Effects of a high-fat diet on insulin receptor kinase and the glucose transporter in rats

Mikiko Okamoto, Motozumi Okamoto, Shigeo Kono, Gen Inoue, Tatsuya Hayashi, Atsushi Kosaki, Ikuko Maeda, Masashi Kubota, Hideshi Kuzuya, and Hiroo Imura

The Second Division, Department of Medicine, Kyoto University School of Medicine, Kyoto, Japan

A high-fat (HF) diet causes insulin resistance. To characterize this type of insulin resistance, autophosphorylation of the insulin receptor (IR) in vitro and in vivo, phosphorylation of its endogenous substrates (pp190, pp175), and the amount of glucose transporters (GLUT-2, GLUT-4) were determined using muscles and liver from rats fed a HF-diet. Hyperinsulinemia and insulin resistance were observed by oral glucose tolerance test in the HF group. The levels of insulin binding to wheat germ agglutinin-purified insulin receptors from both muscles and liver were similar to those of the controls. Autophosphorylation of IR on tyrosine residues in vitro was decreased both in muscles and liver. Tyrosine-phosphorylation of both IR and an endogenous substrate (pp175) in response to insulin injection in vivo was decreased in the liver. However, a decrease in the phosphorylation of pp190 in muscles was not found. The levels of both GLUT-4 from muscles and GLUT-2 from liver in the crude membrane were comparable to those of the controls. These data suggest that insulin resistance in rats fed a HF-diet is a post-receptor defect in muscles, although tyrosine-phosphorylation of both IR and its endogenous substrate (pp175) were decreased in liver.

Keywords: insulin receptor; endogenous substrates; glucose transporter; high-fat diet; liver; muscle

Introduction

A change in dietary composition affects glucose metabolism. In particular, a high-fat diet causes insulin resistance. Glucose clearance[1] and glucose uptake in muscles and in brown adipose tissue[2] decreases in vivo. Glucose transport in muscles[1,3] and adipocytes[1,4] decreases in vitro.

Signal transduction of insulin is initiated by insulin binding to the extra-cellular α-subunit of its receptor. The exact mechanisms of signal transduction from insulin binding to subsequent insulin actions are not yet completely established. However, one of the earliest detectable responses to insulin binding is autophosphorylation of the β-subunit of the receptor,[5] and tyrosine-phosphorylation of exogenous[6] and endogenous proteins.[7,8] Cell lines with site-directed insulin receptor mutants lacking the tyrosine-phosphorylation sites[9] or ATP-binding site[10] had an impaired effect of insulin on 2-deoxy-glucose uptake. These data indicate that tyrosine kinase activation is required for the metabolic effects of insulin.[11] The expression of glucose transporters is also altered under various conditions,[12-14] and both the translocation of the glucose transporter from an intracellular pool to the plasma membrane[15] and its intrinsic activity[16] are regulated by insulin.

Any disorder in these and the subsequent steps may cause insulin resistance. A decrease in insulin binding to its receptor,[1,3,4] and a decrease in insulin receptor autophosphorylation,[17-19] have been reported. The present study was undertaken to clarify the mechanisms of the defects caused by a high-fat diet on insulin action. Besides the insulin binding to its receptors and the autophosphorylation of solubilized insulin receptors from muscles and liver, we studied the autophosphorylation of the insulin receptor and the phosphorylation of its endogenous substrates (pp190 in muscles, pp175 in liver) in rats using an anti-

Address reprint requests to Dr. Hideshi Kuzuya, at the Second Division, Department of Medicine, Kyoto University School of Medicine, 54 Kawaharacho, Shogoin, Sakyo-ku, Kyoto 606, Japan.
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phosphotyrosine antibody. Furthermore, we quantified the muscles/adipocytes type glucose transporter (GLUT-4) in muscles[20] and the liver type glucose transporter (GLUT-2) in liver,[21] using specific antibodies.

Materials and methods

Materials

[^{125}I]-^{14}A-insulin and [^{125}I]-protein A were purchased from Amersham (Arlington Heights, IL, USA); wheat germ agglutinin (WGA)-sepharose and protein A were from Pharmacia (Uppsala, Sweden); goat anti-rabbit IgG horseradish peroxidase conjugate, 4-chloro-1-naphthol, Tween-20, reagents for $NaDodSO_4$/polyacrylamide gel electrophoresis (SDS-PAGE), and the Bradford protein assay were from Bio-Rad Laboratories (Richmond, CA, USA); N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (HEPES), phenylmethylsulfonyl fluoride (PMSF), aprotinin, p-nitrophenylphosphoric acid (PNPP), N-acetyl-D-glucosamine, polyethylene glycol (PEG 6000), bovine serum albumin (BSA), and bovine γ-globulin were from Sigma Chemical Co. (St. Louis, MO, USA); human and porcine insulin were from NOVO (Bagsvaerd, Denmark); nitrocellulose paper was from Schleicher & Schuell (Dassel, Germany); nonfat milk protein was from Yukijirushi (Sapporo, Japan); and Triton X-100 was from Wako Chemical Industries (Osaka, Japan).

Antibodies

A monoclonal antibody to the insulin receptor (Ab-3) was purchased from Oncogene Science Inc. (Manhasset, NY, USA). The anti-phosphotyrosine antibody (α-pTyr) was prepared by rabbit-immunization according to Pang et al.[22] The anti-insulin receptor antibody (α-IRc) was raised against the COOH-terminal sequence (1314–1324) of the human insulin receptor,[23] which is identical to residues 1328–1338 of the rat insulin receptor except for two peptides (Ser^{1315}, Glu^{317} in human, Thr^{1329} Asp^{1331}in rat). The anti-GLUT-2 antibody (α-GLUT-2) was raised against an intracellular loop sequence (259–266) of human GLUT-2,[21] which is identical to 257–264 of the rat GLUT-2 sequence except for one amino acid (Tyr^{266} in human, Thr^{264} in rat). The anti-GLUT-4 antibody (α-GLUT-4c) was raised against the COOH-terminal sequence (498–509) of the rat GLUT-4.[20] These antibodies were prepared by immunization of rabbits with synthetic peptides coupled to keyhole limpet hemocyanin (KLH). Antibodies to the peptides were affinity-purified through a peptide-coupled Sepharose 4B column.

