured in Akt-1/2 immunoprecipitates using crosstide, a peptide containing a GSK-3 motif as substrate. We found that Akt activity is decreased (−40%, $P < 0.05$) in insulin-stimulated muscle of high-fat–fed rats consuming casein or soy protein, as compared with rats fed dietary cod protein (Fig. 3*A* and *B*). Insulin-mediated Akt activity was virtually identical between rats fed a high-fat diet with cod protein and rats fed standard chow diet (Fig. 3*A* and *B*). Basal Akt kinase activity tended to be lower in muscle of casein- and soy protein–fed animals but it was not statistically different from chow or cod-fed groups. Defective Akt activity in high-fat–fed rats consuming either casein or soy protein was further confirmed by the observation that insulin-stimulated GSK-3β phosphorylation (Ser9), an in vivo index of Akt activation, was also impaired in the muscle of these rats, whereas cod protein–fed animals were fully protected against this defect (Fig. 3*A* and *C*). In contrast, we did not find evidence that basal or insulin-induced phosphorylation of GSK-3α (Ser21) is modulated by high-fat feeding or the source of dietary proteins (data not shown).

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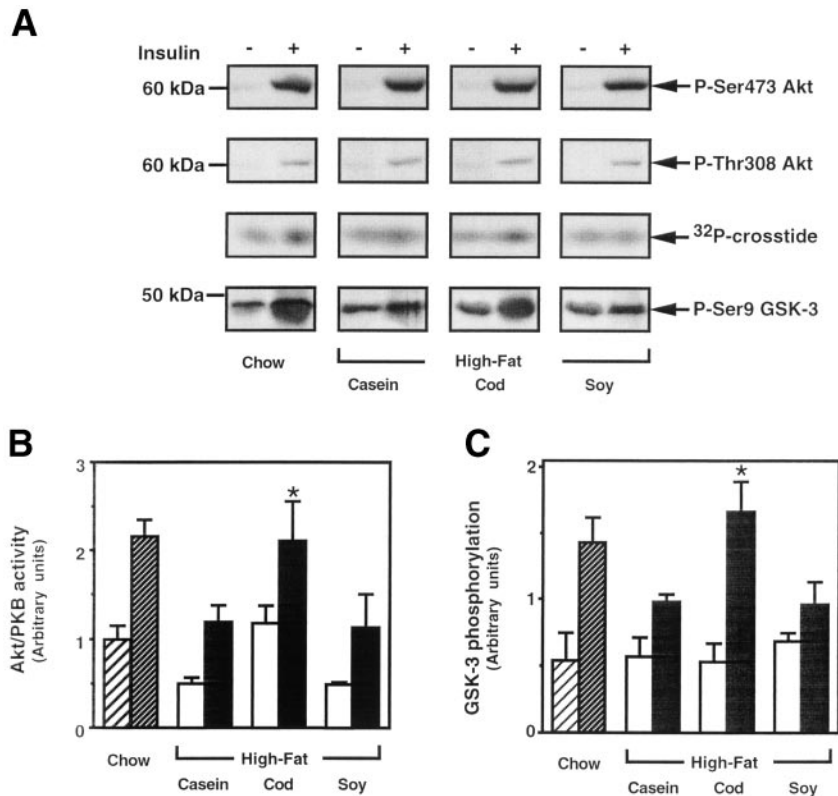

FIG. 3. Effect of dietary proteins on Akt/PKB activation and GSK-3 phosphorylation. Overnight-fasted rats were injected with either saline or insulin (8 units/kg) for 4 min. *A*: Phosphorylation state of Akt (Ser473/Thr308) and GSK-3β (Ser9) was measured in muscle homogenates. Proteins (50 μg) were separated on 7.5% or 8% SDS-PAGE, respectively, and immunoblotted with anti-phosphospecific antibodies against Akt (Ser473 and Thr308) and GSK-3β (Ser9) as described in RESEARCH DESIGN AND METHODS. Akt kinase activity was measured in anti-Akt-1/2 immunoprecipitates (^{32}P incorporated into Crosstide) as described in RESEARCH DESIGN AND METHODS. *B*: Quantification of ^{32}P incorporated into Crosstide (autoradiographs) was expressed relative to chow-fed basal values. *C*: Quantification of GSK-3β phosphorylation was expressed relative to an internal standard (muscle extract). The means ± SE from 4–6 determinations from different animals are shown. □, basal; ■, insulin. **P* < 0.05 vs. casein- and soy protein–fed animals (insulin).