Animals and experimental diets

Male Wistar rats, weighing 200 g, were kept under controlled lighting (lights on from 0600 to 1800 hours) and temperature (22° C) with free access to standard laboratory food and water. During the experimental period these rats were individually caged and fed with either an experimental high-fat (carbohydrate:corn starch, fat:lard, protein:milk casein) or control diet (standard laboratory food, CE-2, from CLEA, Japan) (*Table 1*) ad libitum for 4 weeks. Several days in advance of experiments, an oral glucose tolerance test (OGTT) (2g/kg body weight) was performed after a 24-hr fast.

Preparation of solubilized insulin receptor

Insulin receptors were prepared from the hind-limb muscles and the liver by the methods of Burant et al.[24] and Kasuga et al.,[25] respectively, with minor modifications as previously described.[26,27] Briefly, the muscles were homogenized in 25 mmol/L HEPES (pH 7.4), 5 mmol/L EDTA, 1 trypsin inhibitor unit (TIU)/mL aprotinin, 2 mmol/L PMSF, and 1% Triton X-100 (10 mL/g tissue), and centrifuged at 10,000*g* for 10 min. The supernatant was stirred at room temperature for 1 hr. Following centrifugation at 150,000*g* for 90 min, the supernatant was passed over a WGA-sepharose affinity column, and the insulin receptor was eluted with 0.3 mol/L N-acetylglucosamine in 50 mmol/L HEPES (pH 7.4), 0.1% Triton X-100.

Table 1 Compositions of high-fat diet and control diet

	High fat diet		Control diet	
	Weight (g/100g)	Calories (%)	Weight (g/100g)	Calories (%)
Carbohydrate	18.3	13.5	50.2	58.9
Fat	36.2	60.0	4.5	11.9
Protein	36.0	26.5	24.9	29.2
Vitamin	2.4	0	2.4	0
Mineral	7.1	0	7.5	0
Cellulose	0	0	4.1	0
Caloric value (kcal/100g)	543.4		340.9	

The liver was homogenized with a loose Dounce homogenizer in 10 vol. 50 mmol/L HEPES (pH 7.4), 0.25 mol/L sucrose, 5 mmol/L EDTA, 1 TIU/mL aprotinin, 2 mmol/L PMSF, and centrifuged (1200 rpm). Triton X-100 was added to the supernatant to give a final concentration of 1%. The resulting supernatant was centrifuged (4° C, 150,000*g*, for 1 hr), and passed over a WGA affinity column. The insulin receptor was eluted as described above.

Insulin binding

Aliquots (20 μg muscle, 10 μg liver protein) of WGA eluate were incubated with ^{125}I-insulin (25 pmol/L) and various concentrations of unlabeled insulin at 4° C for 16 hr in 200 μL of buffer (150 mmol/L NaCl, 50 mmol/L HEPES, 0.1% BSA, pH 7.8). Insulin-bound receptors were precipitated with bovine γ-globulin and PEG, then washed and counted.[27]

Phosphorylation with solubilized insulin receptor

Solubilized, WGA-purified insulin receptors (300 fmol binding capacity) were diluted to 50 μL, at a final concentration of 50 mmol/L HEPES (pH 7.4), 5 mmol/L Mn^{2+}, 0.1% Triton X-100, and incubated for 16 hr at 4° C with or without insulin (10^{-7} mol/L).[27] The phosphorylation reaction was initiated by adding 150 μmol/L of ATP and terminated by adding five-fold concentrated Laemmli sample buffer.[28] The mixture was heated immediately in boiling water for 3 min. The proteins were separated in 7.5% SDS-PAGE under reducing conditions, and transferred electrophoretically onto nitrocellulose paper for Western blotting.

In vivo phosphorylation study

Five minutes (by preliminary experiments) after the injection of human insulin (0 or 5 units) through the portal vein, the liver was excised, frozen in liquid nitrogen, homogenized, and solubilized with 1% SDS as previously described.[7] SDS was partially removed by chilling the mixture for 30 min and centrifuging. Phosphotyrosine-containing proteins were then immunoprecipitated with α-pTyr. The sediments were incubated with 10 mmol/L

PNPP for 5 hr. The supernatants were separated by SDS-PAGE, transferred onto nitrocellulose paper, and Western blotted with α-pTyr.

In the muscle study, rats were injected with 5 units of human insulin through the femoral vein. Five minutes after the injection, the hind-limb muscles were excised, frozen in liquid nitrogen, and homogenized. The total homogenate was solubilized with 1% Triton X-100 in 50 mmol/L HEPES, 100 mmol/L NaF, 10 mmol/L $Na_4P_2O_7$, 2 mmol/L Na_3VO_4, 5 mmol/L EDTA, and immunoprecipitated with Ab-3. The precipitant was separated by SDS-PAGE, transferred onto nitrocellulose paper, and Western blotted with α-IRc for insulin receptors, and with α-pTyr for tyrosine-phosphorylated proteins. The endogenous substrates of the insulin receptor, including pp190, were prepared by the method of Tobe et al.[29] with a slight modification as follows. A portion of the solubilized samples described above was applied to a hydroxylapatite affinity column, washed with 0.1% Triton X-100 in the same buffer, boiled with Laemmli sample buffer, and centrifuged. The supernatant was separated by SDS-PAGE, transferred onto nitrocellulose paper, and Western blotted with α-pTyr.

Preparation of GLUT-2 and GLUT-4

Crude membrane from the liver was prepared by the method of Oka,[30] with a slight modification at the final centrifugation step (200,000*g*, 60 min). The crude membrane (100 μg) suspended in Laemmli buffer (50 mmol/L dithiothreitol[DTT]), without boiling) was separated by SDS-PAGE (10%), and electrophoretically transferred to nitrocellulose paper. The muscles were homogenized with a polytron (30 seconds, setting 5) in 20 mmol/L Tris buffer (pH 7.4, 1 mmol/L EDTA, 250 mmol/L sucrose, 2 mmol/L PMSF, 0.1 TIU/mL aprotinin, 0.1 mg/mL bacitracin), and adjusted to 10 vol/wet weight. KCl and $Na_4P_2O_7$ were added to give a final concentration of 300 mmol/L and 25 mmol/L, respectively, and mixed (4° C, 30 min). Following centrifugation (100,000*g*, 60 min), the pellet was resuspended in Tris buffer, and centrifuged (2000*g*, 15 min). The supernatant was then centrifuged (100,000*g*, 60 min), and the pellet was resuspended in Tris buffer (crude membrane). The membrane (50 μg) suspended in Laemmli buffer (without DTT, without boiling) was subjected to SDS-PAGE (10%) and Western blotting.

Western blotting

The nitrocellulose papers, onto which the proteins were transferred (80 V, 16 hr, 15° C) by Bio-Rad Trans Blot (Bio-Rad Laboratories), were soaked in TBS (10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl) for 30 min, 10% non-fat milk for 2 hr at room temperature, washed three times with TBS (10 min each) and incubated with α-IRc (15 μg/ML) or α-pTyr (5 μg/mL) or α-GLUT-2 (5 μg/mL) or α-GLUT-4 (5 μg/mL) in TBS for 10–16 hr at 4° C. After washing three times with T-TBS (TBS supplemented with 0.05% Tween-20), the sheets were incubated with ^{125}I-protein A (5×10^5 cpm/mL, 10% non-fat milk in T-TBS) for 3 hr at room temperature, extensively washed with T-TBS, dried, autoradiographed, and densitometrically scanned.

Table 2 Characteristics of experimental animals

		High-fat diet (N = 5)	Control diet (N = 4)
Initial BW	(g)	206.3 ± 1.7	207.5 ± 1.9
Final BW	(g)	378.8 ± 11.8	369.7 ± 5.0
Average food intake	(kcal/day)	82.4 ± 1.3*	92.9 ± 1.5
FBS	(mmol/L)	6.7 ± 0.63	5.8 ± 0.49
FIRI	(pmol/L)	215.0 ± 47.4†	50.9 ± 4.3
T-CHO	(mmol/L)	16.4 ± 1.84	11.3 ± 1.24
T.G.	(mmol/L)	3.86 ± 1.37	2.46 ± 0.43
NEFA	(mg/L)	223.0 ± 20.0	214.0 ± 32.0

Values are means ± SE.
* $P < 0.01$ versus controls.
† $P < 0.05$ versus controls.

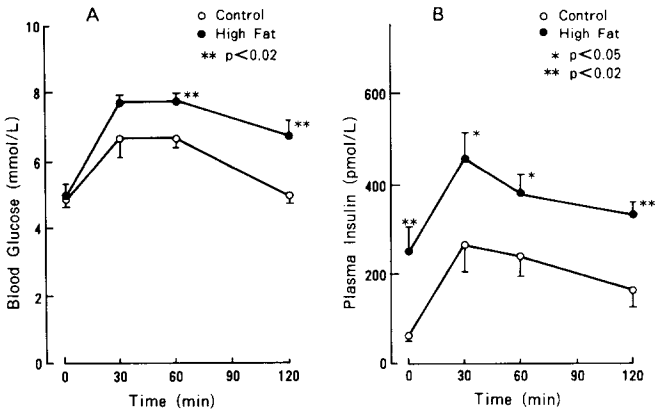

Figure 1 Blood glucose level (A) and plasma insulin level (B) of control rats and rats on a HF diet during an oral glucose tolerance test (2g/kg body weight). Each point represents mean ± SE (control: n = 4; high-fat: n = 5).

Statistical analysis

Data are presented as mean ± SEM and differences between the groups were tested by Student's *t* test.

Results

Physiology of experimental animals

Data concerning the physiologic characteristics of the experimental animals are summarized (*Table 2*). In the high-fat diet group, average food intake in calories was decreased ($P < 0.01$), and fasting plasma immunoreactive-insulin (FIRI) was increased ($P < 0.05$), whereas body weight gain and fasting blood sugar (FBS, over-night fasting) were comparable to those of the controls. Total cholesterol (T-CHO), triglyceride (TG), and non-esterified fatty acid (NEFA) tended to be higher in the high-fat group, but the differences were not significant.

Oral glucose tolerance test

Insulin resistance in the high-fat diet group was demonstrated by OGTT (*Figure 1A,1B*). The basal blood glucose concentration in both groups was lower than the FBS of each group in *Table 2* because the OGTT was performed after 24 hr fasting instead of 12 hr to clarify differences between the groups. Again, the basal blood glucose concentration in the high-fat diet group was comparable to that of the controls. After 2g/kg body weight oral glucose loading, the magnitude of the increase in blood glucose was greater in the high-fat diet group than that of the controls. The differences were statistically significant at 60 and 120 min