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Effect of dietary proteins on aPKC activity and translocation. Another downstream target of PI 3-kinase is the atypical form of PKC. In muscle of chow-fed rats, insulin stimulated both the kinase activity of aPKC (ζ/λ) and its translocation to the plasma membrane (Fig. 4*A–C*). Fat feeding resulted in high basal aPKC activity, regardless of the dietary protein source, and that activity was not further increased by insulin. Accordingly, membrane association of aPKC was already elevated in muscle of high-fat–fed rats, and insulin failed to induce translocation of the kinase in all high-fat–fed animal groups.

Effect of dietary proteins on GLUT4 protein expression in skeletal muscle of high-fat–fed rats. GLUT4 protein expression was measured in muscles enriched in type I (soleus), type IIa (red tibialis), or type IIb (white gastrocnemius) fibers (23). GLUT4 content was selectively decreased in type IIa fiber–enriched muscle of high-fat–fed rats as compared with chow-fed animals (Fig. 5). However, GLUT4 protein expression was not modulated by the dietary protein source. This observation was also confirmed in muscles used for the GLUT4 translocation protocol (mixed gastrocnemius and quadriceps), which are enriched (~50%) with type IIa fibers where the GLUT4 content was reduced in all high-fat–fed animals (chow: 105.05 ± 3.85; casein: 67.67 ± 7.89; cod protein: 70.88 ± 10.64; soy protein: 58.78 ± 5.28).

Effect of dietary proteins on GLUT4 translocation. We next examined the effects of dietary proteins on GLUT4 translocation from a GLUT4-enriched intracellular membrane (IM) fraction to the plasma membrane (PM) and the T-tubules (TT) isolated by subcellular fractionation of control and insulin-stimulated muscles. Protein recoveries and 5′-nucleotidase activity of membrane fractions were similar between dietary groups (Table 1). We found, as expected, that the α1 subunit of the Na/K ATPase was highly enriched in the PM, while being undetectable in the TT or IM. The DHPr, a specific marker of T-tubules, was mainly recovered in the TT fraction with much lower amounts in the PM and IM fractions. Furthermore, we used the IRAP as a marker of the intracellular membranes, and it was only detected in the IM fraction. IRAP was not detected in the PM and TT fractions, even after prolonged exposition of the immunoblots (data not shown). The IM fraction was devoid of sarcoplasmic reticulum (SR) membranes, as revealed by the trace amounts the Ca^{2+}-ATPase recovered in that fraction. The TT fraction contains some level of Ca^{2+}-ATPase, most probably reflecting the recovery of small amounts of triads (where T-tubules appose the SR) in that fraction. Importantly, the recovery of all these markers were neither affected by obesity (chow versus high-fat) nor by the source of dietary proteins.

Insulin stimulation induced GLUT4 translocation from the intracellular membranes to both the plasma membrane and the T-tubules surface compartments in chow-fed rats (Fig. 6*B–D*). However, insulin failed to induce GLUT4 translocation to either cell surface domains in muscle of high-fat–fed rats consuming casein. GLUT4 recruitment to the plasma membrane was detectable in high-fat–fed rats consuming soy or cod proteins. However, plasma membrane GLUT4 content in the insulin-stimulated state still remained much lower in these groups compared with lean control rats. Interestingly, translocation of GLUT4 to the T-tubules was markedly increased by dietary cod protein in high-fat–fed rats and, importantly, the transporter content in the insulin-stimulated state was significantly higher than that of casein- or soy protein–fed rats (Fig. 6*C*). Insulin-induced GLUT4 recruitment from the intracellular membrane fraction of high-fat–fed rats was observed in