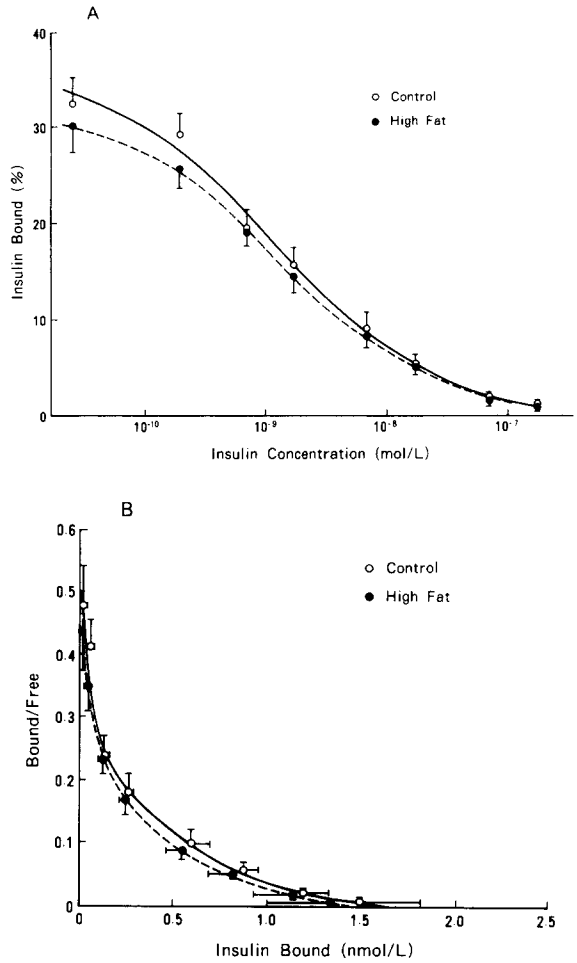

Figure 2 ^{125}I-insulin binding to WGA-purified insulin receptors from the muscles (A), and Scatchard plot analysis of the data (B). Aliquots (20 μg protein) of WGA eluate were incubated with ^{125}I-insulin (25 pmol/L) and various concentrations of unlabeled insulin at 4° C for 16 hr in 200 μL of buffer (150 mmol/L NaCl, 50 mmol/L HEPES, 0.1% BSA, pH 7.8). Nonspecific binding obtained in presence of 200 μg/mL of unlabeled insulin was subtracted from the total binding. Each point represents mean ± SE (control: n = 4; high-fat: n = 5).

after glucose loading (*Figure 1A*). The plasma IRI level was elevated in the high-fat diet group both at the basal state and after the glucose loading (*Figure 1B*).

Insulin binding

Insulin binding to WGA-purified insulin receptors is shown in *Figure 2A* (muscles) and *Figure 3A* (liver). Specific insulin binding at a tracer concentration of insulin (25 pmol/L) was 30.3 ± 3.0% (high-fat) and 32.3 ± 2.9% (controls) in the muscles and 32.0 ± 2.7% (high-fat) and 34.2 ± 2.8% (controls) in the liver. There was no significant change in the insulin binding at all insulin concentrations both in the muscles and the liver. When the data were analyzed by the Scatchard analysis, there was no significant change in receptor number (Ro) or affinity constant (Ke) (high-fat versus control; Ro = 14.5 versus 16 fmol/μg protein, Ke = 3.0 versus 2.9 × 10^8 L/mol in muscles; Ro = 29.5 versus 32.5 fmol/μg protein, Ke = 3.2 versus 3.2 × 10^8 L/mol in liver; *Figures 2B,3B*).

Autophosphorylation of WGA-purified insulin receptor

The amount of insulin receptors in each sample from the muscles (30 μg protein) and liver (15 μg protein) was quantified by Western blotting using α-IRc. *Figure 4* shows typical data from the muscles. Again, there was no significant change in the amount of insulin receptors in the WGA eluate from both the muscles and the liver between the high-fat diet group and controls. The same amount of insulin receptors from the control and high-fat groups was used for the following phosphorylation studies. When WGA-purified muscle insulin receptor was incubated with or without insulin (10^{-7} mol/L) and subjected to SDS-PAGE and Western blotting with α-pTyr, a 95 kDa protein was detected (*Figure 5A*). Tyrosine-phosphorylation of this protein was stimulated by insulin. The magnitude of the insulin-stimulated tyrosine-phosphorylation in the band was decreased in the high-fat diet group (32.4 ± 11.5% decrease, $P < 0.05$, n = 4, one-sided t test).

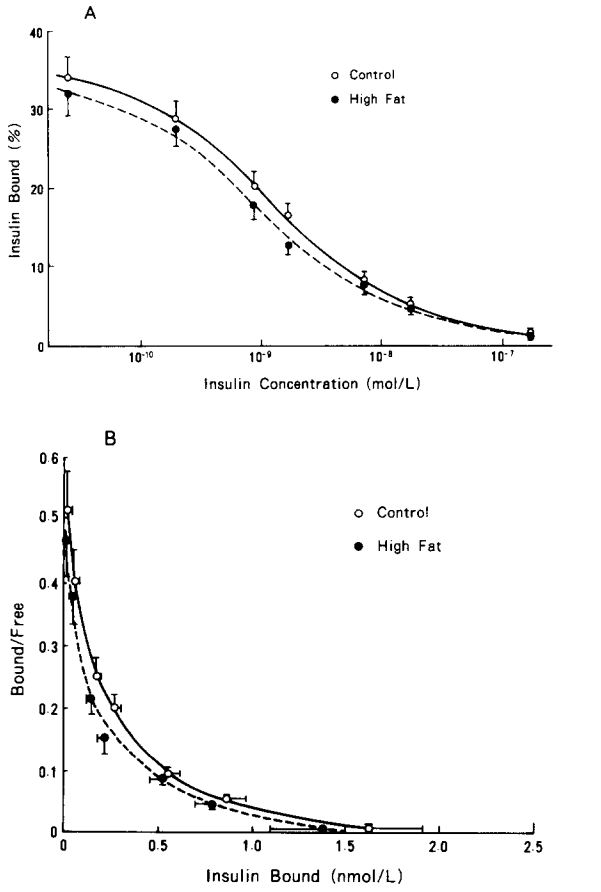

Figure 3 ^{125}I-insulin binding to WGA-purified insulin receptors from the liver (A), and Scatchard plots analysis of the data (B). Aliquots (10 μg protein) of WGA eluate were incubated under the same conditions as those of the muscles. Each point represents mean ± SE (control: n = 4; high fat: n = 5).