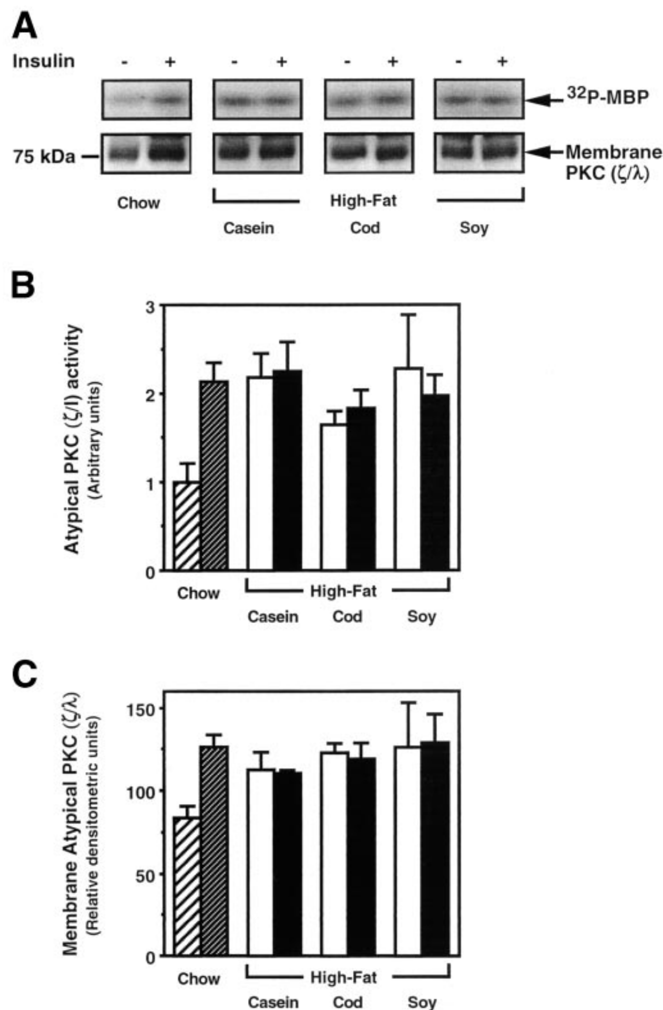

FIG. 4. Effect of dietary proteins on aPKC activity and translocation. Overnight-fasted rats were injected either with saline or insulin (8 units/kg) for 4 min. *A* (*upper panel*): Representative autoradiograph. *A* (*lower panel*): representative immunoblot. *B*: Quantification of aPKC kinase activity was measured in anti-PKC (ζ/λ) immunoprecipitates as described in RESEARCH DESIGN AND METHODS. Quantification of ^{32}P incorporated into myelin basic protein was expressed relative to chow-fed basal values. *C*: PM recovery of aPKC in control and insulin-infused rats was assessed by Western blotting as described in RESEARCH DESIGN AND METHODS. The means ± SE from 4–6 determinations from different animals are shown. □, basal; ■, insulin.

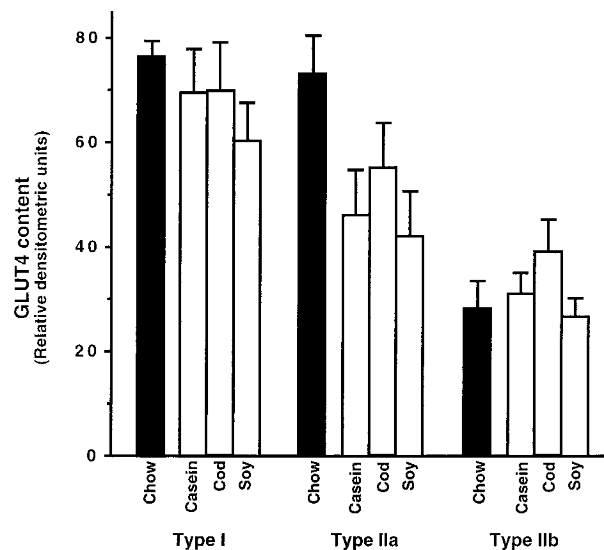

FIG. 5. Effect of dietary proteins on GLUT4 protein expression in skeletal muscle enriched in different fiber types. GLUT4 protein expression was measured in muscle homogenates from type I- (soleus), type IIa- (red tibialis), and type IIb- (white gastrocnemius) enriched muscles of chow- and high-fat–fed rats. Proteins (50 μg) were resolved on 7.5% SDS-PAGE and immunoblotted with polyclonal anti-GLUT4 as described in RESEARCH DESIGN AND METHODS. The means ± SE from 4–10 determinations for each muscle are shown.

cod- and soy protein–fed rats, but not in casein-fed rats (Fig. 6*D*). Furthermore, a positive and statistically significant relationship was found between GLUT4 content in the T-tubules and the glucose infusion rate ($r = 0.68$; $P = 0.01$), an index of peripheral insulin sensitivity. However, no correlations were obtained when the glucose infusion rate was compared with GLUT4 content in the plasma membrane ($r = -0.37$; $P = 0.25$) or the intracellular membrane ($r = 0.15$; $P = 0.66$) fractions.