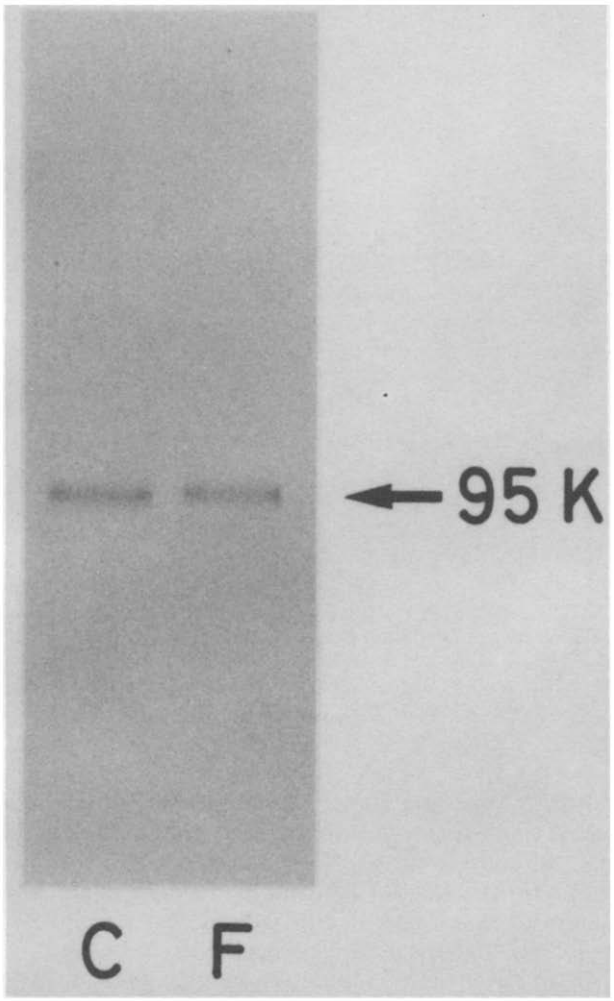

Figure 4 Quantification of insulin receptor in the eluate from WGA-affinity column (muscles). Eluate was separated by SDS-PAGE, and the insulin receptor was quantified by Western blotting using α-IRc as described in Materials and Methods. (C, control; F, high-fat group). Results show one experiment representative of four independent preparations.

The dominant component of the band is supposed to be the insulin receptor β-subunit on the basis of its molecular weight, immuno-precipitability by α-IRc, insulin-stimulated tyrosine-phosphorylation, and previous studies.[5,25] However, we cannot exclude the possibility that significant amounts of IGF-I receptors present in muscles affect the data. Autophosphorylation of the insulin receptor was then studied in the liver, which contains only a minimal amount of IGF-I receptors.[17,31]

A typical autoradiograph of Western blots of tyrosine-phosphorylated proteins from the liver is shown in *Figure 5B*. In contrast to the muscles shown in *Figure 5A*, several tyrosine-phosphorylated proteins (170 kDa, 95 kDa, and others) were detected. Tyrosine-phosphorylation of the 95 kDa protein was stimulated by insulin. In the high-fat diet group, the magnitude of the insulin-stimulated increase in phosphorylation was decreased (19.0 ± 6.7% decrease, $P < 0.05$, n = 4, one-sided t test). Tyrosine-phosphorylation of the 170 kDa protein was not stimulated by insulin. This protein was supposed to be the EGF receptor as previously described using anti-EGF receptor antibody.[32] The characteristics of the other proteins detected in *Figure 5B* remain unknown.

In vivo phosphorylation study with muscles and liver

To further clarify the mechanisms of the insulin resistance in high-fat diet rats, phosphorylation of the insulin receptor and its endogenous substrates in muscles and liver were studied in vivo. Rats were injected with human insulin (0 or 5 units) through the femoral vein, then the muscles were homogenized and solubilized as described in Materials and Methods. Samples were

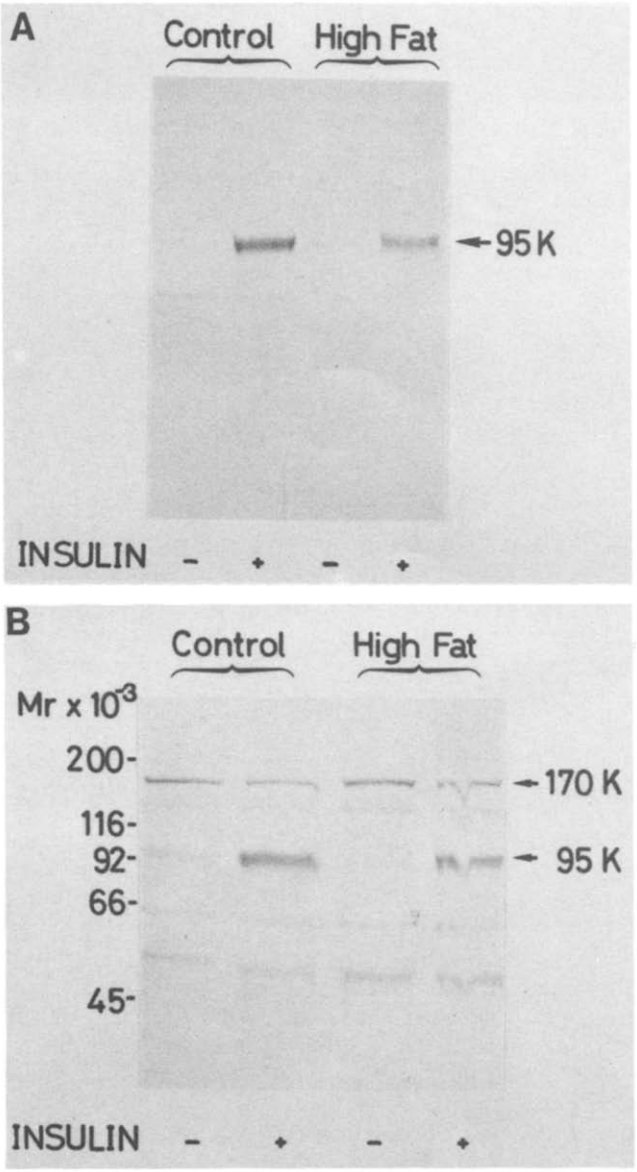

Figure 5 Autophosphorylation of WGA-purified insulin receptors from muscles (A) and liver (B). The same concentration of insulin receptors was stimulated by insulin (10^{-7} mol/L) with 150 μmol/L of ATP, and separated by SDS-PAGE. Phosphotyrosine-containing protein was analyzed by Western blotting with α-pTyr. Results show one experiment representative of four independent preparations.

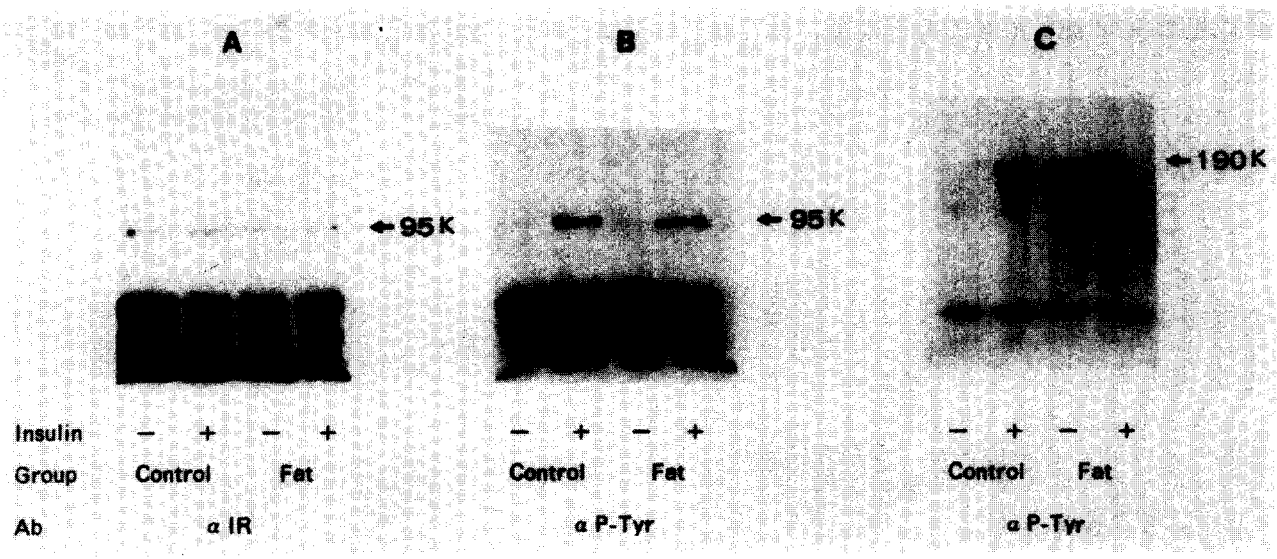

Figure 6 Quantification of the insulin receptor (A), autophosphorylation of the insulin receptor (B), and phosphorylation of the endogenous substrate (pp190) (C). Human insulin (0 or 5 units) was injected into the rats via the femoral vein. After 5 min, the muscles were solubilized and immunoprecipitated with Ab-3 (A,B). Precipitates were separated by SDS-PAGE and Western blotted with α-IRc to quantify the insulin receptor (A), and with α-pTyr for tyrosine-phosphorylation of the receptor (B). A portion of the solubilized samples was applied to a hydroxylapatite affinity column, washed, boiled with Laemmli sample buffer, separated by SDS-PAGE, then Western blotted with α-pTyr to detect the tyrosine-phosphorylation of the endogenous substrate. Results show one experiment representative of three independent preparations.

separated for three experiments; quantification of the insulin receptor, autophosphorylation of the receptor, and phosphorylation of its endogenous substrates.

To quantify the receptor, each sample was immunoprecipitated with Ab-3, transferred onto nitrocellulose paper, and analyzed by Western blotting using α-IRc (*Figure 6A*). There was no difference between the insulin-treated and -untreated samples in the amount of insulin receptor detected. The amount of insulin receptors tended to decrease in the high-fat group (92.0 ± 6.6% of the controls). However, the difference between the groups was not statistically significant.

For the autophosphorylation study of the receptor, solubilized insulin receptors from the rat muscles were immunoprecipitated with Ab-3, separated by SDS-PAGE, and transferred onto nitrocellulose paper as described above. Tyrosine-phosphorylation of the insulin receptor was quantified by Western blotting using α-pTyr (*Figure 6B*). Phosphorylated tyrosine of the insulin receptor β-subunit (95 kDa) was detected after insulin stimulation. In the high-fat diet group, the magnitude of the insulin-stimulated increase in phosphorylation tended to be slightly decreased (94 ± 5.6% of controls; not significant). However, the ratio of the tyrosine-phosphorylation to the amount of insulin receptor quantified in *Figure 6A* was not different between the groups.

For the phosphorylation study of endogenous substrates, phosphorylated proteins were concentrated using the hydroxylapatite affinity column. The proteins were then separated with SDS-PAGE, and tyrosine-phosphorylation of an endogenous substrate (pp190) was also quantified by Western blotting with α-pTyr (*Figure 6C*). Phosphotyrosine on pp190 was detected after insulin stimulation. There was no decrease in tyrosine-phosphorylation on pp190 in the high-fat diet group.

We further studied tyrosine-phosphorylation of the insulin receptor and its endogenous substrate (pp175) in the liver,[7] in which the phosphorylation of the IGF-I receptor by insulin stimulation is negligible.[17,31] After an insulin injection, the liver was homogenized and solubilized by SDS. Tyrosine-phosphorylated proteins were immunoprecipitated with α-pTyr, separated by SDS-PAGE, and detected by Western blotting with α-pTyr (*Figure 7*). Three proteins (200, 120, and 75 kDa) were detected at basal conditions. After the insulin stimulation, the 95 kDa insulin receptor β-subunit and the 175 kDa endogenous substrate appeared. Changes in the phosphotyrosine on pp75 and pp120 were minimal. Phosphotyrosine on pp200 was somewhat decreased by the insulin stimulation. In the high-fat diet group, the magnitude of the increase in phosphotyrosine on both pp175 and pp95 was significantly decreased (pp95, 16.7 ± 5.3% decrease, $P < 0.05$; pp175, 27.6 ± 7.3% decrease, $P < 0.05$; n = 3, one-sided t test).