DISCUSSION

Nutrients such as carbohydrates and lipids are important modulators of insulin action on glucose metabolism (18). However, much less is known about the impact of dietary proteins on insulin sensitivity and glucose homeostasis. Our recent finding that cod protein, but not casein or soy protein, prevents the development of skeletal muscle insulin resistance in high-fat–fed obese rats indicates that dietary proteins can also have a significant impact on insulin action (20). It is therefore important to delineate the cellular mechanisms by which dietary proteins influence insulin sensitivity in muscle.

Characterization of the insulin signal transduction pathway in skeletal muscle of chow-fed lean and high-fat–fed obese rats revealed that feeding cod protein completely normalized insulin-induced activation of IRS-1–associated PI 3-kinase as compared with rats fed either casein or soy protein. Consistent with previous reports (14,24), high-fat feeding did not alter insulin-induced tyrosine phosphorylation of the insulin receptor and IRS-1. Therefore, it appears that the protective effect of cod protein on insulin signaling to PI 3-kinase cannot be explained by increased IRS-1 tyrosine phosphorylation. It cannot be excluded, however, that high-fat feeding reduced the phosphorylation of only a small subset of tyrosine residues, not detectable by immunoblotting, and that dietary cod protein normalized phosphorylation on these residues. This has been recently proposed to explain normal IR/IRS-1 tyrosine phosphorylation in the face of impaired IRS-1–associated PI 3-kinase activity in mice overexpressing the leukocyte-antigen related (LAR) tyrosine phosphatase in muscle (25). Similarly, glucosamine-infused rats displayed normal insulin-stimulated tyrosine phosphorylation of these proximal elements of insulin signaling, but impaired activation of PI 3-kinase (26). Another possible mechanism of action of cod protein is through reduction of a serine kinase activity, which was shown to be elevated in muscle extracts from insulin-resistant animals (27). Indeed, serine phosphorylation of IRS-1 (28), but also of the p85 regulatory subunit of PI 3-kinase (29), impairs the lipid kinase activity of the p110 catalytic subunit. In this regard, we have recently showed that certain amino acids inhibit the insulin-induced activation of PI 3-kinase and glucose transport by increasing serine/threonine phosphorylation

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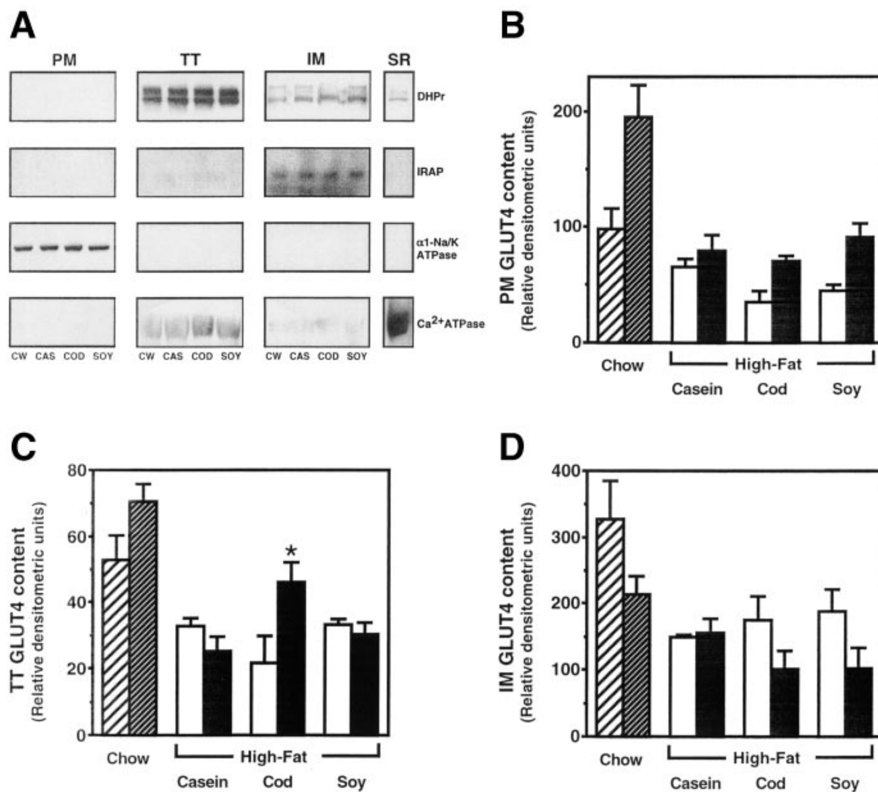