Quantification of glucose transporters (GLUT-2, GLUT-4)

Glucose transporters in the muscles (GLUT-4) and the liver (GLUT-2) were quantified by Western blotting. A crude membrane fraction of muscles was prepared as described in Materials and Methods, separated by SDS-PAGE and transferred onto nitrocellulose paper. GLUT-4 was detected with α-GLUT-4c. Typical results are shown in *Figure 8A*. Only a 45 kDa band was

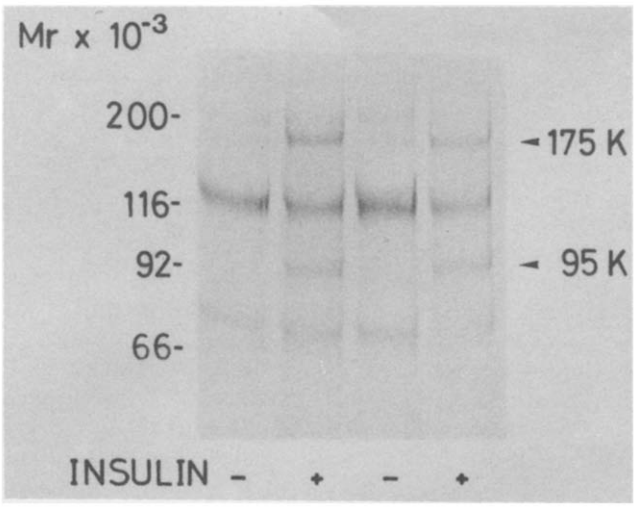

Figure 7 Quantification of insulin receptor autophosphorylation and phosphorylation of endogenous substrate (pp175) by Western blotting with α-pTyr. Human insulin (0 or 5 units) was injected into the rats via the portal vein. After 5 min, the liver was solubilized with 1% SDS, and tyrosine-phosphorylated proteins were immunoprecipitated with α-pTyr as described in Materials and Methods. The sediments were incubated with 10 mmol/L PNPP for 5 hr, then the supernatant was separated by SDS-PAGE, followed by Western blotting with α-pTyr. Results show one experiment representative of three independent preparations.

detected. This protein was identified as GLUT-4 on the basis of its molecular weight, and immunoreactivity to α-GLUT-4c. Furthermore, a similar band was detected in 3T3-L1 adipocytes, which was partially translocated from the low density microsomal membrane to the plasma membrane by insulin stimulation (data not shown). No statistically significant decrease was detected in the amount of GLUT-4 from the high-fat diet group compared with that of controls.

The amount of GLUT-2 in the liver was also determined with α-GLUT-2 (*Figure 8B*). Two peptides (57 kDa and faint 114 kDa) were detected. The 57 kDa protein was considered to be GLUT-2 on the basis of its molecular weight and immunoreactivity to α-GLUT-2. However, it is not clear whether the 114 kDa protein is a dimer of GLUT-2 or other protein which cross-reacts with the antibody. There was no significant difference in the amount of both pp57 and pp114 in the liver between the high-fat diet group and the controls.

Discussion

A high-fat diet causes insulin resistance in humans and rats.[1,3,4] Several studies have been reported about the mechanisms of this type of insulin resistance. A HF-diet causes a change in the composition of plasma membrane,[33] which has a major effect on the transmembrane signalling by various hormones. A decrease in the insulin binding to its receptor, and a decrease in insulin receptor autophosphorylation have been reported in various tissues.[1,3,4,17-19] However, changes in the subsequent steps are still unknown. The present studies attempted to extend knowledge gained from previous studies regarding the mechanisms of insulin resistance in rats fed a high-fat diet.

Glucose intolerance associated with hyperinsulinemia was observed by OGTT in rats fed a high-fat diet, confirming data obtained by others.[3,17,19] Insulin binding to the WGA-purified insulin receptor from muscles and liver was comparable in both diet groups. This is in agreement with the data of Boyd et al.[18] In contrast, Maegawa[1] and Grundleger[3] have shown decreased insulin binding to the intact soleus muscle of high-fat–fed rats. Solubilization of the membrane might remove the effect of the high-fat diet from the receptors.

Our data indicated that autophosphorylation of the insulin receptor was decreased in rats fed with a high-

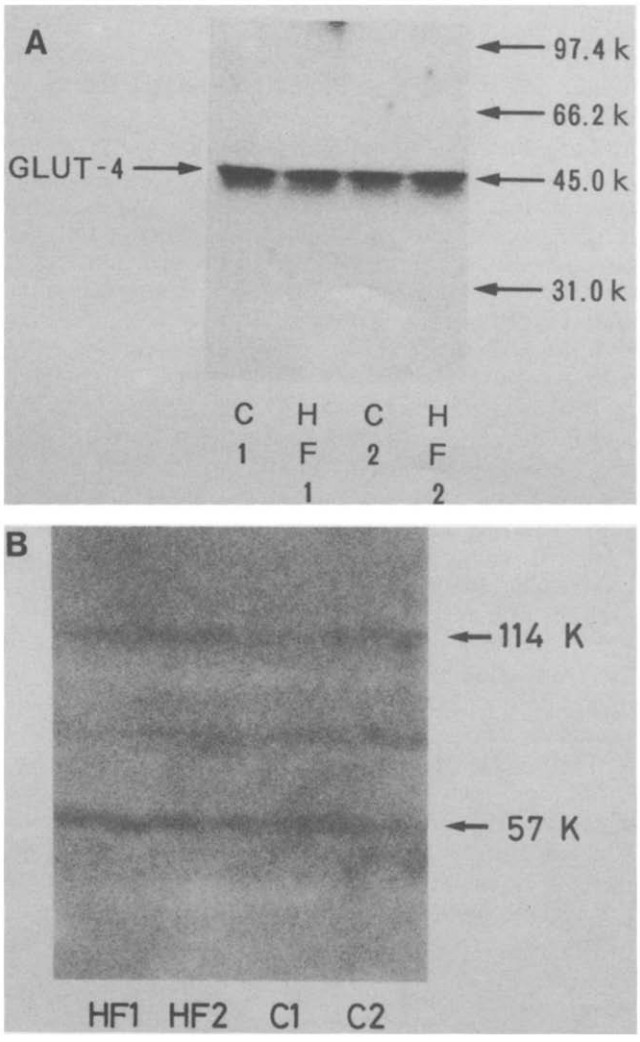

Figure 8 Quantification of Glut-4 in muscle crude membrane (A), and GLUT-2 (B) from the liver. Crude membranes prepared as described in Materials and Methods (50 μg muscle, 100 μg liver protein) were separated by SDS-PAGE, then Western blotted with α-GLUT-4c (A) and α-GLUT-2 (B), respectively. (C1 and C2, controls; HF1 and HF2, high-fat group). Results show one experiment (two sets of controls and high-fat groups) representative of four independent preparations.