FIG. 6. Effect of dietary proteins on GLUT4 translocation in skeletal muscle. Rats were clamped with either saline (basal) or insulin (4 $mU \cdot kg^{-1} \cdot min^{-1}$) for 2 h. Immediately after the clamp, muscles (mixed gastrocnemius and quadriceps) were quickly excised, cleaned of extraneous tissues, and frozen in liquid nitrogen. *A*: Representative immunoblots of membrane markers from 3–5 animals per dietary groups (DHPr, IRAP, α1-Na/K ATPase, and Ca^{2+}-ATPase) of PM, T-tubules, IM, and SR isolated from muscle of saline-infused rats are presented. GLUT4 content was assessed by Western blotting in PM (*B*), T-tubules (*C*), and IM (*D*) isolated from saline- or insulin-infused rats as described in RESEARCH DESIGN AND METHODS. The means ± SE from 4–5 individual membrane preparations are shown. □, basal; ■, insulin. *$P < 0.05$ vs. casein- and soy protein–fed animals (insulin).

of IRS-1 through activation of the mTOR/p70 S6 kinase pathway without affecting IRS-1 tyrosine phosphorylation (30). It is therefore tempting to speculate that the insulin-sensitizing action of cod protein may be linked to a reduced ability of cod protein–derived amino acids to induce mTOR/p70 S6 kinase activation as compared with casein- or soy protein–derived amino acids, but this hypothesis remains to be tested.

The involvement of defects in either Akt or aPKC activation in the pathogenesis of skeletal muscle insulin resistance remains controversial. Both circumstancial and direct evidences exist implicating both downstream effectors of PI 3-kinase in the development of impaired insulin action in muscle (14,31–34). Insulin-induced Akt activity was reported to be impaired in muscle of diabetic Goto-Kakizaki (GK) rats, and restored following correction of hyperglycemia (33). On the other hand, Kanoh et al. (31) showed that aPKC activity, but not Akt, was decreased in muscle of GK rats and that this defect could be alleviated by thiazolidienedione treatment. Insulin-induced activation of Akt in skeletal muscle of type 2 diabetic subjects has been found to be reduced in one study (35) but normal in another one (36). The recent finding that Akt2 knockout mice develop muscle insulin resistance is consistent with the view that this enzyme plays a key role in insulin action in that tissue (32). Using the high-fat–fed rat model of insulin resistance, we found that both Akt and aPKC kinase activation by insulin are reduced in skeletal muscle. In accordance with our PI 3-kinase data, feeding cod protein restored the stimulatory effect of insulin on Akt activity in high-fat–fed rats. Restoration of Akt activity in high-fat fed rats consuming cod protein was confirmed by measurements of GSK-3β phosphorylation in muscle, an in vivo index of Akt activity. In contrast, cod protein failed to restore the activation of aPKC in muscle of high-fat–fed rats. Taken together, our data suggest that normalization of insulin-induced Akt activity contributes to the beneficial action of cod protein on muscle insulin sensitivity in high-fat–fed animals. Whereas aPKC is not modulated by dietary cod protein, its constitutively elevated state of activity in muscle of high-fat–fed rats may be required for complete normalization of insulin-stimulated glucose uptake in these animals.

Activation of Akt by PI 3-kinase is known to be dependent on the phosphorylation of both Thr308 and Ser473 residues by PDK-1 (37,38) and the putative PDK-2 (39), respectively. However, we found that both high-fat feeding and cod protein consumption modulated Akt activity without affecting the phosphorylation state of the enzyme on Ser473 and Thr308. Thus, although Akt activation by insulin appears unaffected based on Akt phosphorylation status, the actual ability of the kinase to phosphorylate an exogenous substrate was significantly altered. This apparent discrepancy may be explained by the fact that PI 3-kinase was activated at least threefold by insulin in all dietary groups (Fig. 2), perhaps allowing sufficient production of PI(3,4,5) P_3, for full activation of PDKs and phosphorylation of Akt, suggesting that another mechanism may account for modulation of Akt kinase activity by high-fat feeding and dietary proteins.