fat diet both in muscles and liver in vitro. This is in agreement with previous studies.[17-19] In extending this observation, the present study has demonstrated that the autophosphorylation of the insulin receptor and the phosphorylation of the endogenous substrate from the liver (pp175) was decreased in rats fed with a high-fat diet. However, the autophosphorylation of the receptor from the muscles and the phosphorylation of pp190 in the high-fat group were comparable to those of the controls. Hind-limb muscles consist of groups with different fiber-type composition, which may have affected the results. The doses of insulin used in the in vivo studies are very high, and these findings may not necessarily reflect the sensitivity of the insulin receptor to physiologic levels of insulin. However, our data suggest that the insulin resistance in the high-fat group is due to post-receptor defects in muscles, which is in agreement with the conclusion by Boyd et al.,[18] although tyrosine-phosphorylation of both IR and its endogenous substrate (pp175) were decreased in liver.

The pp190, which is rapidly phosphorylated by insulin at tyrosine residues, is a Triton X-100 soluble protein. Insulin-stimulated phosphorylation of an approximately 185 kDa protein in intact cells has been reported in a number of tissues.[8,34,35] Although the molecular values appear similar, the major difference between pp190 and pp185 is immunoreactivity to α-pTyr. The pp190 was barely immunoprecipitated with α-pTYr before SDS-PAGE. However, after concentration with hydroxylapatite and separation by SDS-PAGE, it was clearly detected by Western blotting with α-pTyr (*Figure 6C*), suggesting that the pp190 can immunoreact with α-pTyr after some conformational change. This characteristic is similar to the pp195 reported by Komori et al.[36] The non-insulin stimulated form of pp195 binds to WGA, while insulin-stimulated pp190 did not bind to WGA, which was similar to pp185. The relationship among these proteins remains unclear. We cannot rule out the possibility that pp195 is a glycosilated form or is anchored to glycosilated proteins, that pp190 is a cleaved or released form, and that pp185 is a conformationally changed form.

The pp175, which is also rapidly phosphorylated by insulin and IGF-I, is observed only in SDS-extracts, suggesting that it is a cytoskeleton-associated protein.[7] The reasons for the differences in the effect of high-fat diet on the phosphorylation state of pp190 and pp175 remain unknown. The organ and substrate specificity of the effects of the high-fat diet might cause the differences.[19,37] As the IGF-I receptor concentration in the liver is negligible,[17,31] the IGF-I receptor in the muscles might have affected the present results. If the IGF-I receptor also phosphorylates pp190 to pp185,[34,35,37] the phosphotyrosine, detected on pp190 in *Figure 6C*, might represent that catalyzed by both the insulin receptor and the insulin-stimulated IGF-I receptor. This could induce an overestimation in the ratio of pp190 phosphorylation against pp95. To prevent overestimating the ratio, the sum of the phosphotyrosine of 95 kDa proteins catalyzed by both the insulin receptor and insulin-stimulated IGF-I receptor was quantified. For this purpose, we used Ab-3 instead of α-IRc for the first immunoprecipitation in *Figure 6B*, because the latter does not cross-react with the IGF-I receptor. The ratio of phosphorylation of pp190 against that of pp95 was not decreased in the high-fat group. The phosphorylation of 95 kDa proteins tended to be decreased in the high-fat group, but the change was not statistically significant.

Glucose transporters are important effectors of insulin action for glucose metabolism, and several studies have been reported concerning the regulation of glucose transporters in various models.[12-14] Using similar procedures, both GLUT-4 in the muscles and GLUT-2 in the liver were quantified. The amounts of both GLUT-2 and GLUT-4 in the crude membrane from rats fed the high-fat diet were comparable to those from the controls. Further studies, including the changes in the intrinsic activity and the translocation of the glucose transporters, will be required to reveal the insulin resistance at this step.

Two tyrosine residues (1316,1322) are included in the sequence against which α-IRc was raised.[23] The phosphorylation of these residues by insulin stimulation might affect the immunoreactivity of the insulin receptor. However, no statistically significant differences were observed in the amount of insulin receptor from insulin-stimulated and noninsulin-stimulated rats (*Figure 6A*). These data are in agreement with those of Boyd et al. that there was no difference in binding after insulin injection or perfusion.[18]

The mechanism of the decrease in the autophosphorylation of the insulin receptor and the phosphorylation of its endogenous substrate in the high-fat–fed rats remains unclear. The alterations in the membrane composition[33] might change the signal transduction of the insulin receptor from the α-subunit to the β-subunit. Phosphorylation of serine and threonine residues on the insulin receptor might affect the kinase activity.[38,39] In this study, tyrosine-phosphorylation, which is important for signal transduction of insulin using site-directed insulin receptor mutants[9,10] and α-pTyr,[40] was selectively quantified by Western blotting using α-pTyr in intact rats. However, serine kinases, which also appear to be involved in the signal transduction[38] and dephosphorylation of enzymes, also mediate some insulin-induced metabolic changes. Selective detection of phosphoserine and phosphothreonine,[41] and measurement of phosphatase activity will be important as a next step.

In summary, the results presented here show that a high-fat diet induces a decrease in kinase activity on the insulin receptor purified from liver and muscles. The phosphorylation of an endogenous substrate (pp175) was also decreased in the liver. These changes in the phosphorylation states may, at least in part, cause the altered metabolic state that was observed, although no change was detected in the amount of GLUT-2 and GLUT-4 in liver or muscle crude membrane.

Abbreviations

DTT dithiothreitol
SDS sodium dodecyl sulfate
PMSF phenylmethylsulfonyl fluoride
PAGE polyacrylamide gel electrophoresis
pp190 a phosphoprotein of 190 kDa in muscles, found by Siego Kono (manuscript submitted)
pp175 a phosphoprotein of 175 kDa in liver
TBS Tris-buffered saline

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