The ultimate step involved in the activation of glucose transport by insulin in muscle cells is the translocation of GLUT4 to the cell surface domains, namely the plasma membrane and the T-tubules. We first examined whether GLUT4 protein expression was modulated by dietary proteins, since we previously found a selective reduction in GLUT4 content in type IIa fiber–enriched muscles in high-fat–fed rats (14). Cod protein failed to prevent the

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reduction in GLUT4 content in type IIa fibers, suggesting that this defect is not the primary cause of impaired insulin action in muscle of high-fat–fed rats. The beneficial effect of cod protein rather appears to be linked to an increased number of GLUT4 transporters at the muscle cell surface. Interestingly, GLUT4 content was selectively increased in the T-tubules but not in the plasma membrane. This indicates that improved GLUT4 translocation to the T-tubules, even in the face of reduced GLUT4 content in the plasma membrane, is sufficient to confer insulin sensitivity in muscle of obese rats fed cod protein. The determining role of T-tubules in the regulation of insulin-stimulated glucose transport is not without precedent. Indeed, we have shown that GLUT4 translocation to the T-tubules, but not to the plasma membrane, is impaired in muscle from insulin-deficient diabetic rats (40). Furthermore, disruption of the T-tubules by glycerol shock completely abolished insulin-stimulated glucose transport in isolated muscle (41). More recent studies have shown that adenovirus-mediated overexpression of Munc18c, a protein that binds and inhibits the function of the t-SNARE syntaxin 4, abrogated insulin-induced GLUT4 recruitment to the T-tubules, but not to the plasma membrane (42). This is consistent with the fact that the plasma membrane content of syntaxin 4, which is required for the docking and fusion of GLUT4 at the cell surface, is greater than that of T-tubules and could not be saturated by Munc18c overexpression (42). Yang et al. (43) further showed that syntaxin 4 heterozygous knockout mice display impaired insulin-stimulated glucose transport and GLUT4 translocation in muscle. Since the T-tubule membrane is more likely to be affected by a 50% reduction of syntaxin 4, it was proposed that a reduced GLUT4 translocation to the T-tubules was leading to the development of insulin resistance in these mice. Collectively, these results strongly argue for an important role of the T-tubules in the regulation of insulin-stimulated glucose transport and, possibly, in the development of insulin resistance.

A puzzling aspect of this study is the observation that insulin was able to mobilize similar amount of GLUT4 from the IMs isolated from soy protein– and cod protein–fed animals, while translocation of the transporter to the T-tubules was only observed in the latter group. It is now recognized that GLUT4 cycles through a complex network of intracellular organelles including the *trans*-Golgi network and the endosomal system and that insulin influences movement between these organelles (rev. in 44). It is unlikely that the IM fraction isolated by our procedure contains all of these organelles. Thus, it is possible that despite the fact that GLUT4 disappearance in the IM isolated from cod protein– and soy protein–fed rats were similar, the targeting of these vesicles was not the same. For instance, it is possible that GLUT4 vesicles leaving the IM pool in soy protein–fed animals are mistargeted to other intracellular compartment(s), resulting in impaired mobilization to the cell surface.

Another issue that deserves attention is the ability of soy protein–fed animals to maintain the capacity to translocate GLUT4 to the plasma membrane, whereas casein-fed rats were completely irresponsive to insulin with respect to translocation. Despite this, insulin-stimulated glucose transport into muscles as well as insulin signaling to the PI 3-kinase/Akt pathway was similarly impaired in these two groups. One possible explanation is that although the "classical" insulin signaling profile was almost identical between casein- and soy protein–fed groups, it could be argued that other signaling pathway(s) are affected in muscle of the former group. For example, the PI 3-kinase-independent CAP-Cbl-TC10 pathway was also shown to participate in the translocation of GLUT4 to the cell surface (rev. in 45). Therefore, it is possible that this pathway is more active in soy protein–fed animals as compared with those fed casein. One should note, however, that translocation of GLUT4 to the plasma membrane, with no translocation to the T-tubules, was not sufficient to confer insulin sensitivity in soy protein–fed animals.

In summary, this study provides convincing evidence that dietary proteins are important modulators of insulin signaling and action in rat skeletal muscle. Furthermore, we showed that dietary cod protein is a potent and natural insulin-sensitizing agent that normalizes the activation status of the PI 3-kinase/Akt pathway coupled to an increased translocation of GLUT4 to the T-tubules in obese high-fat–fed rats. Identification of the precise molecular mechanism by which dietary cod protein improves insulin signaling to PI 3-kinase/Akt will help defining novel therapeutic tools for the prevention and treatment of insulin resistance.

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